Head and Neck Paragangliomas

Genetics, Heredity and Clinical Characteristics

Erik Frans Hensen

Head and Neck Paragangliomas

Genetics, Heredity and Clinical Characteristics

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Im Geister-Labyrinth, in scheinbaren Begriffen
Kann auch der Klügste sich in fremde Bahn vertiefen;
Und wann sein sichrer Schritt sich nie vom Pfad vergisst,
Am Ende sieht er doch, daß er im Anfang ist.

From: Gedanken über Vernunft, Aberglauben und Unglauben,
Albrecht von Haller, 1729.

voor Henk
en Tineke
dankzij Iris
en Koen

Cover photograph: fluororescent in situ hybridisation image of the nucleus of a paraganglioma chief cell of a SDHD-linked patient. The red fluorescent label is hybridized to the centromere of chromosome 1, the green label is hybridised to the centromere of chromosome 11. In this nucleus, only one chromosome 11 centromere can be identified as opposed to two centromeres of chromosome 1, indicating the loss of an entire copy of chromosome 11.

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1. The paraganglion system

Paraganglia are small bodies of chromophil cell clusters associated with the ganglia of the autonomic nervous system. The paraganglion system consists of the adrenal medulla, the largest paraganglion in the human body, the sympathetic paraganglia, and the parasympathetic paraganglia[1,2]. The sympathetic paraganglia are associated with the ganglia of the paravertebral sympathetic trunk, the organ of Zuckerkandl, and the celiac, renal, suprarenal and hypogastric plexuses (figure 1)[2].

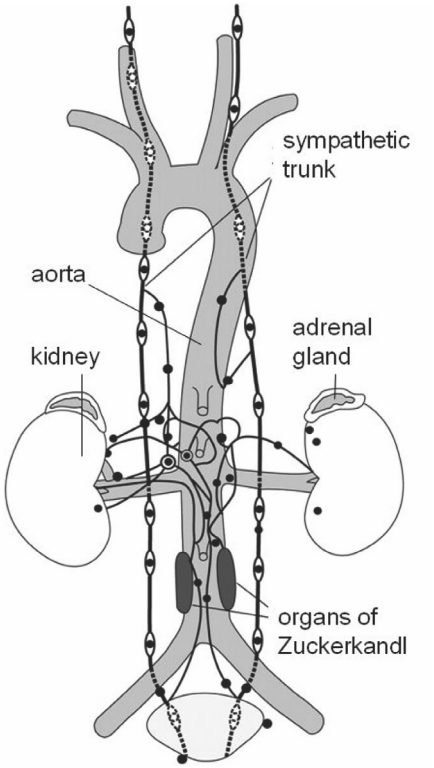


Figure 1. The adrenal medulla and extra-adrenal sympathetic paraganglia. Adapted from: Lee et al. Am. J. Roentgenol. 187 (2006) 492-504.

The parasympathetic paraganglia consist of the intravagal bodies and the branchiomeric paraganglia in the mediastinum and head and neck region, most notably located in the carotid bifurcation, the jugular foramen and on the promontory of the middle ear (figure 2)[2].

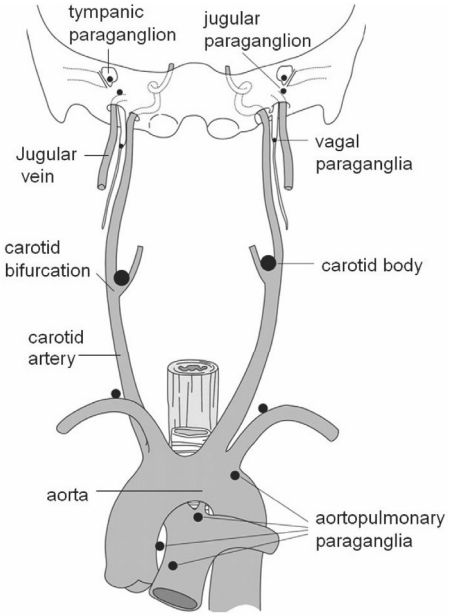


Figure 2. The parasympathetic branchiomeric paraganglia. Adapted from: Lee et al. Am. J. Roentgenol. 187 (2006) 492-504.

Paraganglia contain a parenchymal and a stromal component. The parenchymal component is of neuroectodermal origin. During embryogenesis, neuronal precursor cells migrate from the neural crest to locations along the cranial nerves, sympathetic trunk and greater blood vessels, where they develop into the paraganglionic type 1 or chief cells. The stromal component is of mesenchymal origin en contains the type 2 or sustentacular cells, as well as other stromal components such as blood vessels[3]. Type 1 and 2 cells form a specific configuration known as the “Zellballen”: small clusters of type 1 cells surrounded by type 2 cells and other stromal components (figure 3)[4].

The exact function of the paraganglion system is not fully known. The adrenal medulla, the inner part of the adrenal gland, produces the catecholamines adrenalin, noradrenalin and dopamine: hormones that regulate heart rate, blood pressure, metabolism, and cause vasoconstriction and bronchiole dilatation. The organs of Zuckerkandl are thought to be important regulators of the embryonic homeostasis and blood pressure through the production and release of catecholamines during early gestation, and they normally start to regress in the third trimester. The carotid and aortic bodies function as peripheral chemoreceptors sensitive to changes in arterial oxygen levels, and to a lesser degree also

to carbon dioxide levels and arterial pH. Arterial hypoxia, hypercapnia and acidosis cause excitation of the paraganglionic type 1 cells. This signal is relayed by the afferent fibers of the glossopharyngeal and vagal nerves to the central cardiorespiratory centers in the medulla oblongata, which regulate cardiac output and respiration (see paragraph 4.2.3: oxygen sensing at the carotid body, and paragraph 5.1: the discovery of the carotid body function)[5].

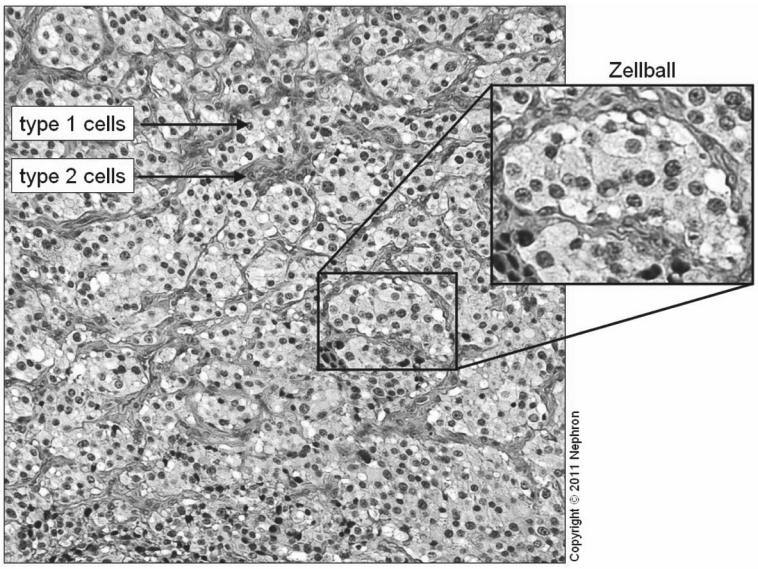


Figure 3. Microscopy of hematoxylin and eosin (H-E) stained carotid body paraganglioma tissue showing the type 1 and 2 cells in the classic Zellballen configuration. This characteristic architecture is usually preserved in the progression from normal paraganglion tissue to paraganglioma.

2. Neoplasia of the paraganglion system

The nomenclature of the neoplasia arising from the paraganglion system is equivocal and has changed over time. The terms 'chemodectomas', 'chromaffin tumors', 'glomus tumors', 'paragangliomas' and 'pheochromocytomas' have all been used interchangeably. The current classification according to the World Health Organization (WHO) designates tumors originating from the paraganglia in the head and neck region as 'paragangliomas', accompanied by the site of origin, i.e. 'carotid body paraganglioma'. The term 'pheochromocytoma' is reserved for tumors arising in the adrenal medulla, and 'extra-adrenal paraganglioma' for tumors developing in sympathetic paraganglia elsewhere in the retroperitoneal space, the abdomen, or the thorax[6].

However, some authors argue that the WHO classification is too refined, and as the distinctions between the subgroups are largely based on arbitrary conventions, consensus on the terminology of paraganglion tumors has thus far remained elusive. As a result, the term 'pheochromocytoma' is also used to describe all paraganglion tumors located outside of the head and neck region, or all paraganglion tumors located within the abdomen, or it is reserved for paraganglion tumors that secrete catecholamines and cause associated symptoms (see paragraph 2.3: 'functional paragangliomas')[6-8]. The use of the term 'glomus tumor' when referring to a head and neck paraganglioma, a remnant of the 19th century terminology for head and neck paraganglia, is quite persistent among physicians, but is better avoided because this term describes a completely different histological entity (namely a painful cutaneous tumor arising from neuromyoarterial glomus cells, characteristically located under the finger nails)[6,9,10].

Paragangliomas are usually slow growing and highly vascular tumors. The typical architecture of normal paraganglion tissue, the 'Zellballen' configuration consisting of type 1 and type 2 cells (figure 3), is usually maintained in the tumor, although in pheochromocytomas it may be less prominent[4,6,11]. It has been demonstrated that the tumorigenic component is formed by the type 1 or chief cells, and that the type 2 stromal cells show expansion under the influence of the type 1 cells[12].

2.1 Paragangliomas of the head and neck

Paragangliomas of the head and neck are rare tumors, representing approximately 0.6% of all head and neck neoplasms[13]. The incidence is estimated to be between 1:1.000.000 and 1:100.000, based on pooled data from Dutch pathology laboratories and surgical patients[4,14,15]. Due to the benign natural course of the disease, paragangliomas will not be surgically removed in a substantial proportion of the patients and it is therefore

likely that these figures represent an underestimation of the actual incidence[15,16]. Necroscopy rates for carotid body paragangliomas of 1:13.4000 to 1:3.860 also point towards a higher incidence[15,16]. The incidence of paragangliomas seems to be influenced by environmental factors that facilitate paraganglioma formation, such as high altitude, and by genetic factors, such as the regional clustering of paraganglioma patients due to a common hereditary trait, as can be seen in the Netherlands (see: 'genetics of paraganglioma', 'tumor biology of paragangliomas', and chapters 2, 3 and 4).

The majority of head and neck paragangliomas comprises of carotid body tumors, arising in the carotid bifurcation (approximately 61%). Approximately 19% is located along the vagal nerve, 12% are found in close relation to the jugular bulb or tympanic nerve, and 8% is located elsewhere in the head and neck region, most frequently along the larynx, the trachea or the aortic arch[4].

Symptoms of head and neck paragangliomas vary with the tumor localization. Most tumors are characterized by slow and expansive growth, but approximately 10-15% of the head and neck paragangliomas show a more aggressive, rapidly progressive behavior[17]. The most common symptom is a non-painful palpable neck mass or pharyngeal bulging. In addition, cranial nerve invasion or compression and subsequent dysfunction may occur, especially of the facial, glossopharyngeal, vagal, spinal accessory and hypoglossal nerves, because of their close anatomical relations with the jugulotympanic, vagal and carotid paraganglia. In case of tympanic or jugulotympanic tumors there may be conductive hearing loss and tinnitus, which is pulsatile in typical cases. Patients with functional paragangliomas can present with symptoms and signs of catecholamine excess (see paragraph 2.3: 'functional paragangliomas')[18]. A number of paragangliomas do not produce any clinical symptoms, and 6-16% are found as incidentalomas on imaging studies or through screening of paraganglioma families[18,19].

Diagnosis

The diagnosis of head and neck paragangliomas is based on the patient and family history, clinical investigation of the ears, the pharynx and the neck, biochemical screening for catecholamine excess (see paragraph 2.3: 'functional paragangliomas'), and radiology.

Detailed radiological examinations are essential for the diagnosis. The classic way of visualizing head and neck paragangliomas is digital subtraction angiography (DSA), which shows paragangliomas as highly vascular lesions. It is considered the gold standard in the diagnosis of small head and neck paragangliomas and for the identification of the vascular anatomy and main contributing blood vessels[20,21]. It is especially useful when surgery

is considered, because in addition to its role in the evaluation of the vascular structures, the angiography procedure may also be used to eliminate the main blood supply to the tumor or to perform a preoperative balloon occlusion test of the internal carotid artery (see paragraph 2.1: 'therapy')[20-22]. The disadvantages of the DSA technique are the need for catheterization (usually through the femoral artery) and the lack of visualization of the exact extension of the tumor and its relations to surrounding structures.

Nowadays DSA has largely been replaced by high resolution computed tomography (HRCT or CT) and magnetic resonance imaging (MRI). While both techniques are useful in assessing tumor extension, evaluating its anatomical relations, and detecting multiple paragangliomas within the head and neck region if present, MRI is the preferred modality because of its better visualization of soft tissues[20]. In addition, the CT imaging exposes the patient to radiation (albeit in a very low dose) and the contrast used might provoke catecholamine release in patients that are not pre-treated with alpha- or beta-blockers, although this complication was not found in recent studies[23-25]. The most accurate MRI technique in the detection of head and neck paragangliomas is a pre-and post-contrast enhanced 3D Time of Flight (TOF) MR angiography[20,26]. In both CT and MR imaging, it is essential to assess tumor extension in the axial as well as in coronal planes.

Functional imaging techniques like ^{131}I-metaiodobenzylguanidine (MIBG) scintigraphy, ^{18}F-fluorodopamine or ^{18}F-fluorodihydroxyphenylalanine positron emission tomography (FDA-PET and FDOPA-PET, respectively) have a high specificity for paragangliomas because they detect abnormal isotope uptake by noradrenalin transporters in paraganglioma tissue[27,28]. They are useful when in doubt of the diagnosis and in whole-body screening for functional paragangliomas and pheochromocytomas, but a reduced sensitivity of MIBG and FDA-PET has been described in extra-adrenal and malignant paragangliomas[27-30]. ^{18}F-fluorodeoxyglucose (FDG)-PET is efficient in whole-body screening for metabolically active tissue. As such, it is not very specific for paragangliomas or pheochromocytomas, but it is useful in screening for multiple tumors and has been shown to be a superior tool in the detection of paraganglioma metastases[27,28,30]. The lack of anatomical detail in the images, a disadvantage of PET imaging, can nowadays be overcome by combining PET and CT techniques, creating a single superposed image[20].

Definitive confirmation of the diagnosis is obtained by histopathology and the identification of the pathognomonic 'Zellballen' configuration within the tumor tissue. However, because of the high vascularity of these tumors and the risk of profuse bleeding upon biopsy, tissue samples for histopathology are rarely available prior to the surgical resection of the tumor. Nevertheless, if the origin of the lesion is uncertain and the diagnosis can not

be reliably made upon physical examination and imaging alone, one may consider fine needle aspiration biopsy (FNAB). Although the cytologic features of a paraganglioma are not very specific and cytology alone is therefore not sufficient for a reliable diagnosis of paraganglioma, the FNAB technique has been found to be safe and is sometimes required in order to rule out other types of malignancy[31,32].

Classification

Different classification systems exist for different primary paraganglioma sites in the head and neck region. For carotid body tumors, the classification according to Shamblin et al. is widely used[33]. Shamblin type I tumors are localized within the carotid bifurcation but do not involve the internal or external carotid artery; Shamblin type II tumors are adherent or partially surround one or both of these vessels; Shamblin type III tumors encase the internal and external carotid arteries, and extend to the hypoglossal nerve (figure 4). The Shamblin type can be evaluated preoperatively using CT or MRI imaging. The Shamblin type is positively correlated with the size of the tumor as carotid body tumors become more adherent to carotid vessels as they increase in diameter, and there is a correlation between the Shamblin classification and outcome after surgery, as cranial nerve injury (particularly to the vagal, the superior laryngeal, hypoglossal or facial nerve) is more likely to occur in larger tumors[33-35].

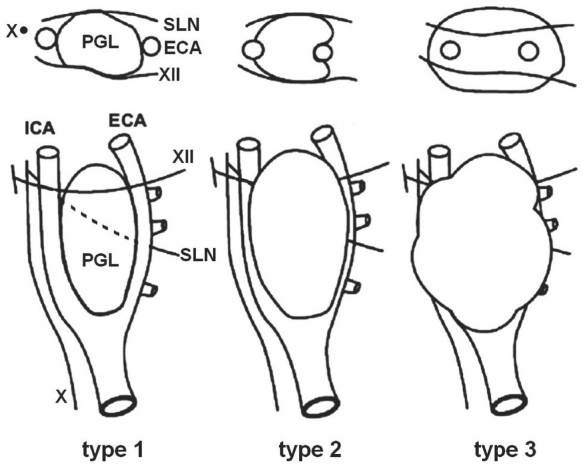


Figure 4. The classification of carotid body tumors according to Shamblin. The top row shows the axial views; the bottom row shows the sagittal views of Shamblin type 1, 2 and 3 paragangliomas (PGL). The classification is based on the relations of the tumor with the internal carotid artery (ICA), the external carotid artery (ECA), the vagal nerve (X), the hypoglossal nerve (XII) and the superior laryngeal nerve (SLN). Adapted from Arya et al. Am J Neuroradiol 29 (2008) 1349-1354.

Paragangliomas involving the temporal bone (tympanic and jugulotympanic tumors) are generally classified according to Fisch (table 1 and figure 5)[36,37]. This classification is primarily based on the extension of the tumor in the temporal bone and the involvement of the internal carotid artery, the jugular bulb, and the intracranial space. Jugulotympanic paragangliomas can be classified preoperatively using CT for Fisch type A and B paragangliomas, and a combination of CT and MRI for type C and D tumors. The Fisch type dictates the surgical approach necessary for tumor removal[37,38].

As of yet, no universally accepted system exists for the classification of vagal body tumors. The key features in the tumor description of vagal body tumors include the tumor size and its relations to the skull base and the internal and external carotid arteries[39].

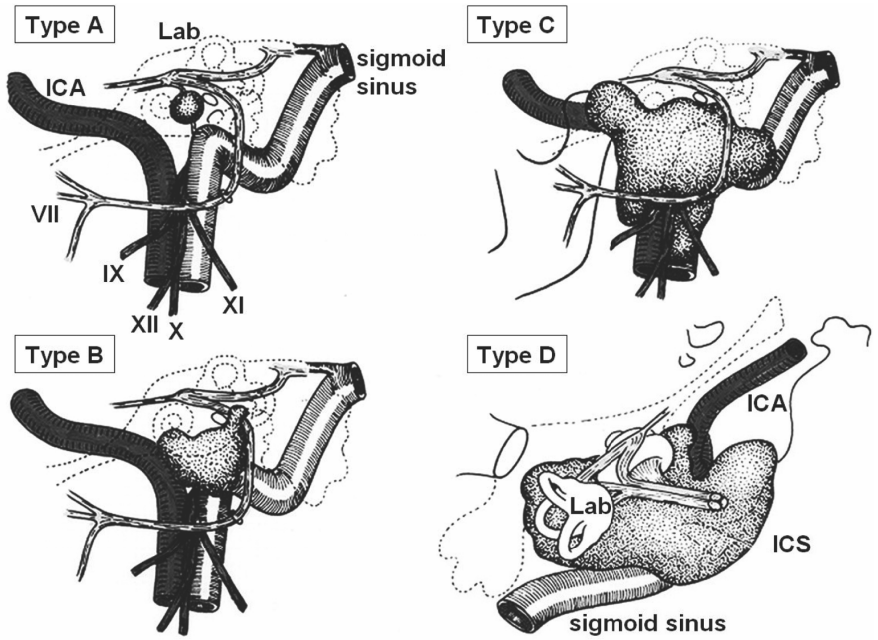


Figure 5. Schematic representation of the classification of temporal bone paragangliomas according to Fisch[36]. Type A, B, and C paragangliomas and their relations to the sigmoid sinus, the facial (VII), glossopharyngeal (XI), hypoglossal (XII), vagal (X) and spinal assessory (XI) nerves, the internal carotid artery (ICA) and the labyrinth (Lab) are shown in a sagittal view, type D is depicted in an axial view. Type A tympanic paragangliomas originate on the cochlear promontory and are limited to the mesotympanic space in the middle ear. Type B tympanic paragangliomas are limited to the middle ear and mastoid. Type C jugulotympanic paragangliomas show erosion of the bone covering the jugular bulb and extend along the ICA. Type D jugulotympanic paragangliomas extend into the intracranial space (ICS).

Table 1. The classification of temporal bone paragangliomas according to Fisch[36]. Combinations of C and D types are used to classify extended jugulotympanic paragangliomas.

Type	Name	Extension
A	Tympanic paraganglioma	Limited to mesotympanum. No bone erosion.
B		Limited to hypotympanum, mesotympanum and mastoid. No erosion of jugular bulb.
C	Jugulotympanic paraganglioma	Erosion of jugular bulb. Subclassification by degree of carotid canal erosion:
	C1	no invasion of carotid canal
	C2	invasion of vertical trajectory of carotid canal
	C3	invasion of horizontal trajectory of carotid canal
	C4	invasion of formane lacerum and cavernous sinus
D		Intracranial extension, either extradural (De) or intradural (Di).
	De1	intracranial extradural extension < 2cm
	De2	intracranial extradural extension > 2cm
	Di1	intracranial intradural extension < 2cm
	Di2	intracranial intradural extension > 2cm

Therapy

Today, there are 4 main strategies in the management of head and neck paragangliomas: surgical excision, embolization, radiotherapy, and watchful waiting.

The obvious benefit of surgical resection of paragangliomas is the removal of the tumor mass and the possibility of histological evaluation of the resection specimen, allowing for definitive confirmation of the diagnosis. Furthermore, future morbidity or progression to malignancy may be prevented. The surgical approach depends on the location of the paraganglioma within the head and neck region, the extension of the tumor, and its relations to adjacent structures. Surgery varies from relatively uncomplicated resections of Shamblin type 1 carotid body tumors and relatively straightforward middle ear and mastoid approaches in Fisch A and B type tympanic tumors, to extended head and neck surgery and infratemporal fossa approaches in Shamblin type 3 carotid body tumors and Fisch C and D type jugulotympanic tumors[34,35,37,38]. Due to the high vascularity of paragangliomas and their close anatomical relationships with the carotid artery, the jugular vein, multiple cranial nerves, and/or the skull base, there is a definite risk of surgical complications. Complete removal of the tumor is not always possible or may result in significant morbidity or even mortality, especially in larger tumors and tumors invading the skull base[15,21,37,40,41]. The cranial nerves that are most at risk when a surgical resection is performed are the glossopharyngeal, vagal, spinal assessory and

hypoglossal nerves in vagal and carotid body tumors; and the facial, vestibulocochlear, glossopharyngeal, vagal, spinal assessory and hypoglossal nerves in jugulotympanic tumors[35,37,39,41]. In vagal body tumors, the vagal nerve is almost always transected as vagal body tumors arise from its nodose ganglion[39,41]. The risk of peroperative cranial nerve injury is not always readily assessable preoperatively, as even in patients without preoperative cranial nerve deficit, infiltration or encasement of cranial nerves by paraganglioma tissue is present in 50% of the cases[42]. Cranial nerve deficit is especially incapacitating when cranial nerves are bilaterally affected, as this precludes the compensation of function from the contralateral side.

A possible additional complication of bilateral resections of carotid body paragangliomas specifically is the loss of the regulation of the hypoxic ventilatory response, resulting in immediate hypoventilation and respiratory acidosis (see paragraph 4.2.3: 'the hypoxia pathway')[43].

In larger vagal, carotid body and temporal bone paragangliomas, intraoperative control of the carotid arteries and jugular vein is compulsory, and grafting or sacrifice of these structures is sometimes necessary[41]. Whereas unilateral sacrifice of the jugular vein is generally well tolerated, bilateral resection or ligation may lead to elevated intracranial pressure and neurological sequelae[44,45]. If the need for ligation or partial resection of the internal carotid artery is anticipated, a preoperative intraluminal balloon occlusion test is recommended to evaluate the effects on the brain[20].

In order to minimize surgical difficulty and risk of uncontrollable bleeding, embolization of the main contributing blood vessels prior to the excision of the paraganglioma can be very helpful, especially in vagal and jugulotympanic tumors. In tympanic and carotid body tumors, the benefits of this procedure are not so clear[20,21,37,46-48]. Embolization is also performed as a primary palliative therapy for symptomatic or malignant paragangliomas as it may reduce tumor size, however the effects are almost always temporary, as alternative blood supply will develop and the tumor retains its potential to grow[49,50].

The third treatment option, radiotherapy, can be used as a primary treatment or as an adjuvant therapy after incomplete surgical resection of a paraganglioma[21,51]. As a single modality therapy, it has a much reduced risk of intraoperative bleeding and cranial nerve injury[21,52,53]. Because eradication of head and neck paragangliomas is not achieved by radiotherapy, the objective is local control of tumor growth, and one of the obvious drawbacks is therefore the persistence of the tumor mass[20]. Other disadvantages include the possible long-term effects of irradiation such as osteoradionecrosis of the skull base,

potential induction of malignancy, and an increased surgical difficulty and risk if resection proves to be necessary at a later stage[54,55]. Furthermore, there is evidence from histopathology that the tumor response to radiotherapy is unpredictable[56]. With the advent of stereotactic radiotherapy and 'gamma-knife' strategies, irradiation of adjacent normal tissue has been reduced while reported local tumor control rates (90-92%) are comparable to conventional radiotherapy[51,52,57]. However, as the natural course of most paragangliomas is characterized by no or very slow growth, it is difficult to ascertain whether a non-growing paraganglioma after radiotherapy is the result of tumor control by successful radiotherapy or due to the indolent natural course of the disease[20,39,58].

The fourth option in the management of head and neck paragangliomas consists of a policy of watchful waiting, also called 'wait and scan'. No intervention is performed, and tumor growth is monitored regularly with repeated MRI. Surgery or radiotherapy is undertaken only if there is evidence of tumor growth or impending complications. The disadvantage of this strategy is the persistence of the tumor and its potential to grow, however, most head and neck paragangliomas are characterized by slow growth, a substantial number of head and neck paragangliomas will not become symptomatic, and the effects of cranial nerve palsy are often better mediated if the paresis is slowly progressive due to tumor growth as opposed to sudden paralysis due to surgical injury[19,20,39,58].

Not surprisingly, the optimal treatment strategy for head and neck paragangliomas is subject of much debate in the literature[21,22,53,58,59]. The choice of treatment modality and timing require a multidisciplinary approach, and are tailored to the individual patient, depending on symptoms, tumor location, tumor stage, mutlifocality, catecholamine excess (see below), heredity and the causative gene mutation[15]. Surgical resection of paragangliomas is considered either when the tumor is small and total removal is not likely to cause significant cranial nerve injury and associated morbidity, or when cranial nerve deficit has already been caused by the tumor and complete resection is unlikely to cause additional problems. A rationale for surgical intervention is the anticipation of complications due to progression of tumor extension towards cranial nerves, the skull base, or the carotids[15,20,21]. Furthermore, surgical resection is the therapy of choice in functional paragangliomas (see below). Radiotherapy, stereotactic radiotherapy or 'gamma knife', although considered by some as the primary treatment of choice in all paragangliomas, are primarily used as a palliative treatment in malignant paraganglioma, or in progressive paragangliomas in which surgery is deemed to confer a high risk of significant morbidity[15,20,21,52]. A conservative treatment strategy consisting of closely monitoring the natural course of the disease without intervention (´wait and scan'), seems, at least initially, appropriate for many asymptomatic patients, elderly patients,

patients with multiple and bilateral paragangliomas, tumors with extensive temporal bone involvement and tumors caused by a gene mutation that is characterized by a mild disease phenotype[15,20,21,39].

2.2 Pheochromocytomas and extra-adrenal paragangliomas

Pheochromocytomas and extra-adrenal paragangliomas, together described as sympathetic paragangliomas, are tumors closely related to head and neck paragangliomas. Sympathetic paragangliomas are rare tumors, with an estimated incidence between 1:500.000 and 1:50.000[60,61]. About 80% of the sympathetic paragangliomas originate from the adrenal medulla and are called pheochromocytomas. The remaining 20%, called extra-adrenal paragangliomas, occur elsewhere in the sympathetic paraganglia, most frequently in the abdomen and pelvis, less frequently in the thorax[29]. Extra-adrenal paragangliomas more often progress to metastatic disease than either pheochromocytomas or head and neck paragangliomas [62].

The symptoms of pheochromocytomas are usually caused by the secretion of catecholamines or their metabolites by the tumor, and include hypertension (in about 60%) which may be fluctuating or sustained, paroxysmal palpitations, headache, agitation, excess sweating and pallor.

The diagnosis of pheochromocytomas and extra-adrenal paragangliomas is based on biochemical screening for catecholamine excess (see paragraph 2.3: 'functional paragangliomas') and radiology. Both abdominal CT and MRI are sensitive modalities for the detection of abdominal masses. As described above, some authors prefer MRI because no iodine containing contrast is needed to adequately visualize the tumor.

The mainstay of pheochromocytoma therapy is surgical resection. The preferred technique is a partial or cortical sparing adrenalectomy via a laparoscopic or retroperitoneoscopic approach, as this minimizes surgical risk and morbidity[63-66]. Bilateral endoscopic cortical sparing adrenalectomies should be considered in bilateral pheochromocytomas or in case of a genetic predisposition for developing bilateral disease, although there is debate as to whether partial adrenalectomy is associated with an increased long-term recurrence rate in hereditary cases[63,66-68]. Total adrenalectomy is indicated in malignant pheochromocytomas and sometimes unavoidable in benign pheochromocytomas, especially in large tumors or recurrent disease[66,68]. If performed bilaterally, total adrenalectomy carries the risk of potentially life-threatening post-operative Addisonian crises and necessitates lifelong corticoid supplementation therapy[66,69]. Adrenalectomy via an open laparotomy is nowadays rarely indicated, even in large tumors (i.e. tumors with a

diameter exceeding 6 cm)[70]. There is debate as to whether open procedures should be performed in malignant pheochromocytomas and extra-adrenal paragangliomas. Most authors agree that it is indicated in case of large malignant tumors, local invasion, or if resections of neighboring organs are required[66,71-73]. Because of the risk of catecholamine excess during, and catecholamine depletion after an adrenalectomy procedure, peri-operative treatment with α- and β- adrenoreceptor antagonists, calcium channel blockers and/or catecholamine synthesis-inhibitors is mandatory (see paragraph 2.3: 'functional paragangliomas').

2.3 Functional paragangliomas

A proportion of the neoplasia of the paraganglion system is 'functional', i.e. they secrete vasoactive catecholamines like dopamine, adrenalin and noradrenalin and/or their metabolites. Excess catecholamine secretion is a well-known feature of adrenal pheochromocytomas and extra-adrenal paragangliomas, but relatively rare in head and neck paragangliomas (1-5%)[18]. The majority of functional paragangliomas produce noradrenalin, a few secrete dopamine or adrenalin[74,75]. Catecholamine secreting tumors are best detected through the evaluation of the urine or plasma concentrations of metanephrine and normetanephrine, metabolites of catecholamines. Plasma free metanephrine measurements are the most accurate diagnostic tool, with a superior sensitivity (97-99%) and specificity (86-97%)[15,74,76,77]. The next best technique, 24-hours urinary metanephrine and normetanephrine measurements, has a comparable high sensitivity (96-97%) but lower specificity (45-82%)[15,74]. The latter is still widely used in the Netherlands due to better availability[15].

Biochemical screening should be performed if a patient's signs or symptoms indicate a functional paraganglioma, in case of a genetic risk for the development of paraganglioma-pheochromocytoma syndrome, and in all pheochromocytomas and extra-adrenal paragangliomas (see: 'genetics of paragangliomas')[15,27,29]. If catecholamine secretion is present in head and neck paragangliomas, it causes the same symptoms as it does in pheochromocytomas (hypertension, palpitations, headache, agitation, excess sweating and pallor). Prolonged exposure to high levels of catecholamines can result in hyperglycemia, electrolyte disturbances and cardiovascular complications such as cardiac hypertrophy, myocardial infarction or heart failure. Multiple organ failure, shock and sudden death by stroke or cardiac arrest due to catecholamine excess have been reported[29,78,79]. Because of these potentially life-threatening conditions, surgical excision is the treatment of choice in functional paragangliomas[15,27]. Peri-operative measures consisting of pre-operative volume expansion by intravenous saline, stringent intra-operative monitoring, and treatment with α- and β- ardrenoreceptor antagonists, calcium channel blockers and/

or catecholamine synthesis-inhibitors are compulsory to counter critical hypertensive crises and compensatory hypotensive episodes due to manipulation and removal of the tumor[29,65].

2.4 Malignancy

Most paragangliomas are benign tumors, i.e. they do not metastasize and are characterized by an expansive rather than an invasive growth pattern. However, some paragangliomas, especially those within the petrous bone, show erosion of the surrounding bone, some show microvascular invasion, and some do metastasize. As of yet, no definite histologic criteria for malignancy have been established in paragangliomas[6]. Even in malignant paragangliomas and their metastases, the well differentiated architecture of normal paraganglion tissue is usually maintained[4,11]. Factors such as a higher mitotic rate, tumor cell spindling, altered nuclear morphology, aberrant DNA-ploidy, necrosis, and capsular or microvascular invasion are reported to be more prevalent in malignant paragangliomas, but all are also found in benign paragangliomas[4,6,11]. Immunohistochemical markers such as Ki-67, Cyclin-D1, p53, p21, p27, BCL-2 and MDM-2 have been shown to be of little use in predicting malignant behavior in paragangliomas[6,11]. Malignancy in paragangliomas is therefore defined as the occurrence of metastatic paraganglioma cells in non-neuroendocrine tissue.

Paraganglioma metastases are most frequently confined to cervical lymph nodes (69%). Distant metastases are identified in 31% of malignant head and neck paragangliomas, and the distant predilection sites include bone, lung and liver[80].

Several studies have assessed clinical factors that may predict malignancy in paraganglioma patients. Features such as a young age at diagnosis, pain as an accompanying symptom, a rapidly enlarging tumor mass, a large tumor size, and a mediastinal or extra-adrenal abdominal tumor localization all seem to be associated with an increased risk of malignancy, but none of these features are proof of malignancy in themselves[11,81,82]. Tumors that secrete catecholamines may be malignant or benign in nature. There is some debate as to whether dopamine secretion is indicative of extra-adrenal tumor localization and malignancy, but recent studies show that dopamine secretion is not uncommon in benign head and neck paragangliomas (19-23%), and that it is not related to metastatic disease or outcome[75,83-85]. The risk of developing malignant paraganglioma or pheochromocytoma is however correlated with the causative gene defect (see: ‘genetics of paragangliomas’).

The management of malignant paragangliomas is challenging. More aggressive treatment strategies are aimed at eradication and/or control of tumor growth both at the primary and metastatic site. In case of head and neck paragangliomas with metastases limited to regional lymphe nodes, surgical resection of the primary tumor combined with a neck dissection is the treatment of choice if feasible. In this patient group, no clear beneficial effect of adjuvant radiotherapy has been found if resection margins are negative[80]. In case of incomplete resections, adjuvant therapy may consist of embolization, radiotherapy, systemic chemotherapy (with cyclophosphamide, vincristine and dacarbazine) or combinations thereof[80,86-88]. In case of incurable metastatic disease, palliative treatment strategies include surgical tumor debulking, embolization, pharmacological blocking of catecholamine secretion, palliative conventional radiotherapy, metabolic targeted radiotherapy with ^{131}I-MIBG, and/or systemic chemotherapy[80,86-88]. A recent development is the advent of possible targeted molecular therapies. Currently, several are being investigated in patients with malignant paraganglioma and pheochromocytoma[71,72,89-93]. Promising results have been reported of temozolomide and thalidomide combination therapy, of sunitinib (a tyrosine kinase inhibitor), and of somatostatin analogues, but their effectiveness has not yet been validated by clinical trials[71;72;90-93]. As surgical resections confer a risk of surgical complications as mentioned above, and the non-surgical interventions can be complicated by bone marrow depression and a fatal sudden increase in catecholamine levels due to tumor necrosis, treatment of incurable metastatic disease should only be considered if the quality of life is threatened by symptoms caused by local tumor extension or catecholamine excess[86-88]. A policy of watchful waiting may be considered a viable option in patients with stable metastatic disease and mild symptoms[86-88].

Due to the rarity of malignant paragangliomas and the number of different strategies and regimens that have been applied over time, data on the outcome of these interventions are largely retrospective, not fully comparable, and often biased, and the lack of controlled prospective trials hampers the recommendation of specific therapies[80,86,87].

Without taking treatment strategies into account, patients with malignant head and neck paragangliomas have a reported overall five year survival rate of 55-60%[80,86,94]. Survival is greatly influenced by the site of the metastasis, as the five year survival rate of patients with metastatic disease limited to regional lymph nodes (77%) is significantly better than of those with distant metastasis (12%)[80]. Furthermore, survival seems to be influenced by the causative gene, as the five year survival rate after first metastasis is 37% in patients carrying a mutation in the *SDHB* gene, whereas it is 67% in the absence of *SDHB* mutations (see: 'genetics of paragangliomas')[94].

3. Genetics of paragangliomas

3.1 NF1, RET, and VHL

The knowledge of paraganglioma genetics has long been limited to mutations in genes causing neurofibromatosis type 1 (the *NF1* gene), multiple endocrine neoplasia (MEN) type 2a and 2b (the *RET* gene), and Von Hippel-Lindau syndrome (the *VHL* gene)[6]. Whereas pheochromocytomas are a well-known element of the tumor spectrum of these syndromes, head and neck paragangliomas caused by *NF1*, *RET* or *VHL* mutations are rare and almost never occur as the sole manifestation of the disease. *NF1*, *RET* or *VHL* mutations and their associated syndromes are discussed briefly with a focus on their relevance in head and neck paragangliomas and pheochromocytomas.

NF1

Neurofibromatosis type 1 (NF1) is caused by mutations in the *NF1* gene located on 17q11. It encodes the neurofibromin 1 protein, a negative regulator of the Ras intracellular signaling pathway which is involved in cell growth, differentiation and survival (table 2). NF1 is the most common tumor syndrome of the peripheral nervous system, with a prevalence of 1 in 3000[95]. The syndrome consists of cutaneous and peripheral nerve neurofibromas, and is also associated with gastrointestinal tumors, gliomas and myeloid leukemia. The estimated prevalence of pheochromocytomas in NF1 patients is 0.1- 6%, although necroscopy rates are higher (3-13%)[95]. As a rule, pheochromocytomas do not occur without other manifestations of neurofibromatosis, most often neurofibromas and café-au-lait spots on the skin[95;96]. The mean age at diagnosis of the first pheochromocytoma is approximately 41 years, bilateral adrenal involvement occurs in 27%, extra-adrenal localizations are infrequent (5%), and the malignancy rate is 6%[96]. Head and neck paragangliomas associated with NF1 have been reported but are extremely rare[97,98]. In a recent international study including 809 head and neck paraganglioma patients that were not linked to mutations in succinate dehydrogenase (see paragraph 3.2: 'the succinate dehydrogenase genes'), no NF1 mutations were identified[97,98].

RET

Mutations in the *RET* proto-oncogene (located on 10q11) cause MEN type 2 syndromes. *RET* encodes a receptor tyrosine kinase and functions as the receptor for extracellular signaling molecules of the glial cell line-derived neurotrophic factor (GDNF) family (table 2)[99]. MEN type 2 syndromes can be divided into MEN type 2a, characterized by the occurrence of medullary thyroid carcinoma, pheochromocytoma, and primary hyperparathyroidism, and MEN type 2b, characterized by medullary thyroid carcinoma, pheochromocytoma, mucosal neuroma and a marfanoid habitus[99]. In MEN type 2 syndromes, the risk of

developing a pheochromocytoma is high (50%), and if a pheochromocytoma is found, multifocal or bilateral tumors are common (50-80%), but extra-adrenal paragangliomas are rare (3%), as is pheochromocytoma malignancy (3%)[99,100]. *RET* mutation carriers seldom develop head and neck paragangliomas (in approximately 0.1%)[98].

VHL

Von Hippel-Lindau (VHL) disease is a hereditary cancer syndrome caused by mutations in the *VHL* gene located on 3p25-26 (table 2). *VHL* encodes a subunit of the VHL ubiquitin ligase complex, a key component in the degradation of hypoxia inducible factor (HIF) 1α, 2α and 3α subunits (see paragraph 4.2.3: 'the hypoxia pathway', and figure 8)[101]. Specific homozygous recessive mutations in the *VHL* gene do not cause tumors, but a rare form of hereditary polycythemia called Chuvash syndrome[101]. Patients with the VHL tumor syndrome carry heterozygous mutations and the disease is inherited in an autosomal dominant way[101]. In these heterozygous *VHL* mutation carriers, *VHL* acts as a tumor suppressor gene, i.e. loss of the wild type allele is required for tumorigenesis. Mutations predispose to a variety of tumor types, including hemangioblastoma of the retina and central nervous system, clear cell renal carcinoma, neuroendocrine pancreatic tumors and endolymphatic sac tumors[95,101,102]. Pheochromocytomas are found in 20% of VHL patients, and the mean age at diagnosis of the pheochromocytoma is 28 years[95,102]. In the pediatric pheochromocytoma population, *VHL* mutations are the predominant cause of the disease (accounting for 40% of the cases)[95,102]. Most VHL-linked pheochromocytomas are benign and bilateral[95]. Head and neck paragangliomas are found in less than 1% of VHL cases, and *VHL* mutations account for approximately 2% of the head and neck paraganglioma population[98,103]. Almost all VHL-linked head and neck paraganglioma patients have additional manifestations and/or a positive family history of VHL disease[98].

3.2 The succinate dehydrogenase genes

In 2000, Baysal et al. in collaboration with the Paraganglioma research Group Leiden, discovered that mutations in succinate dehydrogenase subunit D (SDHD), a subunit of the mitochondrial succinate dehydrogenase complex (SDH), cause hereditary head and neck paraganglioma syndrome type 1 (PGL1)[104]. This breakthrough discovery initiated the identification of other SDH genes as the causes of the PGL2, PGL3 and PGL4 paraganglioma syndromes. Parts of this overview have been adapted from chapter 2, which reviews the current developments in paraganglioma genetics.

SDHD

The *SDHD* gene is located on the long arm of chromosome 11 (11q23). The mapping of its locus on 11q23 and its subsequent identification as the cause of PGL1 syndrome was greatly facilitated by the concentration of large PGL1 kindreds in the proximity of the city of Leiden, located in the western part of the Netherlands[104-107]. The discovery of *SDHD*, a nuclear gene encoding an anchoring subunit of SDH, was the first time that a mitochondrial protein was identified as a tumor suppressor (figure 7). It was furthermore the first protein with a role in the intermediary metabolism to be directly linked to tumorigenesis[104]. SDHD-linked paraganglioma syndrome is characterized by the formation of benign head and neck paragangliomas, and metastatic disease is rare (0-10%)[62,108-112]. SDHD-linked patients have a high risk of developing multiple paragangliomas (30-74%), and are also at risk of developing a concurrent pheochromocytoma (7-53%)[62,108-110,113]. The diagnosis is generally made in the third or fourth decade of life (mean age at diagnosis 25- 38 years)[62,108,109]. The penetrance of *SDHD* mutations is high upon paternal transmission (87-100%), although not all paraganglioma patients develop tumor-related symptoms[19,62,109,114-116]. Maternal transmission of disease is extremely rare (see paragraph 4.3: 'inheritance of head and neck paraganglioma syndromes')[117]. In the Netherlands, mutations in SDHD are the major cause of head and neck paragangliomas, probably due to the occurrence of multiple Dutch founder mutations (see also chapters 3 and 4)[118,119]. The incidence of SDHD mutations and the clinical characteristics of SDHD-linked paraganglioma syndrome in the Netherlands are further discussed in chapters 3, 4 and 5.

SDHC

In 2000, *SDHC*, located on chromosome 1 (1q23), encoding another SDH anchoring subunit, was found to be the causative tumor suppressor gene in paraganglioma syndrome PGL3 (figure 7)[120]. Mutations in *SDHC* are primarily associated with benign head and neck paragangliomas, although extra-adrenal paragangliomas, pheochromocytomas and malignancy have been reported in SDHC-linked cases[110,120-123]. The average age at diagnosis is 38-46 years[110,121]. Mutations in *SDHC* are a rare cause of paragangliomas, with only 19 index cases and 30 affected patients reported to date[124]. In the Netherlands, *SDHC* mutations represent less than 0.5% of the mutations found in SDH genes (chapter 3)[125]. The inheritance of SDHC-linked disease is autosomal dominant, and the penetrance of *SDHC* mutations is as yet unknown, but the very low incidence of SDHC-related paraganglioma suggests that it is incomplete.

SDHB

In 2001, *SDHB*, located on chromosome 1 (1p35-36.1), encoding the catalytic iron-sulfur SDH subunit, was linked to paraganglioma syndrome PGL4 (figure 7)[126]. It acts as a tumor suppressor gene and it has been shown to be the dominant cause of hereditary paraganglioma syndrome in many parts of the world[62,108]. SDHB-linked paraganglioma syndrome is characterized by a high rate of extra-adrenal paragangliomas (52-84%), while pheochromocytomas (18-28%) and head and neck paragangliomas (27-31%) are less frequently found[62,108,109]. The mean age at diagnosis is 30-37 years, and up to 38% of *SDHB* mutation carriers develop metastatic disease[62,103,108-110,121]. Most SDHB-linked tumors present with catecholamine excess; 10% of the tumors is biochemically silent or produces dopamine only[62,127]. The inheritance of SDHB-linked disease is autosomal dominant, and the penetrance of *SDHB* mutations is estimated to be 26-35%, much lower than that of *SDHD* mutations, which explains why *SDHB* mutations are more often found in isolated paraganglioma patients[128,129,129-131]. In the Netherlands, *SDHB* mutations seem to be remarkably uncommon, and account for only 3% of the head and neck paraganglioma patients and 6% of all SDH mutations (chapters 3 and 4)[111,125].

SDHAF2

The gene encoding succinate dehydrogenase assembly factor 2 (*SDHAF2*, formerly known as SDH5), is located on the long arm of chromosome 11 (11q13). In 2009, it was linked to head and neck paraganglioma syndrome PGL2 (table 2)[132]. *SDHAF2* acts as a tumor suppressor and does not encode a SDH subunit, but a co-factor related to the function of the SDHA flavoprotein subunit. The p.Gly78Arg missense mutation is the only pathogenic mutation in SDHAF2 known to date[133-135]. SDHAF2-linked paraganglioma syndrome is characterized by the formation of benign head and neck paragangliomas[133,135]. Most patients develop multiple head and neck tumors (70-91%), but no extra-adrenal paragangliomas, pheochromocytomas, or malignant paragangliomas have been reported in association with SDHAF2 mutations[111,133,135]. The mean age at diagnosis is 33-34 years[111,133]. SDHAF2-linked disease is characterized by the same parent-of-origin dependent inheritance pattern as SDHD-linked paraganglioma syndrome, i.e. no paragangliomas develop upon maternal transmission of the SDHAF2 mutation, whereas the risk of disease upon paternal transmission of the mutation is very high (88-100%) [132,133]. The SDHAF2 mutation is currently only found in a large Dutch paraganglioma kindred and an unrelated Spanish family[134]. In the Netherlands, it accounts for 7% of SDH mutation carriers, and approximately 4% of the head and neck paraganglioma patients (see chapters 3 and 4)[111,125].

SDHA

SDHA, located on 5p15, is a highly polymorphic gene encoding the flavoprotein subunit of SDH (figure 7)[136]. Until recently, no association between this major catalytic subunit of SDH and paraganglioma formation could be established. Instead, homozygous recessive SDHA mutations were associated with Leigh syndrome, a rare mitochondrial deficiency resulting in encephalopathy, myopathy, developmental retardation, loss of vision, loss of hearing, and a limited life expectancy[137]. In 2010, a heterozygous *SDHA* germ line mutation was identified in a single patient with a functional extra-adrenal paraganglioma of the abdomen. Loss of the wild type *SDHA* allele was found in the tumor tissue of this patient, suggesting that SDHA can act as a tumor suppressor in paragangliomas too[138].

The prevalence of SDHA mutations in the head and neck paraganglioma population remains to be clarified. Its late identification as a tumor suppressor and the isolated presentation of the reported SDHA-linked patient indicate that SDHA mutations are a rare cause of paraganglioma susceptibility[138]. The reasons for this infrequent association of SDHA mutations with paragangliomas are currently unknown. One explanation might be that mutations that eliminate all SDHA activity are incompatible with life. It has been shown that both the homozygous *SDHA* mutations causing Leigh syndrome as well as the heterozygous *SDHA* mutation causing paraganglioma result in SDH deficiency (see paragraph 4.1: 'succinate dehydrogenase')[137-139]. However, in patients with Leigh syndrome, considerable residual cytoplasmic SDHA immunostaining and activity can still be detected, indicating that SDHA stability is affected but SDHA functionality is not completely lost[137]. There is evidence that SDHA has a cellular function additional to its enzymatic role in the TCA cycle, as a component of the mitochondrial ATP-sensitive potassium channel[140,141]. Possibly, mutations that interfere with this other function are not tolerated.

An alternative explanation for the scarcity of SDHA-linked paragangliomas is a low observed frequency of somatic 5p15 loss[138,142]. Assuming that *SDHA* acts as a tumor suppressor gene in paragangliomas, loss of heterozygosity (LOH) targeting the wild type *SDHA* allele on 5p15 is an essential step in SDHA-linked tumorigenesis. The LOH at 5p15 may be prevented by local epigenetic factors or the LOH may simultaneously affect other genes in the close proximity of *SDHA* that are vital to the survival of the cell[138,140].

3.3 Other genes in paragangliomas and pheochromocytomas

Recently, germ line mutations in other genes that are not directly linked to SDH have also been identified in paraganglioma and pheochromocytoma patients. Although to date they do not seem to be very prevalent in the head and neck paraganglioma population, these discoveries may contribute to our insight in the tumorigenic pathways that are implicated in paraganglioma tumorigenesis (see: 'molecular biology of paragangliomas'), and are therefore discussed briefly.

TMEM127

In 2010, mutations in a three-spanner transmembrane protein, transmembrane protein 127 (TMEM127), have been identified as a cause of familial and isolated pheochromocytomas, and recently also of extra-adrenal paragangliomas and paragangliomas of the head and neck[143,144]. Mutations in *TMEM127*, located on 2q11, cause hereditary paraganglioma-pheochromocytoma syndrome with autosomal dominant inheritance, and LOH of the wild type *TMEM127* allele is observed in tumors indicating that *TMEM127* acts as a tumor suppressor gene (table 2)[143]. The function of TMEM127 is currently not fully known, but initial insights suggest that TMEM127 is a negative regulator of the mechanistic target of rapamycin (mTOR) pathway (see paragraph 4.4: 'other mechanisms in paraganglioma tumorigenesis')[143]. Clinically, TMEM127-linked patients are characterized by the occurrence of pheochromocytomas, which are frequently bilateral (in 35-50%), a comparative late onset of disease (mean age at diagnosis 42.8-45.3 years), and a low malignancy rate (0-5%)[143,145]. The penetrance of *TMEM127* mutations awaits detailed investigation, the first data suggest it is high but incomplete (an age related penetrance of 64% by the age of 43-55 years)[143-145]. The prevalence of *TMEM127* mutations in the paraganglioma and pheochromocytoma population seems low, recently a total of in 20 patients carrying 19 different mutations could be identified in an international cohort of 990 paraganglioma-pheochromocytoma patients (2%)[145].

PHD2

In 2008, a mutation in *EGLN1*, located on 1q42, was reported to be associated with an abdominal extra-adrenal paraganglioma in a single isolated patient (table 2)[146]. *EGLN1* encodes HIF-prolyl hydroxylase 2 (PHD2), an enzyme involved in the degradation of hypoxia inducible factor 1 alpha (HIF-1α). PHD2 mutations have also been associated with erythrocytosis, a rare neoplastic disorder causing an elevated red blood cell count, and the patient who developed the recurrent functional paraganglioma suffered from this condition too[146,147]. LOH of the wild type *EGLN1* allele was observed in the tumor, which indicates that PHD2 may act as a tumor suppressor in paraganglioma tumorigenesis[146]. The disruption of PHD2 function caused by this PHD2 mutation could

result in tumorigenisis through a pathway similar to that of defective SDH (see paragraph 4.2.3: 'pseudo-hypoxic drive' and figure 8)[146].

KIF1B

Also in 2008, kinesin family member 1B (*KIF1B*), located on 1p36, was identified as the causative tumor suppressor gene in a cancer-prone family suffering from multiple tumors, including neuroblastomas, ganglioneuromas, leiomyosarcomas, and pheochromocytomas[148]. It was found to act in a pro-apoptotic pathway downstream of prolyl-hydroxylase 3 (PHD3), a pathway that is involved in the development of neuronal precursor cells. Mutations in KIF1B could protect from apoptosis, causing aberrant survival of neuronal precursor cells that may later give rise to pheochromocytomas (see paragraph 4.3: 'abnormal development of neuronal precursor cells', and figure 9)[148-150]. Transcription analysis suggests that *KIF1B*-linked pheochromocytomas are more closely related to *NF1*- and *RET*-, than to *VHL*- and SDH-associated tumors[149].

MAX

In 2011, exome sequencing identified mutations in the MYC associated factor X gene (*MAX*) in hereditary pheochromocytoma patients that had tested negative for mutations in *SDHA*, *SDHAF2*, *SDHB*, *SDHC*, *SDHD*, *NF1*, *RET*, and *VHL*[151]. In all, 12 patients carrying 8 different *MAX* mutations have been described, with an early mean age at diagnosis of 32 years, and high rates of bilateral pheochromocytomas (67%) and malignancy (25%), although these characteristics might in part reflect the selection criteria of the study[151]. As of yet, no MAX mutations have been reported in head and neck paragangliomas.

MAX, located on 14q23, behaves as a tumor suppressor gene[151]. MAX is a member of the basic helix-loop-helix leucine zipper (bHLHZip) family, which also includes myelocytomatosis viral oncogene homolog (MYC) and MAX dimerization protein 1 (MXD1). This family of transcription factors regulates cell proliferation, differentiation and apoptosis, and is linked with the mTOR pathway, the pathway to which TMEM127 is linked as well[151,152]. Mutations in MAX could lead to pheochromocytoma tumorigenesis through these proliferative pathways or through interference with apoptosis and neuronal development via the same pathway as KIF1B (see 4.3: 'abnormal development of neuronal precursor cells', paragraph 4.4: 'other mechanisms in paraganglioma tumorigenesis', and figure 9)[151,153,154].

Table 2. Summary of genes currently associated with paragangliomas and pheochromocytomas.

gene	locus	protein function	inheritance	associated syndrome	paraganglioma predilection site
NF1	17q11	neurofibromin, RAS pathway regulator	autosomal dominant	neurofibromatosis type 1 (NF1)	adrenal
RET	10q11	receptor tyrosine kinase	autosomal dominant	multiple endocrine neoplasia (MEN 2a/2b)	adrenal
VHL	3p25	part of HIF degradation complex	autosomal dominant*	Von Hippel-Lindau syndrome (VHL)	adrenal
SDHA	5p15	flavoprotein, catalytic subunit of SDH	autosomal recessive**	Leigh syndrome, myopathy, encephalopathy	extra-adrenal
SDHAF2	11q13	assembly factor of SDH	autosomal dominant, parent-of-origin dependent	paraganlioma syndrome (PGL2)	head and neck
SDHB	1p35-36	iron-sulphur catalytic subunit of SDH	autosomal dominant	paraganlioma syndrome (PGL4)	extra-adrenal, adrenal
SDHC	1q23	anchoring subunit of SDH	autosomal dominant	paraganlioma syndrome (PGL3)	head and neck
SDHD	11q23	anchoring subunit of SDH	autosomal dominant, parent-of-origin dependent	paraganlioma syndrome (PGL1)	head and neck
TMEM127	2q11	mTOR pathway regulator	autosomal dominant	paraganlioma-pheochromocytoma	extra-adrenal, adrenal
EGLN1	11q23	prolyl-hydroxylase 2, HIF degradation	autosomal dominant	familial erythrocytosis	extra-adrenal
KIF1B	1p36	developmental culling, apoptosis	autosomal dominant	neural crest-derived tumor syndrome	adrenal
MAX	14q23	member of bHLHZip family of transcription factors	autosomal dominant	hereditary pheochromocytoma	adrenal

*Von Hippel-Lindau (VHL) syndrome is caused by heterozygous VHL mutations and inherited in an autosomal dominant way. Homozygous recessive VHL mutations are found in Chuvash syndrome, a rare form of hereditary polycythemia. **Inheritance of the reported SDHA-linked paraganglioma appears to be autosomal dominant, whereas homozygous recessive SDHA mutations may cause Leigh syndrome.

Considerable differences in the proportion of hereditary cases and relative mutation frequencies have been reported in different patient cohorts and different parts of the world. In recent studies that have evaluated patients with at least one head and neck paraganglioma, approximately 18-33% are reported to present with a positive family history, and pathogenic gene mutations can be identified in 31-55% of the head and neck paraganglioma patients, most frequently in *SDHD* (19-79%) and *SDHB* (9-34%), and less frequently in *SDHC* (0-14%), *VHL* (0-2%) or *RET* (0-0.1%)[17,103,110,155,156].

In the Netherlands, the incidence of head and neck paraganglioma and the percentage of paraganglioma patients with a positive family history appear to be disproportionately high, due to the prevalence of Dutch founder mutations in SDH genes[118,119]. The relative frequency of SDH mutations in the Netherlands and the genetics of Dutch paraganglioma patients are further evaluated in chapters 3 and 4.

3.4 Inheritance of head and neck paraganglioma syndromes

The inheritance pattern of paraganglioma syndrome differs considerably depending on the causative gene involved (Table 2). While TMEM127-, SDHB- and SDHC-linked paraganglioma families show normal autosomal dominant inheritance, SDHD and SDHAF2-linked families show a virtually exclusive paternal transmission of tumor susceptibility[115,116,132,133]. Whereas mutations in SDHD and SDHAF2 can be inherited both via the maternal and paternal lines, tumor formation following maternal transmission of a mutation is exceedingly uncommon[115-117]. The absence of maternal transmission of disease in SDHD-linked paraganglioma families is suggestive of maternal imprinting of the *SDHD* gene[115]. However, the actual blocking of transcription by methylation of the *SDHD* gene itself has never been demonstrated, and *SDHD* shows bi-allelic expression in non-paraganglioma tissue[114,157,158]. Recently, it has been hypothesized that tissue specific hypermethylation of a maternal allele flanking the SDHD gene causes the imprinted inheritance of SDHD-linked disease. This flanking element is presumed to encode an alternative promotor of a non-coding RNA sequence in the vicinity of the SDHD promotor on 11q23[158]. The function of this non-coding RNA sequence however is unknown, and evidence of a regulatory role in *SDHD* expression is lacking. Although differential methylation of its putative alternative promotor was found, differential expression of the non-coding RNA sequence, the predicted result of differential methylation of its alternative promotor, could not be established in the majority of cases (86%). Moreover, no allelic imbalance was found for *SDHD*. In contrast, the report identified bi-allelic expression of *SDHD* in all non-paraganglioma tissues including the adrenal gland, in accordance with previous reports, and it is therefore highly unlikely that the reported differential hypermethylation in the vicinity of *SDHD* actually

affects *SDHD* expression[115,158]. Furthermore, differential hypermethylation was not found in SDHD-linked paragangliomas, and it consequently does not seem to play a role in the inheritance of SDHD-linked disease. An additional argument against hypotheses involving maternal imprinting of *SDHD* itself, or a selective reduction of the expression of the maternal *SDHD* allele due to other epigenetic factors, is that it does not explain the loss of the wild type maternal *SDHD* allele that is observed in SDHD-linked paragangliomas and pheochromocytomas[114,158-160]. If the expression of the wild type allele was already significantly reduced by imprinting phenomena, its loss would not confer an increased predisposition to tumorigenesis. Furthermore, these models do not explain the similar exclusive paternal transmission of disease observed in SDHAF2-linked cases[132,133].

A decisive factor in the parent-of-origin-dependent inheritance of SDHD- and SDHAF2-linked disease seems to be the location of *SDHD* and *SDHAF2* on chromosome 11. Both *SDHD* and *SDHAF2* are located on the long arm of chromosome 11 (on 11q23 and 11q13 respectively), while *TMEM127* (2q11), *SDHB* (1p35-36) and *SDHC* (1q23), genes that do not show a parent-of-origin effect, are not.

Although the *SDHD* and *SDHAF2* genes are not imprinted themselves, chromosome 11 harbors the main cluster of imprinted genes of the human genome, on its short arm at 11p15.5. This region consists of a telomeric and a centromeric imprinted domain, both containing putative tumor suppressor genes. This suggests a model in which a maternally expressed, paternally imprinted gene, located within this imprinted 11p15.5 region, is an essential initiator or modifier of tumor development in SDHD- and SDHAF2-linked paraganglioma syndromes[114]. According to this model, tumor formation is not initiated upon loss of the wild type *SDHAF2* or *SDHD* allele alone, but only upon the combined loss of the wild type *SDHAF2* or *SDHD* allele on the long arm of chromosome 11 and the active maternal tumor suppressor allele located within the imprinted 11p15.5 region. In case of a paternally inherited mutation in *SDHAF2* or *SDHD*, this can be achieved in a single event, i.e. the somatic loss of the whole maternal copy of chromosome 11 (figure 6). The infrequent maternal transmission of tumor susceptibly in SDHAF2 and SDHD-linked families would then be explained by the fact that it takes at least two separate events to eliminate both the paternal wild type *SDHAF2* or *SDHD* allele on the long arm of chromosome 11 and the active maternal copy of a paternally imprinted tumor suppressor gene on the 11p15.5 region (figure 6)[114]. In support of this model, multiple studies have found evidence for LOH targeting the 11p15 region and for the selective loss of maternal chromosome 11 alleles in SDHD-linked paragangliomas and pheochromocytomas[114,158,159,161].

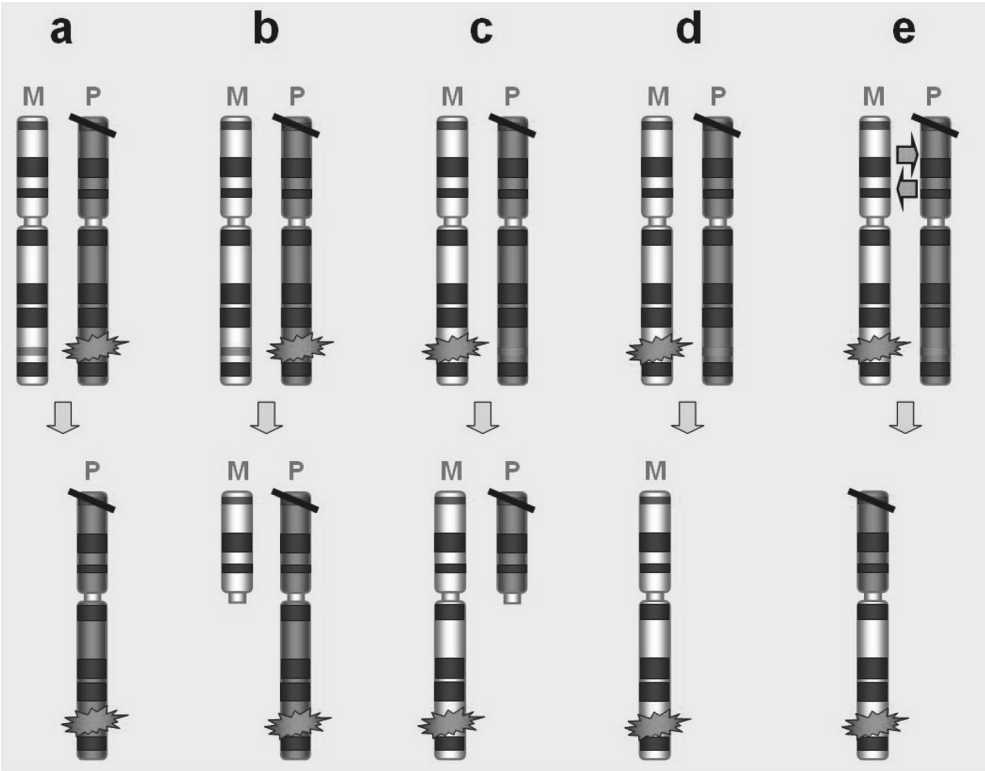


Figure 6. Model for the parent-of-origin-dependent transmission of SDHD-linked paraganglioma syndrome. The model is applicable to SDHAF2-linked disease as well. Maternal (M) and paternal (P) chromosome 11 copies are represented with the wild type (green band) and mutant (green star) *SDHD* alleles located on the long arm (11q23), and the active allele (red band) and imprinted allele (red band struck through) of a tumor suppressor gene on the short arm (11p15.5). (**a**) *SDHD* mutation inherited via the father. Loss of the whole maternal chromosome 11 copy targets both the wild type *SDHD* allele and the maternal 11p region containing the active tumor suppressor allele in a single event, resulting in tumor formation. (**b**) *SDHD* mutation inherited via the father. In case of loss of heterozygosity (LOH) targeting only the wild type maternal *SDHD* allele on 11q, the active maternal tumor suppressor allele on 11p15.5 is not affected and tumor development is inhibited. In case of maternal inheritance of the *SDHD* mutation, a second hit targeting the wild type paternal allele by, for example, a deletion of the paternal 11q region (**c**) or even the whole paternal chromosome 11 (**d**) will leave the maternal 11p15.5 region intact and tumor formation is not initiated. When the *SDHD* mutation is maternally transmitted, at least two separate events are required to inactivate both the wild type *SDHD* allele and the active allele of the imprinted tumor suppressor gene on 11p15.5. To date, true maternal transmission of SDHD-linked disease has been found in association with loss or disruption targeting both the wild type paternal *SDHD* allele and the maternal chromosome 11p15.5 region, either by two separate LOH events (reported by Tobias et al.), or by an altered imprinting status at 11p15.5 in combination with loss of the wild type paternal *SDHD* allele (described by Pigny et al.), or (**e**) through a recombination on chromosome 11 followed by loss of the wild type paternal *SDHD* allele and maternal 11p15.5 region (observed by Bayley et al.). Apparently, these sequences of events occur very rarely in vivo.

To date, very few cases of true maternal transmission of paragangliomas associated with *SDHD* mutations have been reported. Pigny et al. have described a patient that developed a tympanic paraganglioma after inheriting a *SDHD* mutation via his mother. Unlike his unaffected family members, he had also acquired an altered methylation profile and therefore probably an altered imprinted status of *H19*, a known paternally imprinted tumor suppressor on 11p15.5[117,162]. However, in this case the diagnosis of paraganglioma was not confirmed by histopathology, and loss of the paternal *SDHD* allele or alterations in H19 expression could not be evaluated in the tumor tissue[114,117,163]. The second report of maternal transmission identified a pheochromocytoma in a maternally derived SDHD mutation carrier, after two separate LOH events had resulted in loss of the paternal wild type SDHD allele and loss of the maternal 11p15.5 region in the tumor[164]. A third case of maternal transmission of SDHD-linked disease was observed by Bayley and co-workers (presented in 2011 by Bayley et al. at the International Symposium of Phaeochromocytoma and Paraganglioma, Paris, France). In this case, it was demonstrated that an entire copy of chromosome 11 was lost after somatic recombination on chromosome 11, resulting in loss of the paternal wild type *SDHD* allele and the maternal 11p15.5 region in the patients' pheochromocytoma (figure 6). These rare observations, or 'exceptions to the rule', are all consistent with the proposed model, and point to the maternal 11p15.5 region as an essential additional factor in paraganglioma formation. The model explaining the parent-of-origin dependent inheritance of SDHD- and SDHAF2-linked paraganglioma syndrome is presented in chapter 7.

Altered expression of 11p15.5 imprinted genes, especially *H19* and insulin-like growth factor 2 (*IGF2*), has been linked to other tumors and tumor syndromes, such as the Beckwith-Wiedemann syndrome (BWS), a pediatric developmental disease characterized by overgrowth, organomegaly, and a predisposition for various benign and malignant tumors, including, interestingly, pheochromocytomas[165,166]. LOH of 11p15.5 region is also observed in nephroblastoma, embryonal rhabdomyosarcoma, hepatoblastoma and adrenal cortical carcinoma, and in these tumors too, it has been shown that the LOH specifically targets maternal alleles[167]. In VHL-related pheochromocytomas, loss of maternal 11p15.5 alleles is also frequently observed, suggesting that the maternal 11p15.5 region has an important role in the tumorigenesis of paraganglioma-pheochromocytoma syndromes irrespective of the causative gene[168-170]. Moreover, the parent-of-origin-dependent inheritance described in focal hyperplasia of Langerhans islets causing congenital hyperinsulinism (FoCHI), a disease caused by genes that like *SDHD* and *SDHAF2* are located outside of the imprinted region on chromosome 11, and its association with altered expression of 11p15.5 imprinted genes, suggest that this model for parent-of-origin dependent inheritance of disease susceptibility may have implications beyond the spectrum of paraganglioma-pheochromocytoma syndromes[171].

4. Molecular biology of paragangliomas

In recent years, great progress has been made in elucidating the processes that lead from gene mutations to paraganglioma formation. Especially since the discovery of the SDH genes in hereditary paraganglioma syndrome, the understanding of the role of the cellular metabolism and hypoxia in tumor formation has evolved. The genes that cause paraganglioma formation, and the processes they regulate, are diverse and several pathways may be implicated in paraganglioma tumorigenesis. In this paragraph, the current models and insights in the tumor biology of paragangliomas are discussed. These models are not mutually exclusive, probably multiple mechanisms interact, and the relative role of each of these mechanisms in paraganglioma formation is not yet defined.

4.1 Succinate dehydrogenase

Most genes currently known to cause hereditary paraganglioma syndrome, *SDHAF2*, *SDHB*, *SDHC*, *SDHD*, and the recently implicated *SDHA* gene, encode subunits or co-factors of succinate dehydrogenase (SDH). SDH is an enzyme anchored to the inner membrane of mitochondria, which couples the oxidation of succinate to fumarate in the tricarboxylic acid cycle (TCA cycle, also known as Krebs cycle), with the transfer of electrons as the complex II component of the electron transport chain. It thus connects the TCA cycle with the mitochondrial respiratory chain, which places SDH at the center of two of the essential energy producing processes of the cell. SDH consists of a catalytic domain formed by the SDHA flavoprotein, which is involved in succinate binding and oxidation, the SDHB iron-sulfur protein, which is involved in the electron transfer, and a membrane-anchoring domain formed by the hydrophobic SDHC and SDHD subunits that also play a role in passing electrons through the electron transport chain (figure 7). *SDHAF2* encodes a protein that is involved in the incorporation of the flavin-adenine-dinucleotide (FAD) group into the SDHA subunit.

It has been demonstrated that mutations in each of these subunits or co-factors result in compromise of enzymatic function of the SDH complex[132,138,172-174]. The loss of SDH function is thought to interfere both with the TCA cycle as well as the mitochondrial respiratory chain, and it is therefore not readily apparent how defects in SDH can initiate such an energy draining process as tumor formation (see paragraph 4.2: ‘the Warburg hypothesis’)[175].

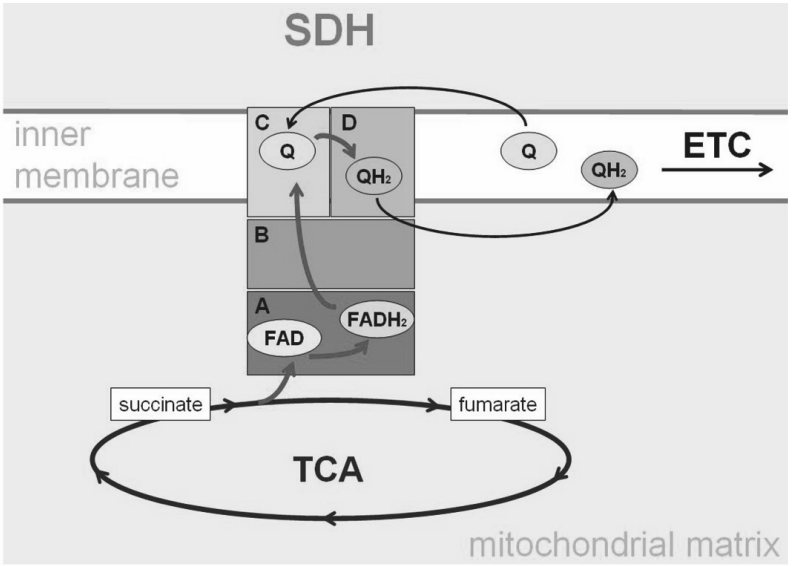


Figure 7. Schematic representation of the succinate dehydrogenase (SDH) complex, or mitochondrial complex II, and its dual role in the tricarboxylic acid cycle (TCA, blue circle) and the electron transport chain (ETC, black arrow). SDH consists of four subunits, SDHA (A), SDHB (B), SDHC (C) and SDHD (D). SDHA and SDHB form its catalytic domain, while SDHC and SDHD anchor the complex to the inner membrane of the mitochondrion. The electron flow within complex II is depicted by the red arrows. During the oxidation of succinate to fumarate by SDHA, its FAD group is reduced to FADH2 by two electrons. The electrons are then transferred through the iron–sulphur groups in SDHB to SDHC and SDHD, where ubiquinone (Q), bound to the mitochondrial inner membrane, is reduced to ubiquinol (QH2). Ubiquinol transfers its electrons to complex III, further in the electron transport chain (not shown).

4.2 The Warburg hypothesis

Already in 1926, the biochemist Otto Warburg postulated that cancer was caused by defects in the oxidative phosphorylation within mitochondria, after observing that cancer cells display high rates of glycolysis even in aerobic conditions, an effect he named 'aerobic glycolysis'. This metabolic shift, known as the 'Warburg effect', has since been recognized as a feature of many cancer types. The concept that disruptions in the mitochondrial respiration can actually cause cancer has long been controversial however (see paragraph 5.3: 'the Warburg controversy'). Today, the discovery of mutations in SDH subunits as a cause of paragangliomas and pheochromocytomas serves as a clear example of the tumorigenic potential of deficient TCA cycle components.

The molecular basis for the initiation of tumor growth by defective SDH has not been fully elucidated. Currently, there are three main models that link SDH disruption with

neoplastic growth: an increase in the formation of reactive oxygen species, a decrease in apoptosis or programmed cell death, and an activation of hypoxia pathway signaling under normoxic conditions (or 'pseudo-hypoxic drive')[175-177].

4.2.1 Reactive oxygen species

Reactive oxygen species (ROS) are highly reactive molecules containing oxygen. ROS are produced by the electron transport chain, predominantly at the site of complex I and complex III, as electrons are transferred to oxygen molecules. Although ROS production does not seem to take place at complex II (SDH) under physiological conditions, mutations in SDH subunits have been associated with increased levels of ROS[175,178,179].

It is currently unknown how mutations in SDH subunits lead to ROS production, but there are several hypotheses. First, mutant SDH might not conduct the electrons that are released in the process of the oxidation of succinate, resulting in a buildup of electrons at the FAD site of the SDHA subunit (figure 7). When sufficient electrons have accumulated, this site could overflow, leading to a direct transfer of electrons to oxygen, thus creating ROS[175,176,180]. Alternatively, the oxidation of succinate to fumarate may be reversed at the SDH complex under hypoxic conditions, generating ROS in the process[175]. Both hypotheses involve an active catalytic domain of SDH, whereas it has been shown that SDH mutations disrupt its enzyme activity[172,176]. A third hypothesis states that the SDH complex has a role in reducing the levels of ROS produced by other electron transfer chain components using its heme group, and that mutations in SDH interfere with this functionality[180,181].

The actions of ROS are diverse and in the light of tumorigenesis seemingly contradictory. On the one hand, ROS are known initiators of apoptosis[175]. On the other hand, the production of ROS can induce neoplastic growth by causing direct damage to the DNA, including strand breaks, cross-links and base modifications which may result in replication errors, altered gene expression, genomic amplification or LOH[175,182]. In addition, increased ROS production is associated with increased hypoxia pathway signaling and hypoxia-induced transcription, processes that have been shown to play a key role in paraganglioma tumorigenesis (see paragraph 4.2.3: 'pseudo-hypoxic drive')[173,183].

In paragangliomas, excess ROS production might be the result of defects in SDH caused by mutations in SDH genes and subsequent LOH of wild type SDH alleles. Alternatively, ROS production may already be increased in heterozygous SDH mutation carriers that have not yet lost the wild type SDH allele, and the high levels of ROS might drive the LOH of SDH genes. Whether or not heterozygous SDH mutations cause ROS excess is still a matter of

debate. Interestingly, heterozygous *Sdhd* mutant mice exhibit slight changes in carotid body physiology that may implicate increased levels of ROS (see paragraph 4.9: 'insights from mouse models')[43,184].

4.2.2 Evasion of apoptosis

In addition to their role in the energy supply of the cell, mitochondria have a central role in programmed cell death or apoptosis. Apoptosis may be induced by increasing the permeability of the mitochondrial membrane, resulting in the release of apoptogenic proteins such as cytochrome-c into the cytosol, or by interfering with the bioenergetic processes within the mitochondria. Evasion of apoptosis is one of the acquired hallmarks of neoplastic growth[185]. Several mechanisms may cause insensitivity to apoptotic signals in paragangliomas. First, in SDH mutant paragangliomas, SDH deficiency causes the oxidative phosphorylation to operate at suboptimal levels. This causes an induction of glycolysis as the alternative energy producing pathway, through upregulation of glycolytic enzymes such as hexokinase and glycerylaldehyde-3-posphatase (GAPD). Hexokinase and GAPD are proteins that are implicated in the regulation of diverse other cellular processes, including apoptosis, and the induction of glycolysis could thus have an anti-apoptotic effect[175,186]. Alternatively, SDH deficiency causes an accumulation of succinate (see paragraph 4.2.3: 'the hypoxia pathway'), which in turn can inhibit the pro-apoptotic activity of prolyl-hydroxylase 3 (PHD3) (see paragraph 4.3: 'abnormal development of neuronal precursor cells')[150,176,187].

A third link between SDH and apoptosis that has been put forward, is a decrease rather than an increase in ROS production under hypoxic conditions due to defects in SDH, which would interfere with the pro-apoptotic signal transduction by ROS in mitochondria[188;189].

A fourth mechanism involves the B-cell lymphoma 2 (BCL-2) family of apoptosis regulating proteins. Two of its members, BCL-2 and BCL-xl, have been shown to counteract the pro-apoptotic signaling by ROS and hypoxia, and prevent the release of cytochrome-c from the mitochondrial membrane[190-192]. The upregulation of BCL-2 and BCL-xl is a known response to hypoxia and has been observed in paragangliomas[193-195]. Another member of the BCL-2 family, BCL-2 interacting protein 3 (BNIP3), acts as a promoter of apoptosis, and has been shown to be repressed in certain SDHB-linked tumors[196].

There are several observations that implicate blocked apoptosis in SDH-linked paraganglioma tumorigenesis. Douwes Dekker et al. found very few morphological stages of apoptosis and no DNA strand breaks suggesting a reduced apoptotic activity in paragangliomas[193]. In addition, it has been shown that whereas short periods of

SDH deficiency can induce apoptosis, prolonged SDH deficiency can result in an absent apoptotic response and induction of tumorigenesis[178,179]. Further evidence for the role of SDH in apoptosis comes from the study of new possible anti-cancer agents such as vitamin E analogs, that are able to induce apoptosis only in the presence of functional SDH and not in SDH deficient cells[197].

4.2.3 The hypoxia pathway

Long before the identification of any of the genes now known to play a role in paragangliomas, it was recognized that living at high altitude increases the risk of carotid body hyperplasia and carotid body tumors[198,199]. In addition, carotid body hyperplasia also occurs in patients suffering from cystic fibrosis or cyanotic heart disease, conditions associated with compromised gas exchange in the lungs[200]. This increased prevalence of paragangliomas in conditions characterized by low oxygen levels and the central role of the carotid body in oxygen sensing suggested that hypoxia or defects in the oxygen sensing mechanism play a role in the tumorigenesis of paragangliomas.

Oxygen sensing at the carotid body

Corneille J.F. Heymans was the first to demonstrate the role of the carotid body as a peripheral arterial chemoreceptor and regulator of respiration and oxygen homeostasis (see paragraph 5.2: 'the discovery of the carotid body function')[5]. Oxygen sensing takes place in the type 1 or chief cells, primarily within the carotid body[201]. Type 1 cells are polymodal chemoreceptors that are sensitive not only to low oxygen, but also to carbon dioxide, extracellular pH, and glucose levels, however the oxygen sensing ability is what makes the carotid body essential in the adaptive hyperventilatory reflex response[200]. Hypoxia elicits the release of two classes of neurotransmitters by the chief cells: conventional neurotransmitters such as acetylcholine, catecholamines, substance P and adenosine triposphate (ATP), and unconventional neurotransmitters such as nitric oxide (NO) and carbon monoxide (CO)[43,202]. Acetylcholine and ATP in particular seem to be responsible for the excitation of afferent endings of the carotid sinus nerve and to increase impulse traffic to the brain stem, thus regulating the hypoxic ventilatory response[43,200,202].

Acute hypoxia response

There are two main models for carotid body oxygen sensing: the 'membrane model', designating potassium channels as the initiators of the response to hypoxia, and the 'mitochondrial model', which involves heme containing proteins like nitric oxide synthetase (NOS), hemoxygenase 2 (HO-2), NADPH oxidase, and/or the mitochondrial complexes such as SDH as the main hypoxia responsive elements[43,202]. Thus far, the identification of

one specific compound as the central oxygen sensor remains elusive. Several interacting molecular mechanisms are believed to be involved but the full complexity of the process is not yet entirely understood.

Central to the membrane model of oxygen sensing is the concept that hypoxia induces an altered conductance of potassium channels located in the membrane of type 1 cells, which causes depolarization and the influx of calcium in the cytosol, triggering the release of neurotransmitters[43,200,202]. There are several pathways that might link hypoxia to potassium channel function and probably different types of potassium channels are involved. It has been hypothesized that low oxygen levels could directly alter the open probability of potassium or calcium channels in type 1 cells, causing depolarization and transmitter release[43,201]. Alternatively, potassium channels can be affected by increased levels of ROS or decreased levels of ATP as a result of hypoxia (see below)[202].

In the mitochondrial model, heme containing compounds such as NOS, HO-2, NADPH oxidase, and/or the mitochondrial complexes such as SDH are viewed as the primary starting-point of the ventilatory response to hypoxia. This response may be regulated by NOS through the production of nitric oxide (NO), which may play a role in suppressing sensory discharge in type I cells under normoxic conditions, and may have modulating effects on hypoxia induced neurotransmitters[201]. A reduced activity of NOS results in low levels of NO, relieving its inhibitory effect on type 1 cells[201]. Whether or not NOS activity and NO levels are actually altered by hypoxia within type 1 cells is yet unclear, but NO produced in nearby nerve terminals might also exert this modulating effect[43,202].

Hemoxygenase 2 could be involved in oxygen sensing through the regulation of carbon monoxide (CO) levels. Hemoxygenase 2 is capable of endogenous production of CO in type 1 cells, in a process that is oxygen dependent. Endogenous CO is thought to exert an inhibitory influence on carotid body function, but the effects of CO on oxygen sensing seem to be of a dual nature: high concentrations of CO inhibit NOS activity and augment sensory discharge in type 1 cells (see above)[201-203].

The mechanism linking the non-mitochondrial enzyme NADPH oxidase or the mitochondrial electron transport chain complexes, including SDH, to oxygen sensing in the carotid body involves the production of ROS by these enzymes as a function of the amount of oxygen available to the cell. Low concentrations of oxygen result in decreased levels of ROS, which would in turn alter the open probability of the potassium channels[43,201]. In addition, mitochondrial complexes could alter the conductance of ATP-dependent potassium

channels, causing depolarization and transmitter release, through a reduced production of ATP in response to low oxygen levels[43,201].

Chronic hypoxia

Chronic hypoxia, i.e. an exposure to hypoxic conditions lasting several hours or longer, induces a number of morphological, electrochemical and physiological adaptations that increase the responsiveness of oxygen sensing mechanisms, promote oxygen delivery to tissues, and adjust the cellular metabolism to limited oxygen availability[43,202]. Most of these effects are regulated through hypoxia inducible factor 1 (HIF-1). HIF-1 is a heterodimeric transcription factor composed of the HIF-1α and HIF-1β subunits. HIF-1β is constitutively expressed, whereas HIF-1α levels increase exponentially as oxygen levels decrease. Under normoxic conditions, HIF-1α levels are reduced, primarily by the activity of prolyl-hydroxylases (PHDs) 1, 2 and 3, which modify HIF-1α so that it can be ubiquitinated by a complex consisting of the Von Hippel-Lindau protein (pVHL) and an E3 ubiquitin-protein ligase, after which it is targeted for proteasomal degradation. Under hypoxic conditions, HIF-1α degradation by PHDs is inhibited either by deprivation of oxygen (a substrate of the PHDs), or as a result of oxidation of the iron group within the PHDs by ROS, resulting in accumulation of HIF-1α. Subsequently, HIF-1α translocates to the nucleus and dimerizes with the HIF-1β subunit, forming the HIF-1 transcription factor[204,205]. HIF-1 binds to hypoxia response elements (HREs) and activates the transcription of a large number of genes that are involved in cell proliferation, survival, apoptosis, glucose transport and metabolism, angiogenesis, cytoskeletal structure and motility, and extracellular matrix metabolism (see also chapter 6)[205-207].

Two other isoforms of HIF-1α exist: HIF-2α and HIF-3α. HIF-2α is a protein with extensive similarity to HIF-1α, its degradation is also regulated in an oxygen-dependent way by PHDs, it also dimerizes with HIF-1β, and it regulates the transcription of an overlapping, but not identical set of genes. HIF-3α is transcriptionally regulated by HIF-1 and acts as an inhibitor of HIF-1[205,206].

The tumorigenic effects of hypoxia

Both HIF-1α and HIF-2α overexpression is associated with the development and behavior of a large variety of neoplasms. It has been shown to increase resistance to chemotherapy, radiotherapy and photodynamic therapy, and to promote tumor growth, vascularization, metastasis, and mortality in melanomas, oligodendromas, astrocytomas, and multiple types of carcinoma[183,206]. The effects of HIF-1α and HIF-2α overexpression are not universal however, and the biological consequences of HIF-1 activation depend on the specific subset of genes that responds[183,206]. The HIF-1 activation can be induced by

intratumoral hypoxia in areas distal to blood vessels and on the border of necrotic cells, or by genetic alterations to hypoxia pathway components (see paragraph 4.2.3: 'pseudo hypoxic drive')[183,205].

Pseudo-hypoxic drive

In some tumors, the tumorigenic effects of hypoxia are induced under normoxic conditions, an effect known as the 'pseudo-hypoxic drive'. This pseudo-hypoxic drive can be caused by genetic mutations that affect pathways regulating the transcriptional activity HIF-1 in an oxygen-independent way[204]. In SDH-linked tumors, there are several possible routes linking the activation of HIF-1 with SDH deficiency (figure 8). The first involves the production of ROS as a result of SDH disruption (see paragraph 4.2.1: 'reactive oxygen species'). When sufficient amounts of ROS accumulate, ROS can oxidize the iron group within PHDs, thereby decreasing PHD activity and increasing HIF-1α (and HIF-2α) stability. As explained above, blocking of HIF-1α degradation will lead to increased HIF-1 mediated transcription and activation of the hypoxia pathway (figure 8)[175]. In addition, there is evidence that ROS can increase HIF-1 stability directly (figure 8)[208]. The second route is through the accumulation of succinate, the substrate of SDH in the TCA cycle. In the cytosol, succinate is also present as a product of the conversion of α-ketoglutarate by PHDs. The accumulation of succinate in the mitochondrion as a result of SDH deficiency, and the subsequent transport of excess succinate to the cytosol, leads to high levels of cytosolic succinate, which prevents the forward hydroxylation of HIF-1α by PHDs, resulting in increased HIF-1 activity (figure 8)[175,209,210].

An alternative pathway linking SDH deficiency with HIF-1 activity is through the reduced activity of other hydroxylases, such as the factor inhibiting HIF (FIH). FIH reduces HIF-1 mediated transcription by preventing the recruitment of co-activators[205,211,212]. Hydroxylation of HIF by FIH, like hydroxylation of HIF by PHDs, requires the co-factors iron and oxygen and α-ketoglutarate as a co-substrate, and FIH activity can be blocked by ROS and high levels of succinate in the same way as PHD activity (see above), resulting in increased HIF-1 transcription[212].

There is ample evidence implicating the hypoxia pathway in paraganglioma tumorigenesis. First, HIF-1α and HIF-2α stabilization, the accumulation of succinate, and its inhibitory effect on PHDs have all been demonstrated in SDH deficient cells and paragangliomas[209,210]. Second, the fact that paragangliomas and pheochromocytomas can be caused by mutations in SDH subunits as well as by mutations in VHL and PHD2, points towards the hypoxia pathway in paraganglioma tumorigenesis because of the role that SDH, VHL and PHD2 all have in the stabilization of HIF-1α and HIF-2α[213]. Third, HIF-1

regulated genes have been shown to be overexpressed in SDH-linked paragangliomas and pheochromocytomas, and this is a very plausible explanation for some of the clinical characteristics of paragangliomas, such as their typical high vascularity (see paragraph 4.4: 'angiogenesis')[173,213].

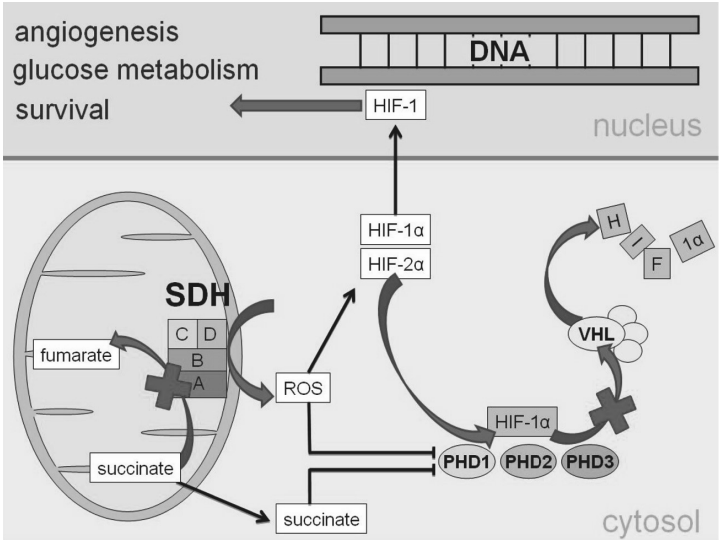


Figure 8. Schematic representation of the pseudo-hypoxic drive. Defects in succinate dehydrogenase (SDH), prolyl-hydroxylase 2 (PHD2) and the Von Hippel-Lindau protein (pVHL) all inhibit the degradation of hypoxia-inducible factors HIF-1α and HIF-2α. The HIF alpha subunits subsequently combine with HIF-1β to form the transcription factor HIF-1. HIF-1 activates the transcription of a multitude of genes involved in glucose metabolism, angiogenesis, survival, cell motility and extracellular matrix metabolism. The stabilization of HIF-1α and HIF-2α subunits can be induced in several hypoxia-independent ways: defects in SDH interfere with the oxidation of succinate to fumarate in the TCA cycle, resulting in accumulation of succinate. Succinate is transported to the cytosol, where it blocks the activity of PHD 1, 2, and 3 by product inhibition. Alternatively, defects in SDH result in the production of reactive oxygen species (ROS), which disable PHDs by oxidizing the iron group within, or may contribute to the stability of HIF-1α directly. Mutations in PHD2 can affect its ability to catalyze the hydroxylation of HIF-1α. Mutations in the Von Hippel-Lindau protein (pVHL) disrupt HIF-1α ubiquitination.

4.3 Abnormal development of neuronal precursor cells

An alternative hypothesis for the development of paragangliomas and pheochromocytomas explains their tumorigenesis not primarily by defects in the cell metabolism or pseudo-hypoxia, but through the faulty development of sympathetic neuronal precursor cells that give rise to the sympathetic nervous system as well as to the paraganglionic type 1 cells. During normal development, damaged or unneeded precursor cells originating from

the neural crest are disposed of by apoptosis in a process called 'developmental culling'. This process is regulated by growth factors, most notably nerve growth factor (NGF) through a pathway involving the c-Jun protein and prolyl-hydroxylase 3 (PHD3, encoded by *EGLN3*) (figure 9). Under normal circumstances, the precursor cells undergo apoptosis when NGF becomes limiting, but it has been demonstrated that pheochromocytoma cells might escape the developmental culling by blocking apoptosis through the c-Jun/PHD3 pathway[150]. This model for paraganglioma and pheochromocytoma development is attractive because the SDH genes, *NF1*, *RET*, *VHL*, *KIF1B* and *MAX* can all be linked to this essential mechanism in the development of the paraganglion system (figure 9)[149,151]. Moreover, there is some evidence from knockout mouse models that associates the developmental stages of the nervous system with pheochromocytoma formation (see paragraph 4.5: 'insights from mouse models'). However, the hypothesis implies that the second hit targeting the wild type allele of these tumor suppressor genes occurs very early in life, or that heterozygous mutations already exert an inhibitory effect on this pathway. In case of SDH mutations, there is currently no evidence to support that heterozygous SDH mutations result in succinate accumulation, and it is therefore uncertain whether heterozygous SDH mutations can induce aberrant survival of neuronal precursor cells (figure 9).

4.4 Other mechanisms in paraganglioma tumorigenesis

Cell cycle arrest

The process of the replication of cells, called the cell cycle, comprises of multiple phases: the G1- or growth phase, the S- or DNA replication phase, the G2 phase in which microtubules are formed, and finally, the M- or mitotic phase in which the actual division of nuclear DNA takes place. Each transition into the next phase is guarded by a checkpoint that is very tightly regulated through multiple complex mechanisms. In order to attain uncontrolled proliferation, a hallmark of cancer, the neoplastic cell must evade these checkpoints, and in most forms of cancer the mitotic rate, i.e. the proportion of replicating cells, is high. In paragangliomas however, a very low mitotic rate has been observed, and a large number of cells seem to be stranded in the G2 phase of the cell cycle, indicating that some cell cycle regulation is still operative[193]. The low mitotic rate might explain the indolent behavior and slow growth that characterizes most paragangliomas. Cell cycle arrest can be caused by hypoxia, through upregulation of tumor protein 53 (p53), one of the most well-known regulators of the cell cycle and apoptosis, which regulates the G2/M checkpoint through a complex cascade. Mutations in p53 confer a growth advantage as the cell is less able to respond to hypoxia or DNA damage with cell cycle arrest and apoptosis, and p53 mutations are implicated in a vast array of neoplasms, but are an infrequent finding in paragangliomas[193,214]. It is therefore conceivable that the cell cycle arrest

observed in paragangliomas is the result of the intact response of p53 to pseudo-hypoxia caused by SDH deficiency, however, p53 overexpression also is not a characteristic of most paragangliomas[193,214].

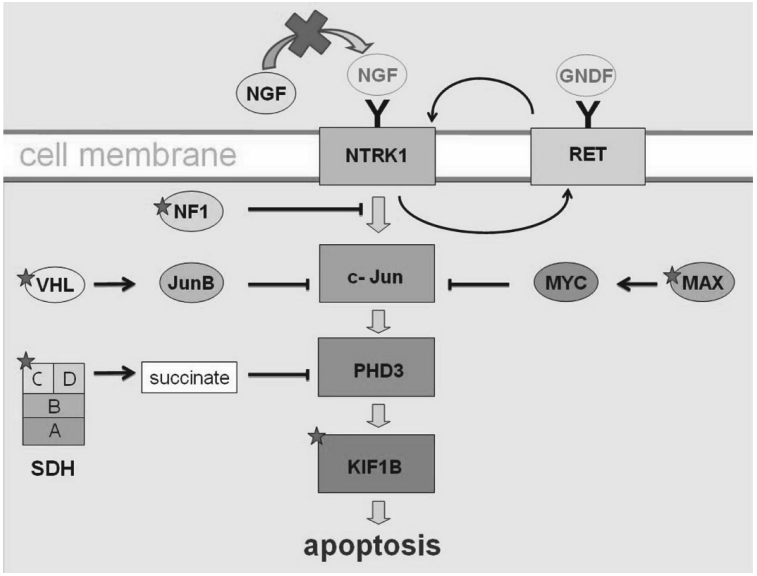


Figure 9. Almost all paraganglioma and pheochromocytoma genes converge on the pro-apoptotic pathway that is involved in the developmental culling of neuronal precursor cells. NTRK1, the receptor for nerve growth factor (NGF), collaborates with the RET tyrosine kinase receptor (RET) to regulate NGF and glial cell line- derived neurotrophic factor (GDNF) signals. Loss of NGF leads to apoptosis under normal conditions. Mutations (red stars) in neurofibromin 1 (NF1) interfere with downstream signaling by the NTRK1 receptor. Mutations in the Von Hippel-Lindau protein (VHL) cause induction of the JunB protein, which in turn antagonizes the pro-apoptotic activity of c-Jun. The upregulation of c-Jun can also be blocked by mutations in MYC associated factor X (MAX) through altered activity of myelocytomatosis viral oncogene homolog (MYC). The hydroxylation activity of prolyl-hydroxylase 3 (PHD3), which is transcriptionally activated by c-Jun, can be blocked through product inhibition by increased levels of cytosolic succinate. The accumulation of succinate can be caused by disrupted enzymatic activity of succinate dehydrogenase (SDH) due to mutations in the SDH subunits. Kinesin family member 1B (KIF1B) regulates apoptosis downstream from PHD3, and KIF1B mutations may therefore also result in blunting of the pro-apoptotic signal through this pathway.

An alternative pathway that links pseudo-hypoxia with cell cycle arrest is through the upregulation of cyclin-dependent kinase inhibitor 1 (CDKN1A or p21) by HIF-1. The p21 protein is regulated by p57, but is also a transcriptional target of HIF-1[207;215]. The p21 protein is capable of inducing cell cycle arrest in G1/S- and G2/M transitions, and has been shown to be expressed in paragangliomas[193,215]. Interestingly, prolonged G2/M

arrest through induction of p21 is also associated with polyploidisation, another feature frequently encountered in the nuclei of type 1 paraganglioma cells[114,193,215].

Whereas p21 and p57 have been studied in paragangliomas, many other pathways that regulate the cell cycle have yet to be investigated, and the exact mechanism of cell cycle arrest in paragangliomas remains to be elucidated[193,214].

Angiogenesis

Paragangliomas are characterized by a highly vascular stroma and close relations to adjacent vascular structures. Histologically, the 'Zellballen' clusters of type 1 and type 2 cells are surrounded by a very prominent capillary network[4,216]. The mechanism by which tumors induce the sprouting and development of blood vessels from existing vasculature, called angiogenesis, is an essential step in neoplastic growth and progression, as the expanding tumor requires increasing amounts of nutrients and oxygen to sustain itself[212,217]. One of the critical triggers of angiogenesis is hypoxia within a tumor, and virtually all of the central mediators in the process, such as vascular endothelial growth factor (VEGF), platelet derived growth factor b (PDGFB), stromal derived factor 1 (SDF1), angiopoietin 2 (ANGPT2), fibroblast growth factor 2 (FGF2), and important receptors are regulated through the hypoxia pathway by HIF-1 (see also chapter 6)[205-207,218]. Both the stabilization of HIF-1α and HIF-2α lead to the expression of angiogenic factors, but there is evidence that HIF-2α has a more prominent role in angiogenesis[212]. In paragangliomas, angiogenesis could be initiated by pseudo-hypoxia signaling (see paragraph 4.2.3: 'pseudo-hypoxic drive'). HIF-1α, HIF-2α and various HIF-1 target genes involved in angiogenesis such as FGF2, VEGF, and VEGF receptor 1, have indeed been shown to be upregulated in type 1 cells of paragangliomas and pheochromocytomas, indicating that the pseudo-hypoxic drive is responsible for the vascular nature of these tumors[196,219,220].

Proliferative pathways

The recent discovery of TMEM127 and MAX as a tumor suppressors in hereditary paraganglioma and pheochromocytoma syndromes, and their roles as regulators of the mTOR pathway and the MYC-MAX-MXD1 family of transcription factors respectively, indicate that HIF-independent proliferative pathways may also play a role in paraganglioma formation (see paragraph 3.3: 'other genes in paragangliomas and pheochromocytomas') [143,151]. Gene expression studies have indicated that pheochromocytomas may be classified into two broad categories: one consisting of VHL and SDH-related tumors, and another consisting of NF1 and RET-related tumors (see paragraphs 3.1 and 3.2)[213]. The VHL/SDH associated tumors are characterized by an expression signature of angiogenesis,

hypoxia, and a suppression of the mitochondrial oxidative response and TCA cycle components, consistent with their role in HIF-1 regulated transcription (see paragraph 4.2.3: 'pseudo-hypoxic drive'). The NF1/RET associated tumors are characterized by upregulation of a distinct set of biological programs, including translation, protein synthesis, and kinase signaling, in agreement with their respective gene functions (see paragraph 3.1: NF1, RET, and VHL) [213]. The transcriptional profile of TMEM127-related tumors shows similarities with the NF1/RET cluster, and mTOR signaling has been shown to be important in NF1 associated tumorigenesis as well[213,221]. However, whereas TMEM127-linked tumors are associated with the mTOR signaling pathway, NF1 and RET-linked tumors are more closely associated with the mitogen-activated protein kinase (MAPK) pathway[143,213]. MAX has been linked to both the mTOR and MAPK pathways through the MYC-MAX-MXN1 network[152,222].

The MAPK and mTOR pathways are highly complex, and regulate a multitude of cellular processes including transcription, cell proliferation and survival, and both pathways have been implicated in various forms of cancer[223,224]. Here, the MAPK and mTOR pathways will not be discussed in detail, but some interesting links to the hypoxia pathway exist. Like the hypoxia pathway, both MAPK and mTOR signaling can be induced by hypoxia and oxidative stress, both MAPK and mTOR signaling can be induced by growth factors that are known HIF-1 target genes, and both MAPK and mTOR pathways have a regulating effect on HIF-1 transcriptional activity (and vice versa), illustrating the intricacy and interdependence of the different pathways that may lead to paraganglioma tumorigenesis[205,218,223-225].

4.5 Insights from mouse models

Several studies have reported on the effects of inactivation of genes associated with paragangliomas and pheochromocytomas in genetically modified mice (knockout mouse models). Whereas paraganglioma development is extremely rare in mice, life-span studies of laboratory mouse strains report a risk of 0-5% for the development of pheochromocytomas, and a higher prevalence in several genetically engineered strains[226]. Mice are the most widely used species for the knockout technique, because they are the closest related animal species in which the technique can be applied with relative ease. Mouse models can be used to study the effect of specific gene mutations and evaluate determinants of tumor behavior. The available models for the paraganglioma and pheochromocytoma genes that have been discussed in former paragraphs will be briefly evaluated here.

Sdhd

Several studies have demonstrated that homozygous disruptions of the *Sdhd* gene (*Sdhd* -/-) in mice invariably result in mortality early in embryogenesis[184,227,228]. In heterozygous *Sdhd* knockout mice (*Sdhd* +/-), the response of the carotid body to acute hypoxia remains largely intact, although a higher rest-excitability and basal catecholamine release has been demonstrated, probably due to potassium channel dysfunction and persistent calcium influx (see paragraph 4.2.3: 'oxygen sensing at the carotid body') [43,184,228]. A possible explanation for this phenomenon, linking Sdhd mutations and potassium channel function, could be an (increased) production of ROS by defective SDH in *Sdhd* +/- mice, however, this has not yet been clearly demonstrated[184].

In heterozygous *Sdhd* mutants, SDH activity is reduced, but no specific disease phenotype develops, although a slight carotid body hyperplasia has been observed[184,227,228]. Apparently, in *Sdhd* +/- mice the loss of one *Sdhd* allele is sufficiently compensated by transcription from the wild type allele in order to escape paraganglioma formation[227]. In addition, a *Sdhd/H19* double knockout mouse model also did not show increased paraganglioma or pheochromocytoma susceptibility, and H19 may thus, at least in mice, not be a modifier gene in paraganglioma development as proposed in the model for parent-of-origin-dependent inheritance (see paragraph 3.4: 'inheritance of head and neck paraganglioma syndromes')[227]. On the other hand, the lack of paraganglioma formation in Sdhd knockout mice may also be explained by a great number of other unknown physiological or genetic factors, and differences in genotype-phenotype correlations between mouse and man are not uncommon[227]. Furthermore, inducible tissue-specific *Sdhd* knockouts are not yet available and the effects of heterozygous germ-line *Sdhd* defects and subsequent LOH in paraganglion tissue have not been studied[227].

Vhl

In Vhl knockout mice, the loss of both *Vhl* alleles (*Vhl* -/-) results in embryonic lethality during mid-gestation, due to lack of placental vasculogenesis[229,230]. A specific homozygous *Vhl* mutation at codon 200 is compatible with life and results in mild polycythemia, which resembles the homozygous recessive *VHL* mutation causing hereditary polycythemia in man (Chuvash syndrome)[229]. Heterozygous *Vhl* knockout mice (*Vhl* +/-) develop cavernous liver hemangiomas and sometimes renal cysts (both rarely associated with *VHL* mutations in humans), but paragangliomas or pheochromocytomas are not reported[226,229,230]. The reason for this phenotypic divergence between mouse and man is currently unknown.

Nf1

Like *Sdhd* and *Vhl*, the *Nf1* null state (*Nf1* -/-) is lethal in mice[231]. Heterozygous knockout mice (*Nf1* +/-) do not develop neurofibromas and astrocytomas, the hallmark tumors of human *NF1* mutations, but heterozygous mutations involving exon 31 of the mouse *Nf1* gene have been associated with pheochromocytoma formation[226,231]. In the Nf1-linked tumors, the wild type *Nf1* allele is lost, indicating that *Nf1* is a pheochromocytoma tumor suppressor gene in mice[226]. Microarray gene expression studies of *Nf1* knockout pheochromocytomas show an expression signature of early central and peripheral nervous system development, in line with the concept of persistent neuronal precursor cells and disrupted developmental culling as a cause of pheochromocytoma formation (see paragraph 4.3: 'abnormal development of neuronal precursor cells')[232].

Ret

The human MEN type 2B syndrome, consisting of medullary thyroid carcinoma, pheochromocytoma, mucosal neuroma and marfanoid skeletal changes, is predominantly caused by one specific mutation at codon 918 in exon 16 of the human *RET* gene (see paragraph 3.1: 'NF1, RET and VHL')[95]. The corresponding Ret mutation in mice produces a phenotype with comparable lesions in the mouse thyroid and adrenal medulla[233]. Homozygous (Ret^{MEN2B}/Ret^{MEN2B}) mice develop bilateral adrenal chief cell hyperplasia early in life, which invariably progresses to pheochromocytoma[233]. A minority of heterozygous mutants ($Ret^{MEN2B}/+$) also develop adrenal chief cell hyperplasia (in 16%) and pheochromocytomas (in 2%), but later in life[233]. Interestingly, extra-adrenal chief cell nodules were also observed in homozygous mutant mice, but the head and neck region was not investigated in this model[233]. The Ret^{MEN2B}/Ret^{MEN2B} mice display the highest frequency of pheochromocytoma formation of any mouse model to date. Based on observations in the Ret^{MEN2B} mouse model, it has been hypothesized that gain-of-function Ret mutations such as Ret^{MEN2B} cause adrenal tumors through abnormal migration, proliferation and survival of neuronal precursor cells (see paragraph 4.3: 'abnormal development of neuronal precursor cells')[233].

5. Historical notes

The major steps, and the people that took them in the development of our current understanding of the paraganglion system and the neoplasms that stem from it, deserve some special consideration, because (in the words of Goethe): "*die Geschichte einer Wissenschaft ist die Wissenschaft selbst*".

5.1 The discovery of the carotid body

The discovery of the carotid body and its recognition as an anatomical entity is widely attributed to Albrecht von Haller (1708-1777), who named it the 'exiguum caroticum', although it was his student Taube who in 1743 published the first anatomical description of what he called the 'ganglion minutem'[234,235].

Albrecht von Haller (1708-1777)

Von Haller was a Swiss scholar and veritable 'homo universalis', with contributions in the fields of anatomy, medicine, botany, physiology, philosophy, politics and poetry. He started his studies in medicine in 1724 in Tübingen, but went to Leiden in 1725 to continue under the famous Herman Boerhaave. While in Leiden, he also studied anatomy and surgery with Bernhard Siegfried Albinus. In 1727, at the age of eighteen, he graduated doctor medicinae under Boerhaave after writing a thesis on an otolaryngological topic, proving that a recently discovered 'salivary duct' was in fact a blood vessel. He wrote an extensive seven-volume book about his learning in Leiden: 'Erläuterungen zu Boerhaaves Institutiones'. He later went on to study in London, Oxford, Paris, and Basel before he was eventually appointed chair of medicine, anatomy, botany and surgery at the University of Göttingen in 1736. Von Haller was an avid researcher and a prolific writer. The body of

work he produced was immense, covering all fields of human knowledge, most notably botany, anatomy and poetry. His most famous contributions to the field of medicine were his recognition of the mechanism of respiration, the autonomous function of the heart, and the description of nerve and muscle activity. Another great passion, botany, led him to mountaineer in the Alps in search of specimen for his enormous plant collection and botanic garden. These forays inspired him to write his most famous poem, called 'Die Alpen', the first example of lyrical appreciation of the natural beauty of the high mountains in European literature[236,237]. At the time, the relation between high altitude and hyperplasia of the carotid body had yet to be discovered.

5.2 The discovery of the carotid body function

After the discovery of the carotid body and its description in 1743, it has taken considerable time before its function was elucidated. The first to hypothesize that the carotid body had chemoreceptive properties was De Castro, who in 1926 stated that the glomus caroticum could 'taste the blood'[238]. It was Corneille J.F. Heymans, a Flemish physiologist, who in the 1920's was the first to fully appreciate the function of the carotid body as a peripheral chemoreceptor of oxygen, carbon dioxide and acidity in the arterial blood and its role in the reflexogenic regulation of ventilation and blood pressure[5].

Corneille J.F. Heymans (1892-1968)

The discovery of the carotid body as a regulator of ventilation occurred rather by chance, as Heymans later claimed, by doing a 'foolish experiment' at the end of a day experimenting on the severed heads of dogs. Spurred on by his father and principle teacher to '*never kill an animal at the end of an experiment if the animal may still be used for any experimental purpose.. even if it looks foolish*', he injected some cyanide that happened to be standing

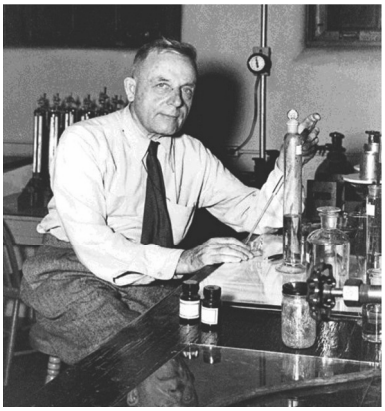
on the laboratory desk into the carotid artery of a dog with- and a dog without intact innervation of the carotid body area, and was surprised by the difference in ventilatory response. This observation lead Heymans to perform the experiments that ultimately won him the Nobel Prize in 1938[239,240].

5.3 The Warburg controversy

In 1926, the biochemist Otto Warburg observed that hypoxia alone was not sufficient to kill cancer cells, and that even in aerobic conditions when oxidative phosphorylation would be more efficient, they display high rates of glycolysis, a process he called 'aerobic glycolysis'[241]. For this discovery of 'the nature and mode of action of the respiratory enzyme', he was awarded the Nobel Prize in 1931. Warburg believed that the metabolic shift form oxidative phosphorylation to glycolysis in cancer cells, now called the 'Warburg effect', was the fundamental cause of cancer (in the words of Warburg at a lecture in of Nobel-Laureates in 1966: "*Cancer, above all other diseases, has countless secondary causes. But, even for cancer, there is only one prime cause. Summarized in a few words, the prime cause of cancer is the replacement of the respiration of oxygen in normal body cells by a fermentation of sugar*")[241,242]. The contention that mitochondrial dysfunction and disrupted metabolism is the 'only one prime cause of cancer' was disputed by many (as summarized by one of Warburg's most prominent opponents, the biochemist Sidney Weinhouse: "*at present the whole conception of cancer initiation or survival by "faulty" respiration and high glycolysis seems too simplistic for serious consideration*" and "*it has led far too many researchers into dead-end avenues of fruitless, ill-conceived attempts at the understanding or treatment of the neoplastic process*")[243]. With the discovery of genetic mechanisms and environmental factors as causes of cancer, Warburg's hypothesis on the origin of cancer faded to the background, much to the dismay of its discoverer, who in 1956 even stated: "*…there is today no other explanation for the origin of cancer cells, either special or general. From this point of view, mutation and carcinogenic agents are not alternatives, but empty words, unless metabolically specified. Even more harmful in the struggle against cancer can be the continual discovery of miscellaneous cancer agents and cancer viruses, which, by obscuring the underlying phenomena, may hinder necessary preventive measures and thereby become responsible for cancer cases*"[242]. Warburg died in 1970, at a time when his hypothesis was largely replaced by the idea that cancer was caused by abnormalities in genes and gene expression, not metabolism.

Although nowadays it is widely accepted that mitochondrial dysfunction is not the 'only one prime cause of cancer', as Warburg stated, it is also recognized that a high rate of glycolysis is a feature of many cancer types, a characteristic that today is exploited by the FDG-PET imaging of tumors (see paragraph 2.1: 'paragangliomas of the head and neck'). Indeed, in

the 2011 revision of their influential publication 'The hallmarks of cancer', Hanahan and Weinberg acknowledge the ability of cancer cells to reprogram energy metabolism as an 'emerging hallmark of cancer'[185,217]. What is more, the identification of defects in the mitochondrial SDH as a cause of paragangliomas and pheochromocytomas, as well as the identification in 2002 of defects in fumarate hydratase (FH), another TCA cycle component, as a cause of leiomyoma, leiomyosarcoma, and clear cell renal carcinoma, have confirmed Warburg's' hypothesis that defects in the cellular metabolism can actually initiate tumor growth. These discoveries have contributed to the revival of the scientific interest in the role of the metabolism in the neoplastic cell, both as a possible cause of cancer as well as a potential therapeutic target, just as Warburg envisioned[176,177,185,197,244].



Otto Warburg (1883-1970)

6. Outline of the thesis

The aim of this thesis is to gain insight in the genetics, inheritance and tumor biology of head and neck paragangliomas and the clinical consequences for paraganglioma patients, with a focus on hereditary paraganglioma syndrome in the Netherlands.

Chapter one consists of a general introduction into the current insights in head and neck paragangliomas, the diagnosis and treatment, the causative genes and their phenotypes, the heredity of paraganglioma syndromes, and an attempt is made to link gene mutations to tumor formation and behavior through the molecular biology of paragangliomas.

In **chapter two**, the current insights in the genetics of paragangliomas are reviewed, with an emphasis on the most recent developments.

In **chapter three**, the mutation frequency of SDH genes in the Netherlands is analyzed, using the data acquired by the Department of Human Genetics and the Laboratory for DNA Diagnostics of the LUMC, the primary Dutch national referral center for SDH mutation scanning. Using this SDH mutation database we evaluate the relative role of each of the SDH genes in the Dutch paraganglioma and pheochromocytoma population and the contribution of Dutch SDH founder mutations.

In **chapter four**, the clinical characteristics of Dutch head and neck paraganglioma patients treated at the Leiden University Medical Center (LUMC) are evaluated and correlated to their gene mutation status. It describes the unusual genetic make-up of the Dutch head and neck paraganglioma population and the consequences for the clinical characteristics of paraganglioma syndrome in the Netherlands.

In **chapter five**, the phenotype of the SDHD.D92Y (Asp92Tyr) Dutch founder mutation, the most prominent cause of paraganglioma syndrome in the Netherlands, is studied in a large multigenerational paraganglioma family, with a focus on the penetrance and the risk of developing symptomatic disease.

In **chapter six**, gene expression of SDHAF2- (formerly known as the PGL2 locus) and SDHD-linked paragangliomas as well as sporadic head and neck paragangliomas is investigated using RNA-microarrays, a high-throughput gene expression profiling technique. An attempt is made to distinguish these genetic subgroups on the basis of their gene expression profile and to link mutations in *SDHD* and *SDHAF2* to specific tumorigenic pathways.

In **chapter seven**, the unusual parent-of-origin-dependent inheritance that is observed in SDHD-linked paraganglioma kindreds is further investigated. A hypothesis is put forward that explains the exclusive paternal transmission of paragangliomas in SDHD-linked families, a pattern consistent with maternal imprinting, in the absence of imprinting of the *SDHD* gene itself.

Chapter eight consist of a summary of the thesis, its general implications for head and neck paragangliomas in the Netherlands and future perspectives of paraganglioma research.

Abbreviations

CT	computed tomography
FAD	flavin-adenine-dinucleotide, cofactor of SDHA
HO-2	hemoxygenase 2
HIF	hypoxia inducible factor
HRCT	high resolution computed tomography
KIF1B	kinesin family member 1B
LOH	loss of heterozygosity
MAPK	mitogen-activated protein kinase
MEN	multiple endocrine neoplasia
MRI	magnetic resonance imaging
mTOR	mammalian target of rapamycin
NF1	neurofibromatosis type 1, may also refer to neurofibromin 1 gene or protein
NOS	nitric oxide synthetase
PET	positron emission tomography
PHD	prolyl hydroxylase
RET	proto-oncogene (REarranged during Transfection)
ROS	reactive oxygen species
SDH	succinate dehydrogenase; complex II in the electron transport chain
SDHA	succinate dehydrogenase subunit A; catalytic flavoprotein subunit of SDH
SDHAF2	succinate dehydrogenase assembly factor 2; factor in the flavination of SDHA
SDHB	succinate dehydrogenase subunit B; catalytic iron sulphur subunit of SDH
SDHC	succinate dehydrogenase subunit C; anchoring subunit of SDH
SDHD	succinate dehydrogenase subunit D; anchoring subunit of SDH
TCA	tricarboxylic acid (cycle), or Krebs cycle
TMEM127	transmembrane protein 127, may refer to gene or protein
VHL	Von Hippel-Lindau, may refer to the VHL syndrome, gene or protein

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Chapter 2

Recent advances in paraganglioma genetics

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Abstract

The last 10 years have seen enormous progress in the field of paraganglioma and pheochromocytoma genetics. The identification of the first gene related to paraganglioma, *SDHD*, encoding a subunit of mitochondrial succinate dehydrogenase (SDH), was quickly followed by the identification of mutations in *SDHC* and *SDHB*. Very recently several new SDH-related genes have been discovered. The *SDHAF2* gene encodes an SDH co-factor related to the function of the SDHA subunit, and is currently exclusively associated with head and neck paragangliomas. *SDHA* itself has now also been identified as a paraganglioma gene, with the recent identification of the first mutation in a patient with extra-adrenal paraganglioma. Another SDH-related co-factor, SDHAF1, is not currently known to be a tumor suppressor, but may shed some light on the mechanisms of tumorigenesis. An entirely novel gene associated with adrenal pheochromocytoma, *TMEM127*, suggests that other new paraganglioma susceptibility genes may await discovery. In addition to these recent discoveries, new techniques related to mutation analysis, including genetic analysis algorithms, SDHB immunohistochemistry, and deletion analysis by MLPA have improved the efficiency and accuracy of genetic analysis. However, many intriguing questions remain, such as the striking differences in the clinical phenotype of genes that encode proteins with an apparently very close functional relationship, and the lack of expression of *SDHD* and *SDHAF2* mutations when inherited via the maternal line. Little is still known of the origins and causes of truly sporadic tumors, and the role of oxygen in the relationships between high altitude, familial and truly sporadic paragangliomas remains to be elucidated.

Introduction

Prior to the year 2000, knowledge of the genetics of paraganglioma and pheochromocytoma was confined to mutations of the *VHL*, *RET* and *NF1* genes. The identification of mutations in the succinate dehydrogenase subunit D gene (*SDHD*) in patients with head and neck paraganglioma was therefore a major breakthrough[1]. The association of paraganglioma with mutations in SDHD, and later with mutations in other SDH subunits, has helped elucidate both the role of the mitochondrial SDH complex and intermediary metabolism in tumorigenesis. The subsequent discovery of SDH mutations in patients with pheochromocytomas and extra-adrenal paragangliomas led to a recognition that paragangliomas and pheochromocytomas share not only similar cellular origins, but can also have a comparable genetic basis[2].

Paragangliomas of the head and neck are generally benign tumors that arise in the paraganglion tissue associated with the autonomic nervous system. Paragangliomas most frequently arise in the head and neck region, as carotid body tumors in the carotid bifurcation (approximately 80%). Other frequently seen locations within the head and neck region are along the jugular bulb or tympanic nerve (17.5%), or the paraganglia along the vagal nerve (4.5%)[3].

Pheochromocytomas and extra-adrenal paragangliomas are tumors associated with the sympathetic nervous system, are commonly described as sympathetic paragangliomas (sPGLs), and show a close embryological and physiological relationship to head and neck paragangliomas. They are most commonly derived from the chromaffin cells of the adrenal medulla (pheochromocytoma). Approximately 10-20% occur elsewhere in the abdomen, but they can occur in any of the sympathetic paraganglia from the neck to the pelvic floor[4]. Extra-adrenal sympathetic paragangliomas show a greater degree of malignancy than either pheochromocytomas or head and neck paragangliomas[5].

Here we discuss recent advances in the understanding of the genetic basis of both head and neck paragangliomas and pheochromocytomas, and further developments relevant to the genetic diagnosis of these tumors.

Genetics

Presently, causative gene mutations can be identified in around 32% of paraganglioma pheochromocytomas[6]. Hereditary tumor syndromes which have pheochromocytoma within their spectrum include the multiple endocrine neoplasia syndromes, MEN2A and MEN2B, caused by mutations of the *RET* (Rearranged in Transfection) proto-oncogene,

subtypes of von Hippel-Lindau (VHL) disease, caused by mutations of the *VHL* tumor suppressor gene, and neurofibromatosis type 1 (NF1) resulting from mutations of the *NF1* tumor suppressor gene[7]. These syndromes account for around 17% of cases but are rarely associated with head and neck or extra-adrenal paragangliomas[6].

More recently, mutations in genes associated with the mitochondrial succinate dehydrogenase (SDH) complex (*SDHA*, *SDHB*, *SDHC*, *SDHD* and *SDHAF2*) have been shown to cause head and neck paragangliomas, extra-adrenal paragangliomas, and pheochromocytomas (Table 1)[1,2,8-11]. These genes account for the remaining 15% of cases[6]. All of these genes are tumor suppressors, showing loss of heterozygosity (LOH), the loss of the normal allele in the tumor, in conjunction with the germline mutation. This results in loss of a protein subunit, which in turn destabilizes the SDH complex and abolishes its enzymatic activity[12].

Succinate dehydrogenase is an enzyme of the mitochondrial tricarboxylic acid cycle, and also plays an important role as the complex II component of the electron transport chain, contributing to the generation of ATP by oxidative phosphorylation. These combined roles place SDH at the center of two of the essential energy producing processes of the cell. SDHA, a flavoprotein, and SDHB, an iron-sulfur protein, together form the main catalytic domain, while SDHC and SDHD are the membrane-anchoring subunits of SDH and play a role in passing electrons through the electron transport chain. Despite the fact that SDH proteins are all components of the same protein complex, mutations lead to clear differences in clinical phenotype. The molecular basis for this clinical divergence is not currently known.

SDHD

Researchers in the Netherlands were the first to successfully tackle the genetics of head and neck paraganglioma and they were greatly assisted by the unusual social and demographic history of the country[13-18]. Until relatively recently, the Netherlands was characterized by significant religious, social, and geographic obstacles to intermarriage, leading to the creation of many genetically isolated populations[19]. Such populations facilitate the proliferation of founder mutations, one of them being the well-known Dutch SDHD founder mutation, p.Asp92Tyr[20]. The increased prevalence of this and other SDHD founder mutations, relative to SDHB mutations, facilitated the initial mapping of the SDHD locus[13,14].

The subsequent identification of the gene in 2000 represented a significant discovery as it was the first time that a mitochondrial protein was shown to be a tumor suppressor[1].

It was also the first protein with a role in intermediary metabolism to be directly linked to tumorigenesis. Mutations in *SDHD* most frequently result in benign head and neck paragangliomas and are much less commonly associated with sympathetic paragangliomas and adrenal pheochromocytomas[21]. The proportion of *SDHD* mutation carriers that will develop a tumor (penetrance) is high (87-100%), although not all carriers with a tumor will develop additional tumor-related symptoms[22,23].

SDHB

The identification of mutations in SDHD as a cause of hereditary paraganglioma syndrome quickly led to the discovery of the role of other SDH subunits. SDHB plays a major role in hereditary paraganglioma syndrome, and is now known to be a significant cause of adrenal pheochromocytomas, but is chiefly associated with extra-adrenal paragangliomas[2,6]. Since its discovery, *SDHB* has been found to be the dominant gene in hereditary paraganglioma syndrome in many parts of the world, despite a relatively low penetrance of *SDHB* mutations of 25-40%[24-26]. Due to their lower penetrance, *SDHB* mutations are often found in apparently sporadic patients[27]. *SDHB* mutations primarily predispose to sPGLs, and around 20% of *SDHB* mutation carriers will develop metastatic disease[5,6].

SDHC

SDHC was the second SDH subunit gene identified as a cause of paragangliomas[11]. Paragangliomas due to mutations in SDHC are much rarer than SDHB- and SDHD-related paragangliomas, accounting for less than 1% of all patients in a recent study[6]. SDHC mutations result primarily in head and neck paragangliomas, but have also been identified in patients with sympathetic paragangliomas[28,29].

SDHAF2

While the role of the *SDHB*, *SDHC* and *SDHD* genes in paraganglioma/pheochromocytoma has been known for a number of years, several novel SDH-related genes have only been identified very recently. The first was a gene encoding a novel protein involved in the addition of the flavin-adenine dinucleotide (FAD) prosthetic group to form the active SDHA flavoprotein[10]. While the approximate location of this paraganglioma-associated gene had been known for over a decade, referred to as PGL2 locus, a yeast screen of respiration deficient mutants facilitated the fortuitous discovery of a conserved mitochondrial protein of unknown function that physically associated with the SDHA flavoprotein[15,16]. Initially named SDH5, the succinate dehydrogenase complex assembly factor 2 (SDHAF2) was shown to be essential for the correct flavination of SDHA and function of the SDH complex. The c.232G>A (p.Gly78Arg) missense mutation in *SDHAF2*, identified in a large

Dutch head and neck paraganglioma kindred, results in the loss of SDHA flavination and activity of the SDH complex[10].

In a follow-up study with the joint aims of identifying new mutation carriers and assessing the frequency of *SDHAF2* mutations amongst 443 paraganglioma and pheochromocytoma patients, it became clear that mutations in this gene make a very modest contribution to the overall genetic burden in these syndromes[8]. No mutations of *SDHAF2* were identified in any patient with a pheochromocytoma, and all currently affected mutation carriers have head and neck paraganglioma exclusively. Only one additional SDHAF2 related family was identified, which interestingly carried the exact mutation, p.Gly78Arg, previously found in the Netherlands, but without evidence of a familial relationship to the Dutch kindred[8]. Although apparently a simple loss of function mutation in yeast, the recurrence of this mutation and absence of other mutations may suggest that the SDHAF2 protein with the specific p.Gly78Arg mutation retains residual activity, allowing the protein to participate in other, currently unknown, cellular activities, most feasibly the addition of FAD prosthetic groups to other flavoproteins[8,10,30].

A striking aspect of *SDHAF2* mutations, and the probable explanation for the rapid identification of all mutation carriers, is the very high penetrance. Of the 42 identified mutation carriers thought to be at risk, 37 are known to have developed a tumor. All currently unaffected mutation carriers are under the age of 45. This level of penetrance will usually lead to a familial presentation and such families will have already come to the attention of clinicians. Seven mutation carriers are known to have inherited the mutation via the maternal line, and are not thought to be at risk of tumor development (see ‘‘Inheritance’’ below).

The studies above suggest that *SDHAF2* mutation screening should only be considered in patients who suffer exclusively from head and neck paragangliomas, who have familial antecedents, multiple tumors, or a very young age of onset, and in whom the *SDHB*, *SDHC* and *SDHD* genes have been shown to be negative for mutations and deletions by sequencing and multiplex ligation-dependent probe amplification (MLPA).

SDHA

The identification of SDHAF2 as a paraganglioma-related tumor suppressor that interacts with SDHA was unexpected, as SDHA itself was the only SDH subunit not known to be mutated in paraganglioma cases. *SDHA* is the largest gene and protein of the SDH complex and is the major catalytic subunit of the enzyme. For 10 years following the discovery of *SDHD*, it remained a mystery why no mutations of *SDHA* could be found in

paraganglioma patients, a mystery which deepened with the identification of *SDHAF2* as a paraganglioma related tumor suppressor gene. Recently the first SDHA mutation was reported, (c.1765C>T, p.Arg589Trp-exon 13) in a patient with a catecholamine secreting extra-adrenal paraganglioma[9]. This patient had no family history of paraganglioma or any related endocrine syndrome.

It remains unclear why *SDHA* mutations in paragangliomas are so rare, but the patient above may suggest that *SDHA* mutations show reduced penetrance and most mutation carriers escape the development of clinical symptoms. Equally, and as suggested above for *SDHAF2*, the scarcity of *SDHA* mutations could be attributable to a secondary cellular function of SDHA, leading to intolerance for missense and truncating mutations that eliminate all enzyme activity.

The most stable of the SDH proteins when soluble, SDHA has been reported to be a component of a mitochondrial ATP-sensitive potassium channel[31]. While SDHB also seemed to be involved in this complex, the main protein interaction was between SDHA and the mitochondrial ATP-binding cassette protein 1 (mABC1), and the complex could be inhibited by 3-nitropropionate (NPA), a specific inhibitor of SDHA[32]. Whether the maintenance of this complex is essential to cell viability remains to be determined.

Alternatively, if we assume that an LOH event which deletes the remaining normal allele is required for tumorigenesis, loss of essential genes in the proximity of *SDHA* may not be tolerated, or other local genomic factors may be preventing the secondary LOH event. An exact molecular description of the LOH event in the case described by Burnichon et al. and in any subsequent cases may provide useful insights[9].

A few rare cases of congenital SDHA deficiency due to homozygous recessive mutations are known[33-35]. While the patients themselves tend to be severely affected by developmental abnormalities or cardiomyopathy early in life, due to mitochondrial deficiency, the heterozygous parents of these patients have never been reported to develop paraganglioma, perhaps suggesting that LOH events are indeed rare in conjunction with mutations of *SDHA*.

Mutations seen in these patients are generally missense and the only known truncating mutation in a patient was found together with a missense mutation on the opposing allele suggesting that complete loss of SDHA function may not be compatible with life[36].
Whether the patient described by Burnichon et al. will prove to be first of many paraganglioma cases related to *SDHA* mutations is presently unclear[9]. The current

significance of SDHA in the clinical management of paraganglioma-pheochromocytoma is minimal, but this may change if future studies identify additional mutation carriers.

SDHAF1

The identification of *SDHAF2* as a paraganglioma gene underlines the curious fact that another recently identified gene is not currently known to be involved in paraganglioma, but may nevertheless further our understanding of the role of SDH in paraganglioma formation. Succinate dehydrogenase complex assembly factor 1 (SDHAF1) is a novel LYR-motif protein; the first SDH assembly factor identified in any organism, and is located within the mitochondrial matrix[37]. Identified in consanguineous families of Turkish and Italian origin, homozygous mutations of the *SDHAF1* gene result in infantile leukoencephalopathy in affected children, and symptoms include rapidly progressive psychomotor regression beginning in the first year of life, reminiscent of the clinical symptoms seen in homozygous *SDHA* mutations carriers[38]. Patients show defective succinate dehydrogenase (complex II), with only 20-30% residual activity in muscle and fibroblasts, and the accumulation of lactate and succinate in the brain white matter. Disruption of the homologous gene or expression of the mutated gene in yeast caused SDH deficiency and failure of oxidative phosphorylation-dependent growth. Because the LYR tripeptide motif found in SDHAF1 is also seen in several iron-related proteins and may be a signature for proteins involved in Fe-S metabolism, this protein may well be associated with the SDHB subunit.

Loss of SDHB is currently thought to be central to tumorigenesis in paragangliomas, but none of the parents in *SDHAF1* families, who are heterozygous mutation carriers, have been reported to develop paragangliomas[39]. The explanation for the lack of tumor development in these mutation carriers and heterozygous *SDHA* mutation carriers may lie in the biochemical activity of SDH-complex II. *SDHA* homozygous mutation carriers generally show retention of complex II activity of at least 20% (range 20-61%), and likewise, homozygous *SDHAF1* mutation carriers show 20-30% residual activity[33,35]. In contrast, SDH related tumors, including those related to SDHD, SDHB, SDHA and SDHAF2 carry an inactivating mutation which, combined with the loss of the wild type allele (LOH), results in almost complete loss of activity[9,12,40]. As *SDHAF1* and most *SDHA* mutations do not eliminate all enzyme function, even allowing for LOH in a specific cell, a residual activity of 10-30% is apparently sufficient to prevent the development of paragangliomas.

A further interesting aspect of the biochemical profile of *SDHAF1* and *SDHA* mutation carriers is the accumulation of succinate. In both cases succinate will accumulate and can lead to the nuclear translocation of hypoxia-inducible factor 1 (HIF-1)[37,41]. The nuclear

translocation of HIF-1 may be an important mechanism in triggering tumorigenesis in paraganglioma progenitor cells, but its occurrence in *SDHAF1* and *SDHA* mutation carriers may suggest that complete loss of SDH activity is required to achieve levels of succinate accumulation sufficient to drive HIF-1 translocation to the extent needed to initiate tumorigenesis[42,43]. For a detailed discussion of these and other recent developments in the understanding of the molecular basis of tumorigenesis, we refer readers to a recent review[43].

Although none of the heterozygous mutation carriers in *SDHAF1* families currently seem susceptible to the development of paragangliomas-pheochromocytomas, the recent example of *SDHA* emphasizes that no SDH-related gene can be entirely excluded when one is considering the genetics of these tumors[9].

TMEM127

In addition to the recently reported genes related to succinate dehydrogenase, a novel tumor suppressor gene associated with a clinical phenotype of exclusively adrenal pheochromocytoma has also been described[44]. The gene encodes a putative transmembrane protein, TMEM127, and is found on chromosome 2q11. TMEM127 is a highly conserved and broadly expressed protein with three transmembrane regions, but has no known functional domains. Transfection experiments showed that the protein is found in both the plasma membrane and the cytoplasm, and suggested that TMEM127 may participate in protein trafficking between the plasma membrane, golgi and lysosomes.

Previous gene expression studies have indicated that pheochromocytomas fall into two broad categories based on the transcriptional profile, which may translate to the molecular pathways leading to tumorigenesis[45]. SDH and VHL associated tumors show a signature of angiogenesis, hypoxia, enhanced expression of the extracellular matrix, and reduced expression of components of the oxidative response and tricarboxylic cycle. Tumors linked to *NF1* or *RET* mutations show an upregulation of biological pathways including genes that mediate translation initiation, protein synthesis, and kinase signaling, and are both associated with the RAS/RAF/MAP kinase signaling pathway[45].

TMEM127-related pheochromocytomas show a transcriptional profile similar to NF1 and RET related tumors[44]. However, neither RAS activation nor AKT phosphorylation was seen, indicating that TMEM127 loss is not identical to either NF1 or RET. The authors focused on the mammalian target of rapamycin (mTOR), which is deregulated on loss of NF1, and could show that the C1 mTOR complex is specifically affected by *TMEM127* knockdown, leading to increased phosphorylation of targets of mTORC1. Knockdown of *TMEM127* also

resulted in larger cells with higher rates of proliferation. Pheochromocytomas carrying a *TMEM127* mutation showed hyperphosphorylation of mTOR effector proteins, all these data together indicating that TMEM127 is a negative regulator of mTOR.

The authors were able to identify mutations in 4 out of 12 families without known mutations in other susceptibility genes, and in 3 of 83 apparently sporadic patients. Of the seven distinct germline mutations identified, six were truncating, and the deletion of the wild-type allele in tumor DNA indicates that this is a bone fide tumor suppressor gene.

The identification of TMEM127 underlines that there are several pathways that can lead to adrenal, extra-adrenal, and head and neck paragangliomas. Whether there are important links between the essential molecular pathways of NF1, RET, and TMEM127 on the one hand and the VHL and SDH-related proteins on the other, is presently unclear, but hypoxia can regulate both HIF-1 and mTORC1, perhaps related to expression of BCL2/Adenovirus E1B 19-KD protein-interacting protein 3 (BNIP3)[46,47]. As each of these genes is associated with patterns of biological and clinical expression that are not yet understood, it is clear that we are only at the beginnings of our knowledge of these syndromes.

Inheritance

Inheritance of paraganglioma syndrome differs significantly dependent on the gene involved. While *SDHB*- and *SDHC*-linked paraganglioma families show normal autosomal dominant inheritance, *SDHD*- and *SDHAF2*-linked families show an exclusively paternal transmission of tumor susceptibility[10,18]. The recognition of this phenomenon was made possible by the same social and demographic factors in the Netherlands that facilitated the initial mapping of the *SDHD* locus, and specifically by the increased prevalence of *SDHD* mutations, relative to *SDHB* mutations. Although mutations in *SDHD* and *SDHAF2* can be inherited via the maternal and paternal lines, tumor formation following maternal transmission of a mutation is extremely rare[18,48].

The failure of maternally transmitted mutations to initiate tumorigenesis initially suggested that an imprinted gene expressed only from the paternal allele could be the underlying cause of the tumor[18]. The subsequent identification of SDHD, with its central role in cell biology, called this assumption into question. It was also established that the gene does not show mono-allelic expression, at least in the tissues analyzed to date[1,49]. The concept of gene expression of *SDHD* exclusively from the paternal allele is also contradicted by the normal development of mutation carriers with a paternally inherited mutation.

Table 1. Summary of genes, known protein functions and related syndromes.

	Locus	Protein function	Gene mechanism	No. of unique mutations[a]	Syndrome	Primary locations
SDHD	11q23	One of the two transmembrane subunits of Complex II of the respiratory chain	Autosomal dominant with LOH + imprinting	110	Hereditary paraganglioma/ pheochromocytoma	Head and neck; parasympathetic trunk
SDHB	1p36.-p35	The iron-sulfur protein catalytic subunit of Complex II	Autosomal dominant with LOH	175	Hereditary paraganglioma/ pheochromocytoma	Abdominal/ thoracic paraganglia, adrenal; sympathetic trunk
SDHC	1q23.3	One of the two transmembrane subunits of Complex II of the respiratory chain	Autosomal dominant with LOH	34	Hereditary paraganglioma/ pheochromocytoma	Head and neck; parasympathetic trunk
SDHAF2	11q12.2	Mitochondrial assembly factor for Complex II – interacts directly with SDHA	Autosomal dominant with LOH + imprinting	1	Hereditary paraganglioma/ pheochromocytoma	Head and neck; parasympathetic trunk
SDHA	5p15	The flavoprotein catalytic subunit of Complex II	Autosomal dominant with LOH	1	Hereditary paraganglioma/ pheochromocytoma	Abdominal paraganglia, sympathetic trunk
SDHA	5p15	The flavoprotein catalytic subunit of Complex II	Autosomal recessive	7	Mitochondrial encephalopathy/ Leigh Syndrome	Systemic
SDHAF1	19q13.12	Mitochondrial assembly factor for Complex II – interacts directly with SDHB?	Autosomal recessive	2	Infantile leukoencephalopathy	Systemic
TMEM127	2q11.2	Transmembrane protein involved in protein trafficking	Autosomal dominant with LOH	8	Hereditary pheochromocytoma	Adrenal; sympathetic trunk

[a] Number of mutations derived from the literature or the TCAC gene mutation database [34].

The additional occurrence of this phenomenon in paraganglioma families linked to *SDHAF2*, (like *SDHD*, located on chromosome 11), while it is absent in *SDHB*- and *SDHC*-related tumors (both genes located on chromosome 1), suggested that chromosomal location could be a factor in *SDHD*- and *SDHAF2*-related tumors.

It is known that the entire maternal copy of chromosome 11 is lost in many paragangliomas[49-51]. Although *SDHD* and *SDHAF2* themselves seem not to be imprinted, the main cluster of imprinted genes in the human genome is located on the same chromosome, at 11p15.5. This suggests a model in which a maternally expressed, paternally imprinted gene is an essential initiator or modifier of tumor development in these syndromes[48,49]. Indeed, the only report to date that has claimed to show the maternal transmission of tumor susceptibly together with an *SDHD* mutation showed that the patient had also acquired an altered methylation profile and therefore probably an altered imprinted status of H19, a known paternally imprinted tumor suppressor gene on 11p15[48,52]. In addition, it is known that VHL-related pheochromocytoma also show loss of the maternal copy of the chromosome 11p15.5 region specifically, indicating that this model may have wider importance[53,54].

High altitude paraganglioma

Long before the identification of any of the genes now known to play a role in paraganglioma, it was recognized that living at high altitude can have a profound influence on the development of carotid body hyperplasia and carotid body tumors[55-57]. A number of mammalian species are known to develop pronounced hyperplasia or tumors with a prevalence of up to 10% in humans and up to 40% in bovines, in contrast to an estimated low altitude prevalence of head and neck paraganglioma of 1 in 500,000 or less[58,59].

This increased prevalence and the central role of the carotid body in oxygen sensing suggested a role for oxygen sensing in the tumorigenesis of paragangliomas. The identification of succinate dehydrogenase and subsequent molecular studies has affirmed this link. A number of studies have linked the central mediator of cellular hypoxia, HIF-1, to defects in succinate dehydrogenase[60]. These studies postulate that a so-called ‘pseudohypoxia’ results from the inhibition of succinate dehydrogenase, leading to the accumulation of succinate, resulting in the activation of HIF-1 through the inhibition of prolyl hydroxylase-mediated degradation[42,61]. The HIF-1 transcription factor complex initiates the transcription of a range of genes that mediate an adaptive response to reduced oxygen[62]. How the activation of the HIF-1 protein may lead to the initiation of tumorigenesis in the carotid body and the exact relation of physiological hypoxia to molecular ‘pseudo-hypoxia’ awaits further investigation. Despite this suggestive link,

the possible role of succinate dehydrogenase mutations in high altitude paraganglioma cases has received little attention and the first genetic analysis failed to identify any mutations[63]. Recently, Cerecer-Gil et al. identified a family with two SDHB-linked cases of high altitude paraganglioma, residing at elevations of up to 2,200 m[64]. These are the first cases to link high altitude paraganglioma to mutations of the succinate dehydrogenase genes. While the occurrence of paraganglioma in this family could be purely coincidental to their place of residence, two factors indicated that elevation may be playing a role in the expression off these tumors. One of the patients showed a remarkably aggressive recurrent tumor, which achieved a volume almost equivalent to the original tumor within 2 months of excision. This behavior is in sharp contrast with the indolent growth pattern normally seen in head and neck paragangliomas, with a mean doubling rate of 4.2 years[65]. In addition, both patients developed head and neck tumors, while abdominal tumors occur much more frequently in SDHB mutation carriers. The identification of SDHB mutations in high altitude paraganglioma may serve to renew interest in this fascinating but underappreciated field of paraganglioma research, and refocus attention on the role of oxygen levels in the initiation and development of these tumors.

New strategies in mutation analysis

The importance of the SDH-related genes in paraganglioma-pheochromocytoma has led to extensive genetic screening of patients, even in the absence of clear familial antecedents. In patients with pheochromocytomas, in addition to the SDH genes, the *RET* and *VHL* genes should also be screened. The costs involved in analyzing all of these genes can be considerable, and are increasing with each new gene identified. Efforts have been made to use clinical data to derive algorithms to guide rational genetic testing, with the aims of efficiency and cost reduction[6,21,66]. Perhaps the most comprehensive of these is that proposed by Mannelli et al., but even this is now in need of updating[6]. Such algorithms are now widely used and assist the rapid identification of mutation carriers, but many patients may provide few useful clinical parameters, or may not conform to the rather broad criteria of these algorithms.

Mutation analysis is generally carried out using DNA sequencing, but this technique can rarely detect large deletions. Both MLPA and similar multiplex PCR methods have been applied in SDH deletion analysis, and have led to the recognition that deletions can represent up to 10% of all mutations[67-69].

While algorithms have improved the efficiency of genetic testing, recently a supplementary approach has been developed with the use of SDHB immunohistochemistry. As originally noted by Douwes Dekker et al., paragangliomas show loss of staining for the

iron protein component of SDH, encoded by *SDHB*[12]. This finding was subsequently explored by van Nederveen et al. who showed that in a series of 220 paragangliomas and pheochromocytomas, 102 tumors with known mutation of one of the SDH genes were negative for SDHB staining while *RET*, *VHL* and *NF1* cases were uniformly positive[39]. Only 6 cases were found to be negative and not explained by a known mutation in one of the SDH genes. This translates to a sensitivity of 95% (C.I. 87-100%) and specificity of 84% (C.I. 60-97%).

The utility of this approach was subsequently confirmed in an independent series of tumors by Gill et al. and was also shown to be useful in identifying the gastrointestinal stromal tumor (GIST) component of the Carney triad (CT)[70,71]. Showing that a GIST is a legitimate constituent of this tumor syndrome would potentially allow earlier diagnosis, when compared to current methods which focus on clinical criteria and require the co-occurrence of paraganglioma and pulmonary chondroma. These authors also showed that some cases of apparently sporadic GISTs also show loss of SDHB staining and propose that these represent a new subtype of GISTs.

The development of a reliable SDHB immunohistochemical procedure and the demonstration that SDHB staining can accurately distinguish SDH-related cases from other groups represents an important advance, where tumor material is available. As head and neck paragangliomas are often not operated for a considerable period after initial diagnosis, while most pheochromocytomas will be removed upon diagnosis, phaeochromocytomas represent the most useful group of tumors for the application of this technique.

Conclusion

The last 10 years have seen enormous progress in the field of head and neck paraganglioma and pheochromocytoma genetics. Six new genes have been added to a list that previously included only *VHL*, *RET* and *NF1*, and the number of patients in whom a gene mutation can be identified has doubled, and now stands at around 30-35%. New techniques related to mutation analysis, including analysis algorithms, MLPA and SDHB immunohistochemistry, have improved the efficiency and accuracy of genetic analysis.

The identification of mutations in *SDHAF2* has revealed that proteins ancillary to succinate dehydrogenase can also be tumorigenic, and the belated identification of a mutation in *SDHA* in a paraganglioma patient has demonstrated that no SDH-related gene can be

entirely excluded from consideration when thinking about the genetics of these tumor syndromes.

Finally, the recent identification of *TMEM127* by Dahia et al. has shown that entirely novel genes may be related to these tumor syndromes and suggests that others may await discovery[44].

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Chapter 3

Mutation spectrum of the succinate dehydrogenase genes in the Netherlands

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Abstract

Mutations in four genes encoding subunits or cofactors of succinate dehydrogenase (*SDH*) cause hereditary paraganglioma and pheochromocytoma syndromes. Mutations in SDHB and SDHD are generally the most common, whereas mutations in SDHC and SDHAF2 are far less frequently observed. A total of 1045 DNA samples from Dutch paraganglioma and pheochromocytoma patients and their relatives were analyzed for mutations of *SDHB, SDHC, SDHD or SDHAF2*. Mutations in these genes were identified in 690 cases, 239 of which were index cases. The vast majority of mutation carriers had a mutation in *SDHD* (87.1%). The second most commonly affected gene was *SDHAF2* (6.7%). Mutations in *SDHB* were found in only 5.9% of samples, whereas *SDHC* mutations were found in 0.3% of samples. Remarkably, 69.1% of all carriers of a mutation in an SDH gene in the Netherlands can be attributed to a single founder mutation in *SDHD*, c.274G>T (p.Asp92Tyr). Moreover, 88.8% of all *SDH* mutation carriers carry one of just six Dutch founder mutations in *SDHB*, *SDHD* and *SDHAF2*. The dominance of *SDHD* mutations is unique to the Netherlands, contrasting with the higher prevalence of *SDHB* mutations found elsewhere. In addition, we found that most SDH mutation-related paragangliomas-pheochromocytomas in the Netherlands can be explained by only six founder mutations in *SDHAF2*, *SDHB* and *SDHD*. The findings underline the regional differences in the SDH mutation spectrum, differences that should be taken into account in the development of effective screening protocols. The results show the crucial role that demographic factors play in the frequency of gene mutations.

Introduction

Mutations in genes encoding subunits or cofactors of succinate dehydrogenase (SDH), an enzyme complex bound to the inner membrane of the mitochondria, are an important cause of hereditary paraganglioma syndrome[1-3]. SDH plays an important dual role as complex II in the electron transport chain and as an enzyme of the tricarboxylic acid (TCA) cycle, catalyzing the oxidation of succinate to fumarate. It consists of four subunits: a flavoprotein (SDHA) and iron-sulphur protein (SDHB), which together make up the catalytic domain, and SDHC and SDHD, both transmembrane proteins. In addition to the *SDHB*, *SDHC* and *SDHD* genes, an additional SDH-related paraganglioma tumor suppressor was recently identified[4]. An important cofactor in SDH stability and functionality, *SDHAF2* resides as a soluble protein within the mitochondrial matrix and plays a role in the attachment of the flavin adenine dinucleotide (FAD) cofactor to SDH[5].

Hereditary paragangliomas in the Netherlands are frequently caused by mutations in the *SDHD* gene, but mutations in *SDHAF2*, *SDHB* and *SDHC* are also found[4,6-9]. Founder mutations in SDHD including the c.274G>T, p.Asp92Tyr mutation and the c.416T>C, p.Leu139Pro mutation play a major role in the prevalence of hereditary paraganglioma in the Netherlands[8]. More recently, two founder mutations in *SDHB* were identified in Dutch paraganglioma and pheochromocytoma families[6,10]. The c.232G>A, p.Gly78Arg mutation is the only *SDHAF2* mutation found in Dutch paraganglioma patients, and all patients share a common ancestor[4]. To date, no *SDHC*-linked paraganglioma families have been described in the Netherlands.

In this study, we describe the frequency of mutations in *SDHB*, *SDHC*, *SDHD* or SDHAF2 in 1045 paraganglioma and pheochromocytoma patients and their relatives. The results were obtained from the Leiden University Medical Center (LUMC), a dedicated referral center for paragangliomas and the primary referral laboratory for SDH mutation analysis in the Netherlands. As almost all Dutch paraganglioma patient samples are analyzed here, the results represent the actual prevalence of mutations in genes encoding subunits of the SDH complex in the Netherlands.

Materials and methods

Patients

Peripheral blood leukocyte DNA samples were collected from patients with paraganglioma and pheochromocytoma, and their relatives, from 1990 to 2009, at the Department of Human Genetics and the Laboratory for DNA Diagnostics of the LUMC, the primary national referral center for SDH mutation scanning. The majority of DNA samples from patients and their relatives were sent for genetic analysis only, with only summary clinical data. The reason for referral was diagnosis of 'paraganglioma', 'pheochromocytoma', 'chemodectoma' or 'glomus tumor' in all cases. As the nomenclature of paragangliomas is not unequivocal and has changed over time, exact data regarding tumor location (i.e. head-and-neck region, adrenal medulla or extra-adrenal) are therefore unavailable for many patients, and are not further discussed. Cases were considered to be familial if two or more affected individuals were identified within the same kindred. An index case is defined as the initial patient who presented with paraganglioma.

Mutation and deletion screening

The *SDHB*, *SDHC*, and *SDHD* genes were scanned for the presence of mutations from 2000 to 2009. All exonic regions of these genes were tested by direct sequencing using the Sanger method on an ABI 377 Genetic Analyzer, starting with the exons containing the known Dutch founder mutations in *SDHD* followed by exons that had previously been found to contain pathogenic mutations in *SDHB*, *SDHC*, and *SDHD* (in that order) in the Dutch population. If this analysis was negative, scanning was completed by analyzing the remaining exons of these genes. After the identification of large founder mutation-related families in the Netherlands, the current strategy is to scan the *SDHB*, *SDHC*, and *SDHD* genes as indicated by the clinical phenotype or as requested by the submitting clinician. In 2007, mutation-negative cases were retrospectively analyzed with multiplex ligation dependent probe amplification (MLPA) for the presence of large deletions in *SDHB*, *SDHC*, and *SDHD*. MLPA has been carried out on all mutation-negative cases since then, using the P226 MLPA kit (MRC Holland, Amsterdam) containing probes for all exons and the promoter of each of these genes (27 different probes), according to the manufacturer's protocol. In cases with a negative *SDHB*, *SDHC* and *SDHD* mutation analysis, *SDHAF2* was tested, as recently described[5]. Informed consent was obtained for DNA testing according to protocols approved by LUMC Ethics Review Board.

Results

A total of 1045 samples from paraganglioma and/or pheochromocytoma patients and their relatives were analyzed for mutations in SDH-related genes. Mutations in *SDHB*, *SDHC*, *SDHD* or *SDHAF2* were found in 690 cases, 239 of whom were index cases (Table 1). No mutations in SDH genes were found in 101 index cases and 254 family members of SDH mutation carriers (Table 1). The majority of SDH mutation carriers in the Netherlands carry a mutation in SDHD (87.1%), followed by mutations in *SDHAF2* (6.7%), *SDHB*(5.9%) and *SDHC* (0.3%). By far the most prevalent mutation is the c.274G>T, p.Asp92Tyr mutation in *SDHD*, accounting for 69.1% of all SDH mutation carriers (Figure 1). Altogether, 88.8% of the SDH mutation carriers in the Netherlands carried one of six Dutch founder mutations in the *SDHD*, *SDHB* or *SDHAF2* genes (Table 2 and Figure 1).

A total of 340/1045 cases tested for SDH mutations were index cases. Mutations in *SDHAF2*, *SDHB*, *SDHC* or *SDHD* were identified in 239/ 340 (70.2%) index cases, most frequently a mutation in *SDHD* (62.1%; Table 1). The most prevalent mutation among index cases was also the c.274G>T, p.Asp92Tyr mutation in *SDHD*, accounting for 157/239 index mutation carriers (65.6%).

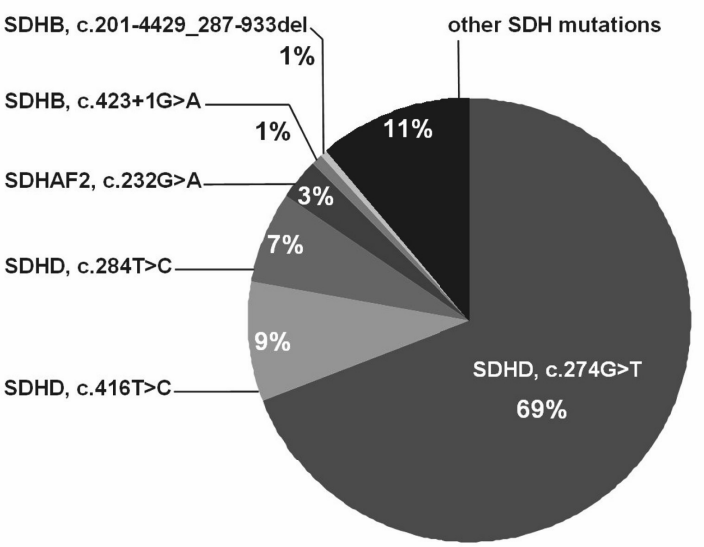

Figure 1. Relative frequencies of Dutch succinate dehydrogenase (SDH) founder mutation carriers. The graph includes a total of 613 carriers of a founder mutation in an SDH-related gene.

Table 1. Mutations in *SDHAF2, SDHB, SDHC*, and *SDHD*. In total, 690 Dutch paraganglioma and pheochromocytoma patients tested positive for a mutation in *SDHAF2, SDHB, SDHC* or *SDHD*, of which 239 are index cases. One mutation was identified in *SDHAF2*, 9 different mutations in *SDHB*, 2 mutations in *SDHC* and 16 mutations in *SDHD*. No SDH mutations were identified in 254 family members of SDH mutation carriers and 101 index cases.

Gene	Exon	DNA mutation	Protein mutation	Cases (n)	Index cases(n)
SDHAF2	4	c.232G>A	p.Gly78Arg	46	4
SDHB	2	c.136C>T	p.Arg46X	3	2
	2	c.141G>A	p.Trp47X	1	1
	3	c.201-4429_287-933del	exon 3 deletion	5	5
	3	c.268C>T	p.Arg90X	5	1
	4	c.343C>T	p.Arg115X	2	1
	4	c.423+1G>A	Splicesite	22	9
	6	c.574T>C	p.Cys192Arg	1	1
	6	c.590C>G	p.Pro197Arg	1	1
	7	c.653G>C	p.Trp218Ser	1	1
SDHC	4	c.214C>T	p.Arg72Cys	1	1
	5 and 6	c.242-241_510+1978del	exon 5 and 6 deletion	1	1
SDHD	1 and 2	c.1-8828_169+442 del	exon 1 and 2 deletion	1	1
	2	c.112C>T	p.Arg38X	8	1
	2	c.120_121insC	p.Glu42ArgfsX27	6	3
	2	c.169_169+9 del TGTATGTTCT	unknown	4	1
	2	c.54_55dupC	p.Leu19ProfsX50	8	1
	2	c.64C>T	p.Arg22X	3	1
	3	c.181delG	p.Ala61LeufsX25	2	1
	3	c.208A>G	p.Arg70Gly	1	1
	3	c.209G>T	p.Arg70Met	1	1
	3	c.242C>T	p.Pro81Leu	16	8
	3	c.274G>T	p.Asp92Tyr	477	157
	3	c.279T>G	p.Tyr93X	7	1
	3	c.284T>C	p.Leu95Pro	4	2
	3	c.287dupC	p.Ala97fs	1	1
	4	c.337_340 del GACT	p.Asp113MetfsX21	3	1
	4	c.416T>C	p.Leu139Pro	59	30

SDH, succinate dehydrogenase.

Discussion

The majority of SDH mutation carriers in the Netherlands harbor the c.274G>T, p.Asp92Tyr mutation in *SDHD*. Several very large families residing in the western part of the Netherlands are known to carry this mutation, all linked by a strong founder effect[12]. The second most widespread SDH mutation in the Netherlands is the c.416T>C, p.Leu139Pro founder mutation in *SDHD*, but this mutation accounts for hardly more than 10% of the number of p.Asp92Tyr mutation carriers, emphasizing the dominant role of the latter mutation. Compared with the high prevalence of *SDHD* mutations, *SDHB* mutations are far less common (87.1% vs. 5.9%), but the majority of *SDHB* mutation carriers also harbor known founder mutations, specifically the intron 4 splice site mutation, c.423+1G>A or the exon 3 deletion, c.201-4429_287-933del (6,9) (Tables 1 and 2).

Table 2. Dutch founder mutations of the *SDHAF2*, *SDHB* and *SDHD* genes.

Gene	DNA mutation	Protein mutation	References
SDHAF2	c.232G>A	p.Gly78Arg	Hao et al.[5] and Bayley et al.[4]
SDHB	c.423+1G>A	Intron4 splicesite	Hes et al.[10]
	c.201-4429_287-933del	exon 3 deletion	Bayley et al.[6]
SDHD	c.274G>T	p.Asp92Tyr	Baysal et al.[2] and Taschner et al.[8]
	c.284T>C	p.Leu95Pro	Dannenberg et al.[11] and Taschner et al.[8]
	c.416T>C	p.Leu139Pro	Cremers et al.[7], Dannenberg et al.[11] and Taschner et al.[8]

SDH, succinate dehydrogenase.

The difference in prevalence between *SDHB* and *SDHD* mutation carriers may in part be attributable to the lower penetrance of *SDHB* mutations[10,13-15]. Despite their common forebears, most patients with a Dutch founder mutation in *SDHB* present without a family history of paraganglioma, suggesting that many more *SDHB* mutation carriers await discovery[13-17].

We noted a remarkable 14-fold difference in the number of *SDHD* and *SDHB* mutation carriers, and even taking only index cases into account, *SDHD* mutation carriers still predominate with a ratio of around 10:1 (Table 1). None of the international studies that have reported variation in the relative frequencies of *SDHB* and *SDHD* mutations in head-and-neck paraganglioma cases have described such a large difference. A recent large Italian study identified a twofold higher prevalence of *SDHD* mutations[18], whereas

a broader European study showed an approximate 1:1 distribution of *SDHB* and *SDHD* mutation carriers[14]. Other studies have shown a 2.7- to 4.5-fold higher frequency of *SDHB* mutation carriers[13,15]. In general, *SDHB* mutations are more common than *SDHD* mutations, indicating that SDHB mutation carriers in the Netherlands only appear to be scarce because of the higher prevalence of *SDHD* founder mutations.

Haplotype studies of the most prevalent founder mutations have shown unequivocally that mutation carriers share a common haplotype surrounding the mutations, and therefore share a common ancestor. The Dutch SDHD mutation, p.Asp92Tyr, is estimated to be 200-960 years old based on coalescence time calculations, and all known Dutch carriers of the SDHAF2 mutation, p.Gly78Arg, share a common haplotype and have also been linked to a common ancestor[12,19].

In addition to mutations of *SDHB* and *SDHD*, we identified 46 carriers of the c.232G>A, p.Gly78Arg mutation in *SDHAF2*. Four large *SDHAF2*-linked paraganglioma families from the south-east Netherlands are now known to share a common ancestor, a male, born in 1771 and who married three times[20]. These families have remained largely in the same area and the p.Gly78Arg mutation is a founder mutation in the south-east Netherlands, accounting for a significant proportion of the paraganglioma cases seen in the region.

Paraganglioma syndrome due to mutations in *SDHC* is extremely rare in the Netherlands. We have identified only two *SDHC* mutations, c.242- 241_510+1978del and c.214C>T, in two patients (0.3%). Like *SDHB*, *SDHC* mutations may have been under-reported because of the often sporadic-like presentation of *SDHC*-linked paraganglioma syndrome[3,21].

The remarkable prevalence of Dutch SDH founder mutations is most probably because of the unusual social and demographical history of the Netherlands. Until only a generation ago, Dutch society was highly segregated, primarily on the basis of religious differences. This segregation affected social, political and cultural life, and was further aided by socio-economic, geographic, and linguistic factors. These factors limited intermarriage until well into the twentieth century, and led to the creation of genetically isolated populations, facilitating the proliferation of Dutch founder mutations, both in SDHD and other disease genes[22]. The p.Asp92Tyr founder mutation in SDHD shows a strong geographic focus even today[8,12].

Mutations of *SDHB*, *SDHC*, *SDHD* and *SDHAF2* each result in distinct hereditary paraganglioma syndromes, with differing modes of inheritance, penetrance, risk

of pheochromocytoma, and risk of malignant paraganglioma, meaning that prior identification of the affected gene is essential to provision of effective genetic counseling to the individual patient[14,15,21]. Several algorithms prioritizing gene-specific mutation testing in paraganglioma patients have been proposed, based on phenotypic characteristics, and with the dual objectives of minimizing mutation screening and cost reduction[23,24]. Although these algorithms represent a useful starting point for genetic analysis, it is doubtful whether the effectiveness and outcome of such algorithms are universally applicable, as the a priori chance of finding a mutation in a specific gene differs from country to country. Recognition of regional differences in the prevalence of mutations will allow the tailoring of genetic screening on the basis of local knowledge.

This study shows that the majority of mutations in SDH subunits or cofactors in the Netherlands involve SDHD, followed by SDHAF2, SDHB and SDHC, and the majority of mutation carriers harbor the Dutch SDHD founder mutation, p.Asp92Tyr. This finding is in stark contrast with the extensive genetic heterogeneity found elsewhere and underlines the importance of regional differences in the mutation spectrum of genes associated with hereditary paraganglioma syndrome.

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Chapter 4

Genetic and clinical characteristics of Dutch paraganglioma patients

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Summary

Objective. Head and neck paragangliomas (HNPGL) are associated with mutations in genes encoding subunits of succinate dehydrogenase (SDH). The aim of this study was to evaluate SDH mutations, family history and phenotypes of patients with HNPGL in the Netherlands.

Design. We evaluated the clinical data and the mutation status of 236 patients referred between 1950 and 2009 to the Leiden University Medical Center.

Results. The large majority of the patients carried mutations in SDHD (83%), and the p.Asp92Tyr Dutch founder mutation in SDHD alone accounted for 72% of all patients with HNPGL. A mutation in SDHAF2 was found in 4%, mutations in SDHB in 3% and a mutation in SDHC was identified in a single patient (0.4%). Over 80% of patients presented with positive family history, of whom 99.5% carried a mutation in an SDH gene. SDH mutations were also found in 56% of isolated patients, chiefly in SDHD (46%), but also in SDHB (8%) and SDHC (2%). The clinical parameters of these different subgroups are discussed: including the age at diagnosis, associated pheochromocytomas, tumor multifocality and malignancy rate.

Conclusion. The majority of Dutch patients with HNPGL present with a positive family history, in contrast to other European countries. The clinical characteristics of patients with HNPGL are chiefly determined by founder mutations in SDHD, the major causative gene in both familial and isolated patients with HNPGL. The high frequency of founder mutations in SDHD suggests a higher absolute prevalence of paraganglioma syndrome in the Netherlands.

Introduction

Paragangliomas are rare, slow-growing and usually benign tumors that arise in the paraganglion tissue associated with the autonomic nervous system. Paragangliomas most frequently arise in the head and neck region, where they originate from the paraganglia in the bifurcation of the carotid artery, the jugular foramen, along the vagal nerve or along the tympanic nerve[1]. The closely related tumor, pheochromocytoma, may also arise in the adrenal medulla or less frequently in the extra-adrenal orthosympathetic paraganglia of the abdomen or thorax where they are generally referred to as extra-adrenal paragangliomas. Recent studies report a positive family history in 10% to 20% of patients with head and neck paraganglioma and indicate that family history can predict aspects of the clinical presentation[2-4]. In the case of familial paraganglioma, the male-to-female ratio is higher, the age at diagnosis lower and patients present more frequently with multiple paragangliomas[5-7]. Hereditary paraganglioma syndrome is caused by mutations in genes encoding subunits or cofactors of the mitochondrial succinate dehydrogenase (SDH): *SDHA*, *SDHB*, *SDHC*, *SDHD* or *SDHAF2*[8-13]. Mutations of *RET*, *NF1* and *VHL* have also been noted in rare cases of head and neck paragangliomas (HNPGL) associated with multiple endocrine neoplasia(MEN2), neurofibromatosis (NF1) and Von Hippel-Lindau (VHL) tumor syndromes[14].

Mutations in the different SDH genes are associated with specific clinical characteristics; head and neck paragangliomas and multiple concurrent paragangliomas are most frequently observed in SDHD-linked cases, whereas extra-adrenal abdominal and thoracic paragangliomas are most frequently found in SDHB-linked cases. Mutations in SDHB, SDHC and SDHD, but not in SDHAF2, are associated with the development of adrenal pheochromocytomas[9,15-17]. Malignancy, defined as metastatic paraganglioma, is most frequently found in SDHB-linked paraganglioma syndrome, but may also occur in SDHD-linked patients[18-23]. SDH mutation-negative paraganglioma cases also show a distinct clinical profile, characterized by a late age at diagnosis and lower risk of multiple tumors[2]. It remains unclear whether there is a genetic basis for differences in initial clinical presentation between clearly hereditary cases and isolated cases in which a genetic factor is later identified. These differences could be attributed to additional, protective genetic factors or to ascertainment bias working against the clinical identification of isolated patients with HNPGL.

In contrast to other European countries, the majority of Dutch patients with HNPGL carry founder mutations, predominantly in SDHD[24,25]. It has been suggested that the

high prevalence of founder mutations in the Netherlands can be explained by a milder phenotype of paraganglioma syndrome because of the low residential altitudes[26].

Here, we evaluate family history, mutation spectrum and clinical characteristics of a series of 236 patients with paraganglioma referred to the Leiden University Medical Center (LUMC), a tertiary referral centre for paraganglioma and pheochromocytoma in the Netherlands. We characterize the clinical presentation and genetic background of these Dutch patients with HNPGL and compare this population to HNPGL populations elsewhere.

Methods

Subjects

We analyzed data on 366 consecutive patients with a diagnosis of head and neck paraganglioma who were diagnosed or referred between 1950 and 2009 to the LUMC; a dedicated tertiary referral centre for patients with paraganglioma in the Netherlands. In all cases, clinical characteristics including gender, age at diagnosis, family history, number of paragangliomas and metastatic disease were recorded. Of the 366 patients screened, 130 patients were excluded because of a lack of data on mutation status (n = 47), incomplete description of clinical data (n = 58) or uncertain diagnosis (n = 25). In total, 236 patients with head and neck paragangliomas were included in this study. The mutation analysis of some of these patients (n = 87) has been described in a previous study of SDH mutations in the Netherlands[25]. The diagnosis of paraganglioma was based on clinical and family history, otolaryngologic examination including otoscopy and laryngoscopy, magnetic resonance imaging (MRI) and/or an angiogram of the head and neck region including the skull base. In cases with resection of the paraganglioma, the diagnosis was confirmed by histopathology. From 1989, all patients with HNPGL were followed up with MRI at intervals of 1-3 years, depending on the clinical status and growth rate of the tumor (1 year for growing tumors, 2-3 years for stable tumors). Extra-adrenal paraganglioma and pheochromocytoma screening was performed using 24-h urine analysis, in duplicate, for excess catecholamines and metanephrines. From 2002, all patients with HNPGL underwent a mutation-specific screening programme consisting of biannual 24-h urine analysis in SDHAF2, SDHC and SDHD mutation carriers, and annual 24-h urine analysis combined with biannual CT or MRI of the abdomen in SDHB mutation carriers. If the result of the biochemical analysis was above the reference limit, an MRI or CT of the abdomen, chest and pelvis was performed in combination with an ^{131}I-meta-iodobenzylguanidine (MIBG) scan to visualize potential extra-adrenal paragangliomas or pheochromocytomas.

If this investigation identified a suspect lesion, a resection was performed and the diagnosis was confirmed by histopathology. In all cases of known or suspected malignant paraganglioma, the diagnosis was confirmed by histology of the tumor material in non-neuroendocrine tissue.

Mutation analysis

All patients with paraganglioma were offered mutation analysis and genetic counseling. If patients consented to DNA analysis, the *SDHB*, *SDHC* and *SDHD* genes were scanned for the presence of mutations at the laboratory for DNA diagnostics at the LUMC. All exonic and adjacent intronic regions of these genes were tested by direct sequencing using the Sanger method on an ABI 377 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA), starting with the exon containing the known Dutch founder mutations in *SDHD* and followed by exons that had previously been found to contain pathogenic mutations in *SDHD*, *SDHB* and *SDHC* (in that order) in the Dutch population. If this initial analysis was negative, the analysis was completed by scanning the remaining exons of these genes. Mutation-negative cases were analyzed for the presence of large deletions in *SDHB*, *SDHC* and *SDHD* by multiplex ligation-dependent probe amplification (MLPA). MLPA was carried out with the P226 MLPA kit (MRC Holland, Amsterdam, the Netherlands), containing probes for all exons and the promoter of each of these genes (27 different probes), according to the manufacturer's protocol. In cases with a negative *SDHB, SDHC* and *SDHD* mutation analysis, *SDHAF2* was tested by sequencing, as recently described[12]. No DNA analysis was performed in 51 patients because the mutation type could be reliably inferred from a positive mutation analysis of the patient's family members and pedigree information. Informed consent for DNA testing was obtained according to protocols approved by the LUMC Ethics Review Board. All tumor specimens were handled according to the ethical guidelines described in the *Code for Proper Secondary Use of Human Tissue in the Netherlands* of the Dutch Federation of Medical Scientific Societies (FEDERA).

Results

Mutations and family history

A total of 236 patients with paraganglioma belonging to 124 different families were eligible for evaluation in this study, of whom 120 were men and 116 women. Of these, 80% presented with a positive family history while the remaining 20% had no known family history (Table 1).

Table 1. Clinical characteristics of head and neck paraganglioma patients with a negative *vs* a positive family history. Screening for pheochromocytomas or extra-adrenal catecholamine producing paragangliomas was performed in 158 patients.

Patient characteristics	Negative family history (n = 48)	Positive family history (n = 188)
Male/female	22/26	98/90
Mean age at onset (years) (95% C.I.)	44.0 (39.7-48.2)	38.1 (36.3-40.0)
Malignant paraganglioma (%)	1 (2)	3 (2)
Multiple paragangliomas (%)	24 (50)	137 (73)
Adrenal pheochromocytoma (%)	3/36 (8)	14/122 (11)
Extra-adrenal paraganglioma (%)	2/36 (6)	8/122 (7)

Pathogenic mutations in SDHAF2, SDHB, SDHC or SDHD were identified in 214 of the 236 patients (91%) and in 102 of the 124 different families (82%). DNA analysis or family history failed to reveal mutations in only 22 patients (9%) (Table 2). The vast majority of patients with HNPGL carried mutations in SDHD (83%), 4% carried mutations in SDHAF2, 3% in SDHB and one mutation in SDHC was identified in a single patient (0.4%) (Tables 2 and 3). The p.Asp92Tyr Dutch founder mutation in SDHD was the most common mutation, accounting for 72% of all patients with HNPGL (Table 3).

Table 2. Clinical characteristics of 235 patients with head and neck paraganglioma. A single *SDHC* mutation carrier is excluded. 'No mutation' is defined as the group of patients without a mutation in *SDHB*, *SDHC*, *SDHD*, or *SDHAF2*.

Patient characteristics	*SDHB* (n = 8)	*SDHD* (n = 195)	*SDHAF2* (n = 10)	No mutation (n = 22)
Male/female	6/2	100/95	5/5	9/13
Mean age at onset (years) (95% C.I.)	47.1 (41.2-53.0)	37.9 (36.3-39.5)	33.6 (21.3-45.9)	52.0 (46.3-57.7)
Malignant paraganglioma (%)	0	4 (2)	0	0
Multiple paragangliomas (%)	4 (50)	144 (74)	7 (70)	7 (32)
Adrenal pheochromocytoma (%)	0/8 (0)	17/127 (13)	0/5 (0)	0/17 (0)
Extra-adrenal paraganglioma (%)	0/8 (0)	10/127 (8)	0/5 (0)	0/17 (0)

Evaluating families only, SDHD mutations were found in 73%, SDHAF2 mutations in 2%, SDHB mutations in 6%, SDHC mutations in 1% and no SDH mutations in 18%. The p.Asp92Tyr mutation accounted for 75 of the 124 families (60%) (Table 3).

Table 3. Mutations identified in *SDHAF2*, *SDHB*, *SDHC* and *SDHD*. Pathogenic mutations in succinate dehydrogenase-related genes could be identified in 214 of the 236 patients (91%); 187 of which had a positive family history (87%). The 214 succinate dehydrogenase (SDH) mutation carriers belong to 102 different families.

Gene	Mutation type	Protein	Positive family history (n = 187)	Negative family history (n = 27)	Families (n = 102)
SDHAF2	c.232G>A	p.Gly78Arg	10	0	3
SDHB	c.423+1G>A	Splice site, intron 4	2	2	4
	c.201-4429_287-933del	del exon 3	1	0	1
	c.574T>C	p.Cys192Arg	0	1	1
	c.590C>G	p.Pro197Arg	1	0	1
	c.649C>T	p.Arg217Cys	0	1	1
SDHC	c.242-241_510+1978del	del exon 5 & 6	0	1	1
SDHD	c.274G>T	p.Asp92Tyr	152	19	75
	c.416T>C	p.Leu139Pro	14	1	8
	Del promoter, exon 1 en 2	Unknown	0	1	1
	c.120_121insC	p.Glu42Argfs	2	1	2
	c.169_169+9delTGTATGTTCT	Unknown	1	0	1
	c.242C>T	p.Pro81Leu	2	0	2
	c.337_340delGACT	p.Asp113fs	2	0	1

In patients with a positive family history, mutations in SDHAF2, SDHB or SDHD could be identified in 99.5% of the patients with HNPGL and 99% of the families with HNPGL. By far the most frequently affected gene was *SDHD*, found in 92% of patients with HNPGL and 89% of families with HNPGL. The p.Asp92Tyr mutation was predominant amongst the familial SDHD mutation carriers, found in 152 of 173 patients with HNPGL (88%) and in 56 of 68 SDHD-linked families (82%) (Table 3). All patients with a negative family history were found to be unrelated. Of these patients, 56% showed mutations in SDHB, SDHC or SDHD, with 46% attributable to mutations of SDHD. The SDHD p.Asp92Tyr founder mutation accounted for 86% of all SDHD-linked patients with an isolated presentation (Table 3). No patient with a negative family history had a mutation in SDHAF2.

Age at diagnosis

The mean age at diagnosis of patients with paraganglioma was 39.3 years (95% CI: 37.6-41.0). Age at diagnosis was higher in patients with a negative family history than in patients with a positive family history (44.0 vs. 38.1 years) (Table 1). Age at diagnosis also differed according to the genetic subgroup, ranging from 33.6 years in SDHAF2-linked patients, 37.9 years in SDHD-linked patients, to 52.0 years without a mutation in SDHAF2, SDHB,

SDHC or SDHD (Table 2). Within the SDHD-linked patient group, the mean age at diagnosis was comparable for patients with an isolated presentation (35.5 years; 95% CI: 30.6-40.4) and for those with a positive family history (38.2 years; 95% CI: 36.3-40.1) (data not shown). Similarly, in SDHB-linked patients, we found no significant difference between the mean age at diagnosis of isolated cases (47.3 years; 95% CI: 31.9-59.6) and that of familial cases (47.0 years; 95% CI: 43.6-50.4) (data not shown).

Multifocality

Multiple synchronous or metachronous paragangliomas were found in 162 of 236 patients with paraganglioma (69%), up to a maximum of six metachronous paragangliomas. The majority of patients with a positive family history were diagnosed with multiple tumors (73%). Significant multifocality was also present in HNPGL patients with an isolated presentation, affecting 24 of the 48 cases (50%) (Table 1), with 14 of those 24 (58%) accounted for by mutations in *SDHD*, while the remaining cases showed either mutations in *SDHB* (three of 24) or no mutation in any of the SDH related genes (data not shown).

We found a clear association between the risk of multiple paragangliomas and genetic subgroup; multiple tumors were most frequently observed in SDHD- and SDHAF2-linked patients (74% and 70% respectively) (Table 2). Within the group of SDHD mutation carriers, multiple tumors were a frequent finding both in isolated patients (14 of 22; 64%) and patients with a positive family history (130 of 173; 75%) (data not shown).

Concurrent pheochromocytomas and extra-adrenal paragangliomas

Screening for concurrent pheochromocytomas and catecholamine producing extra-adrenal paragangliomas was performed in 158 of the 236 patients with HNPGL (Tables 1 and 2). Pheochromocytomas were identified in 17 of these 158 patients (11%), extra-adrenal paragangliomas in 10 of 158 patients (6%). Pheochromocytomas were only found in patients with SDHD mutations and were present in both familial SDHD-linked patients (14 of the 113 screened patients, 12%) and in isolated SDHD-linked patients (3 of the 14 screened patients, 21%) (data not shown). Pheochromocytomas were diagnosed in 14 carriers of the p.Asp92Tyr mutation, in two carriers of the p.Leu139Pro mutation and in one patient with a deletion of exon 1 and 2 of SDHD. Extra-adrenal catecholamine producing paragangliomas were only diagnosed in carriers of the SDHD p.Asp92Tyr mutation, in eight of 113 (7%) familial cases and in two of 14 (14%) isolated cases (data not shown).

Malignancy

Malignant paragangliomas were diagnosed in only four of 236 patients (2%) (Tables 1 and 2). In three cases, the metastatic lesion was discovered on MIBG scan; in one case, the metastatic lesion was discovered on MRI. The metastatic lesion was confirmed to consist of paraganglioma tissue by histopathology of cervical lymph nodes (n = 2) or the pelvic bone (n = 2). All patients developing malignant paraganglioma carried the p.Asp92Tyr mutation in SDHD.

Discussion

In this study, we present the clinical characteristics and genetic background of 236 Dutch patients with paraganglioma, including the largest series of SDHD mutation carriers described to date. Almost 80% of Dutch patients with HNPGL have a positive family history, in contrast to various European studies (performed in France, Italy, Germany and Spain) which identified 11-23% of patients with HNPGL as familial cases[2-4]. A mutation in an SDH gene could be identified in all but one of our familial patients with HNPGL, most frequently in *SDHD* (92%). Even if family members are excluded from the analysis, *SDHD* mutations still represent 89% of paraganglioma families. Mutations in SDH genes were also identified in a surprising number of isolated Dutch patients with HNPGL (56%), contrasting sharply with the 22-25% of isolated cases previously reported to be mutation-positive[4,27].

This predominance of SDHD mutations in patients with HNPGL (82% of all patients with HNPGL and 73% of all families with HNPGL this series) accords well with a recent report on SDH mutations in the Netherlands and with mutation screening in patients with HNPGL performed elsewhere (70-75%)[2,4,24]. In contrast to the study by Burnichon et al. which identified 98 different mutations in a series of 242 mutation carriers, each accounting for a maximum of six cases, the spectrum of *SDHD* mutations in the Netherlands is limited, with only seven different *SDHD* mutations identified in the current study (Table 3)[2]. In accordance with earlier reports, we found that the most prevalent mutations in SDHD are the Dutch founder mutations p.Asp92Tyr and p.Leu139Pro (Table 3)[24,25].

The clinical characteristics found in both familial and isolated SDHD-linked patients were very similar, with a comparable low mean age at diagnosis, a high risk of multiple tumors, and a risk of concurrent pheochromocytomas or catecholamine producing extra-adrenal paragangliomas. As factors such as early diagnosis through family screening and patient

or doctor awareness do not play a role in isolated cases, these characteristics can be seen as a true feature of the SDHD-linked phenotype.

One of the hypotheses put forward to explain the remarkable clustering of SDHD founder mutations in the Netherlands proposes that the high incidence of hereditary paraganglioma syndrome can be explained by low residential altitudes[26]. The relatively high oxygen pressure at sea level was postulated to result in a milder disease phenotype, reducing penetrance and negative selection. This proposal is not supported by our data, as we found the clinical characteristics of the Dutch SDHD-linked phenotype to be comparable to other studies, including the mean age at diagnosis (37.9 vs. 24.9-35.7 years found elsewhere), the risk of developing multiple paragangliomas (74% vs. 23-74% in other studies) and a malignancy rate of 2%, compared to 0-10% found elsewhere[2,3,18,19,26,28,29]. The dominance of SDHD founder mutations in the Netherlands is therefore most probably because of socio-demographic factors. Dutch society was characterized until the middle of the twentieth century by limited intermarriage and a strong segregation by religious affiliation and socio-economic, geographic and linguistic factors. These same factors have contributed to the creation of genetically isolated populations and a high prevalence of many other founder mutations in disease-related genes in the Netherlands[30]. Carriers of the most common Dutch founder mutations share a common haplotype surrounding the mutations and therefore share a common ancestor. Coalescence time calculations have shown that the Dutch SDHD founder mutation, p.Asp92Tyr, is between 200 and 960 years old[31].

A further striking feature of paraganglioma syndrome in the Netherlands is the prevalence of the p.Gly78Arg mutation in SDHAF2, identified in 10 of 236 patients (4%) and three of 124 families (2%) in this study. It is the only pathogenic mutation of SDHAF2 currently known and has been identified in one Dutch kindred and an unrelated Spanish family[32]. All known Dutch carriers of the p.Gly78Arg mutation in SDHAF2 share a common haplotype and have been linked to a common ancestor[31,33]. Like *SDHD*, *SDHAF2* is characterized by an exclusively paternal transmission of symptomatic paraganglioma syndrome. This similarity in inheritance pattern has been hypothesized to be because both genes are located on chromosome 11 and may follow the same route to tumorigenesis[34,35]. In this study, we observed interesting clinical similarities between SDHAF2 and SDHD mutation carriers: both patient groups are characterized by a high percentage of multiple paragangliomas (74% and 70% respectively) and an early mean age at diagnosis (38 and 34 years respectively) (Table 2). However, whereas SDHD-linked patients showed concurrent pheochromocytomas in 13%, extra-adrenal paragangliomas in 8% and metastatic paraganglioma in 2%, SDHAF2 mutation carriers showed no paragangliomas

outside the head and neck region, in accordance with a recent report on a Dutch SDHAF2-linked kindred[17].

The number of HNPGL patients with mutations of SDHB found in this study is remarkably low (3% of patients with HNPGL and 6% of families with HNPGL) compared with recent studies performed elsewhere, which found 22% to 34% of all HNPGL cases to be SDHB mutation carriers[2,4]. This contrast is puzzling, but it is also reflected in the relatively low numbers of mutation-negative cases (9%). As we have recently conducted several studies focused on SDHB mutation carriers, it seems unlikely that these patients have simply escaped our attention[36,37]. This suggests that the apparent relative scarcity of both SDHB-linked and mutation-negative cases may result from excess SDHD-linked cases, compared to surrounding countries. It follows that the absolute prevalence of HNPGL may be higher in the Netherlands than in other European countries. The true prevalence of rare diseases is notoriously difficult to estimate and is prone to a plethora of acquisition biases. Nevertheless, in a recent study on SDH mutation frequencies in the Netherlands, we identified 601 SDHD mutation carriers, while recent large studies from Italy and France identified only 47 and 130 SDHD mutation carriers, respectively, despite the fact that these countries have approximately four-fold higher populations than that of the Netherlands[2,3,24]. While details of the selection and acquisition of each cohort could have a significant impact on prevalence data, the large number of Dutch SDHD mutation carriers identified and the overwhelming predominance of SDHD-linked HNPGL over SDHB and mutation-negative cases are suggestive of a significantly increased prevalence of HNPGL in the Netherlands.

In this study, we evaluate the SDH mutation status and the clinical presentation of a large series of patients with head and neck paraganglioma collected over a 59-year period. In contrast to other European countries, the majority of head and neck paragangliomas are attributable to Dutch founder mutations in SDHD, most prominently the p.Asp92Tyr mutation. SDHD mutations are also a major factor in HNPGL patients with an isolated presentation. We find that the clinical characteristics of SDHD-linked patients with an isolated presentation are identical to those of clearly hereditary cases, and the consequences of the dominance of SDHD mutations are therefore an early age at diagnosis, a high risk of multiple paragangliomas including pheochromocytomas, and an exclusive paternal transmission of disease in the large majority of Dutch patients with HNPGL. Moreover, the very high frequency of familial presentation, the high prevalence of SDHD mutations, the relatively high frequency of mutations in SDHAF2 and the relative lack of mutation-negative cases and SDHB mutation carriers all strongly suggest an increased prevalence of HNPGL in the Netherlands.

Acknowledgement

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Chapter 5

Penetrance and phenotype of the *SDHD.D92Y* mutation

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Abstract

Germline mutations in *SDHD* predispose to the development of head and neck paragangliomas, and phaeochromocytomas. The risk of developing a tumor depends on the sex of the parent who transmits the mutation: paragangliomas only arise upon paternal transmission. In this study, both the risk of paraganglioma and phaeochromocytoma formation, and the risk of developing associated symptoms were investigated in 243 family members with the *SDHD.D92Y* founder mutation. By using the Kaplan-Meier method, age-specific penetrance was calculated separately for paraganglioma formation as defined by magnetic resonance imaging (MRI) and for paraganglioma-related signs and symptoms. Evaluating clinical signs and symptoms alone, the penetrance reached a maximum of 57% by the age of 47 years. When MRI detection of occult paragangliomas was included, penetrance was estimated to be 54% by the age of 40 years, 68% by the age of 60 years and 87% by the age of 70 years. Multiple tumors were found in 65% and phaeochromocytomas were diagnosed in 8% of paraganglioma patients. Malignant paraganglioma was diagnosed in one patient (3%). Although the majority of carriers of a paternally inherited *SDHD* mutation will eventually develop head and neck paragangliomas, we find a lower penetrance than previous estimates from studies based on predominantly index cases. The family-based study described here emphasizes the importance of the identification and inclusion of clinically unaffected mutation carriers in all estimates of penetrance. This finding will allow a more accurate genetic counseling and warrants a 'wait and scan' policy for asymptomatic paragangliomas, combined with biochemical screening for catecholamine excess in SDHD-linked patients.

Introduction

Paragangliomas of the head and neck are rare, usually benign tumors that arise in the paraganglion tissue associated with the parasympathetic nervous system[1]. The carotid body in the carotid bifurcation is most frequently affected, followed by the jugulotympanic bodies at the jugular bulb and tympanic nerve, and the vagal bodies at the ganglions of the vagal nerve. Symptoms are usually mild and tumor progression is characteristically slow, and therefore diagnosis of the disease is often not made before adulthood[2]. An estimated 10-50% of head and neck paragangliomas are hereditary[3]. The natural course of the disease in hereditary cases does not seem to be different from sporadic paragangliomas, but patients with inherited disease are more likely to develop multiple paragangliomas[4]. Hereditary head and neck paragangliomas can be caused by germline mutations in several genes encoding subunits of the mitochondrial succinate dehydrogenase (SDH) complex: the *SDHB*, *SDHC* and *SDHD* gene[5-7]. SDHB (1p36.1-p35), encodes a catalytic subunit, whereas *SDHC* (1q21) and *SDHD* (11q23) encode membrane-anchoring subunits of SDH involved in electron transport. Furthermore, a yet unidentified gene (*PGL2*) on 11q13.1 causes paraganglioma in at least one family[8]. Mutations in *SDHB*, *SDHC* and *SDHD* are also associated with the development of (extra-) adrenal paragangliomas or phaeochromocytomas[9-12].

As paragangliomas can cause incapacitating symptoms, accurate disease risk estimates are of paramount importance in clinical decision making and genetic counseling of paraganglioma patients. The chance of developing disease is dependent on the gene that is affected: in *SDHB*- and *SDHC*-linked families, inheritance is autosomal dominant, whereas in *SDHD*- and *PGL2*-linked families, the inheritance pattern shows a parent-of-origin effect[6,12]. As a rule, individuals are at risk only when they inherit the mutant *SDHD* allele from the father (regardless of his clinical status) and not when the mutation is maternally inherited[13,14]. Thus, proper genetic counseling of *SDHD*-linked paraganglioma families requires knowledge about the risk of developing head and neck paraganglioma upon paternal transmission of a *SDHD* mutation (penetrance), the risk of developing clinical symptoms, the age at onset of the disease, the risk of developing multiple tumors and the risk of developing phaeochromocytoma. To date, two reports have discussed the risk of developing paraganglioma or phaeochromocytoma upon inheritance of a *SDHD* mutation[15,16]. Both studies found that no tumors developed after maternal transmission of the *SDHD* mutation, and that penetrance of disease was 100% at the age of 70 years upon paternal transmission. However, both studies have evaluated a heterogeneous population of *SDHD* mutation carriers with different *SDHD* mutations, relatively large numbers of index cases (16 different *SDHD* mutations in 19 index patients and 15 different

SDHD mutations in 24 index cases, respectively) and small numbers of asymptomatic family members[15,16]. This study design is prone to overestimation of penetrance if the index cases are selected from families with multiple affected individuals and if insufficient asymptomatic family members are included. Moreover, different mutations may confer different risks[17]. In this study, we have therefore evaluated age-specific risk of developing a paraganglioma and/or phaeochromocytoma in an extended family consisting of 243 family members, in which the D92Y germline mutation in the *SDHD* gene segregates. As a significant number of paragangliomas remain asymptomatic even at advanced ages, the age-specific risk of developing paraganglioma-related symptoms is as important in counseling paraganglioma patients as the age-specific risk of developing a paraganglioma. For that reason, we have evaluated the penetrance of symptomatic disease and the penetrance of tumor development separately.

Materials and methods

Clinical status

The disease status of 243 relatives belonging to a seven-generation family with head and neck paragangliomas (family FGT189) was established between 1990 and 2008. The pedigree of this family has been published before and was updated for this study (Figure 1)[18-20]. For the evaluation of clinical characteristics, data from the family members of generations V, VI and VII were used, because patients in older generations were not available for adequate clinical analysis. Family members from generation V, VI and VII underwent magnetic resonance imaging (MRI) of the head and neck if they showed signs or symptoms of paragangliomas. All non-symptomatic carriers of a paternal mutation, who were identified during genetic counseling, were offered clinical evaluation and MRI screening as well. In addition, the data acquired in a previous research protocol were used, in which 83 members of this family were examined with MRI, regardless of their disease status and sex of the carrier parent[18]. To detect occult phaeochromocytomas, head and neck paraganglioma patients were biochemically screened for catecholamine excess. If screening was positive, MRI of the abdomen and ^{123}I-MIBG scintigraphy was performed.

Age at onset

As initial symptoms can be very mild and growth of paragangliomas is usually slow, there may be a substantial delay before a patient comes under medical attention. It can therefore be difficult to establish the exact age at onset of paraganglioma formation. We have defined ‘age at onset’ as the age at onset of complaints and/or symptoms, that

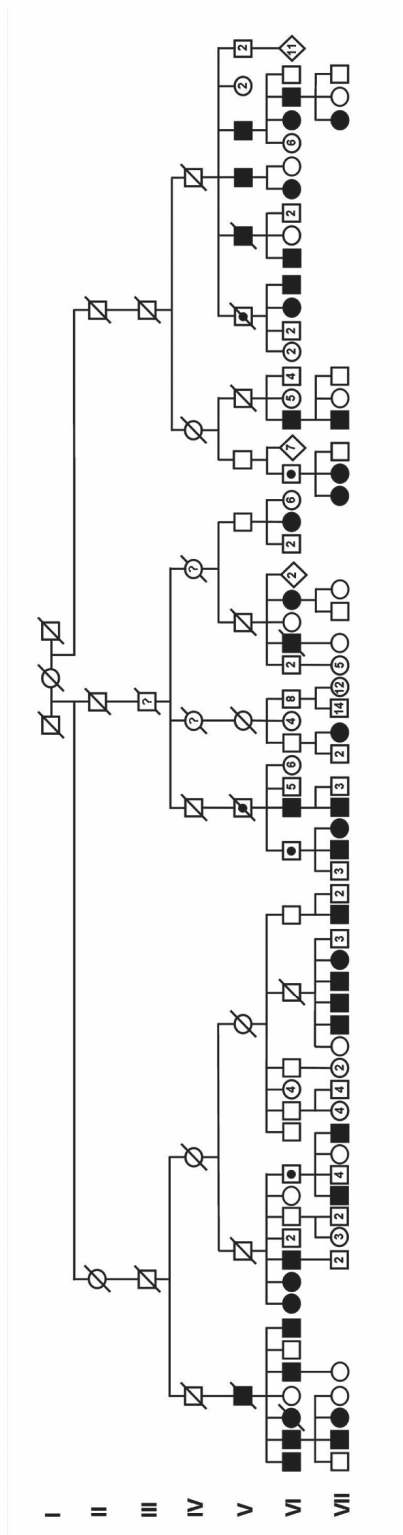

Figure 1. Pedigree of family FGT189. Roman numerals correspond to the subsequent generations of the FGT189 family. Squares depict males, circles depict females and diamonds depict multiple siblings of both sexes. Open symbols represent unaffected family members, dotted symbols represent unaffected obligate carriers of a paternally inherited SDHD mutation and solid symbols represent affected family members. A question mark within a symbol stands for a possibly affected family member, as inferred from carrier status of offspring. A number within a symbol indicates the number of siblings.

is, the age at which the patient retrospectively first experienced complaints of a head and neck paraganglioma or phaeochromocytoma, as opposed to the age at diagnosis, that is, the age at which the patient came under medical attention and the diagnosis of paraganglioma and/or phaeochromocytoma was established.

Genetic status

Paraganglioma patients in family FGT189 were shown to harbor the D92Y missense mutation (g.7882 T>C; p.Asp92Tyr) in the *SDHD* gene[21]. This Dutch founder mutation was detected by direct sequencing of PCR products obtained from peripheral blood lymphocyte DNA, as described previously[7].

Penetrance

For penetrance calculations, only the data from generations VI and VII were used, because insufficient data were available to identify asymptomatic mutation carriers in older generations (Figure 1). In this way, the risk of bias in penetrance calculations is minimized. Given the typical inheritance pattern of *SDHD*-linked paragangliomas, children of female mutation carriers were considered not to be at risk[13,22]. For children of affected fathers, the risk of inheriting the mutation was estimated to be 50%. Penetrance was calculated by comparing the actual number of patients with and without symptoms with the expected number of family members at risk of inheriting the mutation[23]. Next, we combined genetic and clinical data to calculate age-related penetrance in this family. Penetrance was expressed as a Kaplan-Meier curve, representing the probability of a *SDHD* mutation carrier to have developed either paraganglioma-related signs or symptoms, or a detectable paraganglioma at a given age. Calculations were performed for symptomatic and asymptomatic paragangliomas confirmed by radiology and for symptomatic paragangliomas alone.

Results

Clinical status

Figure 1 shows the pedigree of family FGT189. In generations V, VI and VII, paragangliomas were diagnosed in 40 family members (25 men, 15 women). Seven of these individuals (18%) had no signs or symptoms, and their diagnosis was made only after MRI screening. Multiple tumors were present in 26/40 patients (65%), to a maximum of five per patient. The most frequently encountered location was the carotid body (29 patients, 39 tumors) followed by jugulo-tympanic tumors (20 patients, 23 tumors) and the vagal body (13 patients, 16 tumors). Furthermore, 3/40 patients (8%) with head and neck

paragangliomas also had an adrenal phaeochromocytoma as diagnosed by ^{123}I-MIBG scintigraphy and MRI. In 1/40 paraganglioma patients (3%), metastatic paraganglioma tissue was found in the lung and spinal column, and this tumor was thus classified as malignant paraganglioma.

Age at onset of symptoms

In generations V, VI and VII, 33 paraganglioma patients experienced symptoms. The age at onset of symptoms ranged from 14 to 47 years (mean 26.5 years; 95% CI, 23.5-29.6 years), with a mean delay of 2.6 years (95% CI, 1.4-3.7 years) until diagnosis (Figure 2).

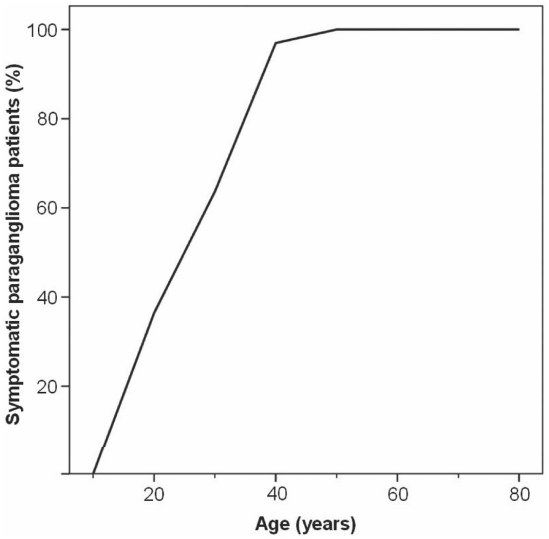

Figure 2. Age at onset of symptoms in SDHD-linked paraganglioma patients. Cumulative chart of the age at which the first symptoms of a head and neck paraganglioma or phaeochromocytoma became evident in the symptomatic family members in generation V, VI and VII (n = 33).

Genetic status

In generation VI and VII, a total of 211 family members were alive at ascertainment and in Mendelian line of inheriting the D92Y founder mutation in the *SDHD* gene. Of these 211, 22 asymptomatic family members declined the invitation to be tested for the mutation. A total of 63 of the remaining 189 family members tested positive, 52 of whom inherited the mutation from their father. One of the 22 asymptomatic family members who could not be tested was identified as an obligate carrier of a paternally inherited mutation,

because of affected offspring. In all, 53 paternal and 11 maternal mutation carriers were thus identified in generation VI and VII.

Penetrance

As expected, penetrance of the disease was parent-of-origin-dependent. No paragangliomas were found by clinical investigation or MRI in the offspring of female *SDHD* mutation carriers, and they were therefore not included in the risk calculations. We identified 11 male mutation carriers in generation V and 17 in generation VI on the basis that they were either affected themselves or had an affected offspring. In total, we identified 138 children of male mutation carriers (83 in generation VI and 55 in generation VII), who were thus all at 50% risk of inheriting the *SDHD* mutation. Of the 138 family members, 36 (26%) had one or more radiologically proven head and neck paragangliomas and/or a phaeochromocytoma. A total of 30 of these 36 patients (83%) experienced symptoms at the time of diagnosis or developed symptoms in the follow-up period. Under the assumption that 50% of the children of paternal mutation carriers are at risk, this corresponds with an estimated overall penetrance of 36/69 (52%) and an estimated overall clinical penetrance of 30/69 (43%)[23].

Using the genetic data, 53 of the 138 children (38%) at risk of a paternally transmitted mutation in generation VI and VII were shown to actually have inherited the mutation (Table 1). A total of 30 of these carriers at risk presented with paraganglioma- or phaeochromocytoma- related symptoms, accounting for an overall clinical penetrance of 30/53 (57%). To correct for the age at onset, a Kaplan–Meier curve was made representing the chance to be symptom free as a function of time (Figure 3). As none of the carriers developed symptoms after the age of 47 years, the penetrance reached a maximum of 57% at this age. Of the 23 clinically non-penetrant carriers of the disease gene, 12 were examined with MRI. In six cases (50%), one or more paragangliomas were diagnosed, raising the overall penetrance to 36/53 (68%) (Table 1). If these cases were included in a Kaplan–Meier curve, the penetrance increased to 87% by 70 years of age (Figure 3).

Table 1. Penetrance in generations VI and VII.

Carriers of a paternally inherited *SDHD* mutation (n)			Symptomatic paraganglioma patients (n)			**MRI diagnosed paraganglioma patients (n)**		
total	*male*	*female*	*total*	*male*	*female*	*total*	*male*	*female*
53	31	22	30(57%)	18	12	36(68%)	21	15

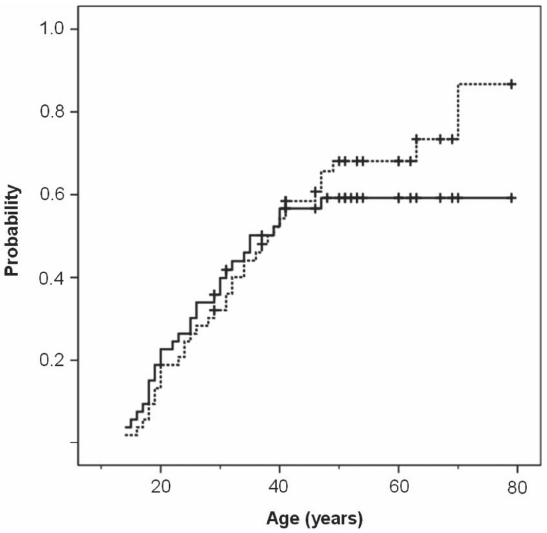

Figure 3. Penetrance of SDHD-linked head and neck paragangliomas. Inverted Kaplan–Meier curve indicating the probability of developing a MRI-detectable paraganglioma (dotted line) or the probability of developing paraganglioma-related symptoms (solid line) at a certain age for carriers of a paternally inherited SDHD mutation. Vertical markers indicate censored patients.

Discussion

SDHD-linked paragangliomas and phaeochromocytomas present a unique tumor syndrome with a specific, parent-of-origin-dependent risk of inheriting disease. In this study, we did not observe the development of paragangliomas or phaeochromocytomas in 11 instances of maternal transmission of the *SDHD* mutation. This parent-of-origin-dependent inheritance seems to be the norm in *SDHD*-linked paraganglioma families, although one case of a tympanic paraganglioma after maternal transmission has been reported[13,14]. Recently, new insights in the mechanisms behind this peculiar inheritance pattern have emerged. We have shown previously that in *SDHD*-linked paragangliomas, not only the wild-type maternal *SDHD* allele on 11q23 but the entire maternal copy of chromosome 11 was consistently lost[22]. A model explaining the parent-of-origin-dependent inheritance in *SDHD*-linked cases was proposed, involving a second, paternally imprinted, tumor-suppressor gene (TSG) located on 11p15[22]. Within this model, paraganglioma formation occurs only when the wild type maternal *SDHD* allele on 11q23 and the active copy of the imprinted TSG on 11p15 are simultaneously lost. This model of inheritance has been supported by the report of Pigny et al., who describe the only known case of maternal transmission of *SDHD*-linked paraganglioma to date[24]. Although at first sight, this unique

case seems to contradict the model, it was shown that the patient had also acquired an altered methylation profile and, therefore, probably an altered imprinted status of H19, a known paternally imprinted TSG on 11p15[24]. This suggests that the parent-of-origin-dependent inheritance of *SDHD*-linked disease is caused by the paternal imprinting of H19, a TSG on the imprinted 11p15 region that seems to be essential for paraganglioma formation. It furthermore suggests that maternal transmission of *SDHD*-linked disease is possible only if the 'second hit' targets the wild-type paternal *SDHD* allele on 11q23 as well as the active status of the maternal H19 allele on 11p15. The fact that these events involve different regions of different copies of chromosome 11 simultaneously is likely to be the reason why maternal transmission of disease is extremely rare.

This has considerable consequences on the genetic counseling of the affected families. Although children of female mutation carriers may not be completely preserved from the risk of developing paraganglioma, maternal transmission of disease remains extremely rare. Offspring of female carriers must, however, be aware that they can be mutation carriers and transmit the disease gene to their children. On the other hand, carriers of a paternally inherited *SDHD* mutation are at risk of developing a head and neck paraganglioma and/or phaeochromocytoma. In this study, the overall risk of developing a paraganglioma upon paternal transmission of the *SDHD* mutation is 68%, when evaluating the results of clinical evaluation and MRI. Age-related penetrance is 54% at the age of 40 years and 68% at the age of 60 years, reaching a maximum of 87% by the age of 70 years, that is, the large majority of patients with a paternally derived disease gene will eventually develop one or more paragangliomas and/or a phaeochromocytoma (Figure 3). These figures may represent an underestimation, because MRI scanning and screening for catecholamine excess was declined by 11 of the 23 asymptomatic paternal mutation carriers. Even in the unlikely event that all these would have had one or more occult paragangliomas or a phaeochromocytoma, overall penetrance is raised only marginally up to a maximum of 89%. Hence, this cannot fully explain why our estimates are slightly lower compared with those reported in the literature. Benn et al. reported an estimated age-related penetrance for *SDHD*-linked disease of 73% at the age of 40 years and 100% at the age of 70 years, whereas Neumann et al. reported a penetrance of 86% at the age of 50 years and 100% at the age of 70 years[15,16]. In the latter two studies, multiple families with multiple index patients and different *SDHD* mutations were investigated. In contrast, we have evaluated a single extended family with the D92Y Dutch founder mutation in the *SDHD* gene for the calculation of penetrance in *SDHD*-linked disease[15,16]. There is evidence that this family based approach yields more accurate estimates, because less index cases and more asymptomatic mutation carriers are included[25]. However, bias may arise if family members share unknown genetic or environmental factors that influence

disease risk[25,26]. This could lead to overestimation of penetrance in *SDHD*-linked disease, especially when high-risk families are used for penetrance calculations[25,27]. In this respect, it is interesting to note that age-related penetrance estimates of *SDHD*-linked disease found in this study are lower compared with those reported by Benn et al. and Neumann et al.[15,16]. This may reflect an upward bias in the latter two studies because of the inclusion of large numbers of index cases and relatively low numbers of asymptomatic mutation carriers. Further positive bias may have arisen in these studies because all risk factors tend to be overrepresented in case patients[28].

The risk of developing head and neck paraganglioma and/or phaeochromocytoma, or penetrance of the disease, is not the only feature that is important in counseling *SDHD* mutation carriers. The risk of developing associated symptoms is at least as relevant in counseling and clinical decision making. In this study, evaluation of the age-related occurrence of clinical symptoms upon paternal transmission of a *SDHD* mutation reveals that a significant number of individuals at risk did not develop clinical symptoms despite the fact that some of them have reached advanced ages. In actual fact, no patients developed first symptoms after the age of 47 years, and clinical penetrance reaches a maximum of 57% at this age (Figure 3). Clinical penetrance might even have been overestimated in our study, because 21 asymptomatic family members at risk of inheriting the *SDHD* mutation through the father were not tested for the *SDHD* mutation and their carrier status could not be inferred from their offspring. Assuming that 50% of these 21 untested family members would have inherited the mutation, the clinical penetrance decreases to 50%. In the unlikely event that all 21 untested family members would have inherited the *SDHD* mutation, clinical penetrance is at least 41%.

Astrom et al. observed that patients with multiple SDHD-linked tumors or a concurrent phaeochromocytoma at the time of diagnosis had lived at higher mean altitudes as compared with those with single tumors[29]. They postulated that the low altitudes found in the western part of the Netherlands cause a milder disease phenotype, manifesting as reduced penetrance and a better fitness of *SDHD* mutations. This would explain the relatively high incidence of hereditary paragangliomas and the remarkable clustering of founder mutations in the *SDHD* gene in the Netherlands[29]. However, despite the fact that most family members of family FGT189 live in the western part of the Netherlands, a region situated at sea level, multiple tumors were ascertained in 65% of its patients, as compared with 30-74% in other studies[15,16,29]. In addition, the risk of developing phaeochromocytoma is 8% for paraganglioma patients in this family, at the lower end of the spectrum of published risk estimates (7-53%), but comparable with that of patients living at higher altitudes (10%)[10,15,16,29]. Remarkably, Astrom et al. did not observe

an effect of altitude on age at onset, although age-dependent carotid body hyperplasia at high altitudes has been observed by others[29,30]. However, they did find a correlation between age at onset and mutation type[29]. Patients harboring missense mutations in the *SDHD* gene seemed to develop symptoms later in life than those harboring nonsense or splicing mutations (mean age at onset of 34.3 years vs. 25.8 years, respectively)[29]. In the present family, patients harboring the D92Y missense mutation had a mean age at onset of 26.5 years (95% CI, 23.5-29.6 years), which was in good agreement with other studies that have evaluated paraganglioma patients with different *SDHD* mutations (25.8-30.6 years)[15,16,29]. Malignancy or metastatic disease, a rare finding in *SDHD*-linked disease with an estimated prevalence of 0-10%, has not been associated with residential altitude, nor with mutation type[15,16,29,31]. In the present family too, only one patient (3%) was diagnosed with a paraganglioma metastasis. All in all, the disease phenotype of the *SDHD.D92Y* mutation in this extended family residing at sea level does not seem to represent a milder or otherwise different phenotype than that of patients living at high altitudes or those carrying other mutation types, and hence altitude is unlikely to explain the observed lower penetrance. Probably, more relevant is the fact that the *SDHD.D92Y* missense mutation has a detrimental effect on the functionality of the SDH-complex[32]. Moreover, as most patients develop first symptoms after reaching reproductive age, and symptoms are usually mild and slowly progressive even at high altitudes, it is doubtful whether negative selection plays a decisive role in the geographical distribution of *SDHD* mutations. Rather, the high incidence of founder mutations in the Netherlands is explained by specific historic and demographic factors, such as migrational patterns, endogamy and rapid population growth, factors that have contributed to the existence of a striking number of Dutch founder mutations in other disease genes[33].

In summary, we have provided risk estimates for a well-defined SDHD-linked population and have shown that penetrance of disease differs considerably depending on whether or not MRI-screening results are included. Whereas the large majority of paternally inherited *SDHD* mutation carriers may eventually develop one or more paragangliomas (87%), symptoms do not occur in substantial proportion of these carriers at risk (43%), probably because of the characteristic indolent growth pattern of paragangliomas[2]. Patients who do develop complaints associated with paraganglioma or phaeochromocytomas generally do so before the age of 50 years; the risk of developing symptoms later in life seems small. This knowledge might reassure especially older non-symptomatic carriers and warrants a ‘wait and scan’ policy for patients with asymptomatic head and neck paragangliomas. Because of the elevated risk of developing a phaeochromocytoma in SDHD-linked disease, surveillance should include screening for the detection of asymptomatic phaeochromocytomas[2,10].

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Chapter 6

Gene expression of head and neck paragangliomas

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Abstract

Background. Paragangliomas of the head and neck are highly vascular and usually clinically benign tumors arising in the paraganglia of the autonomic nervous system. A significant number of cases (10-50%) are proven to be familial. Multiple genes encoding subunits of the mitochondrial succinate-dehydrogenase (SDH) complex are associated with hereditary paraganglioma: *SDHB*, *SDHC* and *SDHD*. Furthermore, a hereditary paraganglioma family has been identified with linkage to the *PGL2* locus on 11q13. No SDH genes are known to be located in the 11q13 region, and the exact gene defect has not yet been identified in this family.

Methods. We have performed a RNA expression microarray study in sporadic, *SDHD*- and *PGL2*-linked head and neck paragangliomas in order to identify potential differences in gene expression leading to tumorigenesis in these genetically defined paraganglioma subgroups. We have focused our analysis on pathways and functional gene-groups that are known to be associated with SDH function and paraganglioma tumorigenesis, i.e. metabolism, hypoxia, and angiogenesis related pathways. We also evaluated gene clusters of interest on chromosome 11 (i.e. the *PGL2* locus on 11q13 and the imprinted region 11p15).

Results. We found remarkable similarity in overall gene expression profiles of *SDHD*-linked, *PGL2*-linked and sporadic paraganglioma. The supervised analysis on pathways implicated in PGL tumor formation also did not reveal significant differences in gene expression between these paraganglioma subgroups. Moreover, we were not able to detect differences in gene expression of chromosome 11 regions of interest (i.e. 11q23, 11q13, 11p15).

Conclusion. The similarity in gene expression profiles suggests that PGL2, like SDHD, is involved in the functionality of the SDH complex, and that tumor formation in these subgroups involves the same pathways as in SDH-linked paragangliomas. We were not able to clarify the exact identity of *PGL2* on 11q13. The lack of differential gene expression of chromosome 11 genes might indicate that chromosome 11 loss, as demonstrated in *SDHD*-linked paragangliomas, is an important feature in the formation of paragangliomas regardless of their genetic background.

Background

Paragangliomas are tumors originating in cells of neural crest origin in the extra-adrenal paraganglia associated with the autonomic nervous system. Most paragangliomas arise in the parasympathetic paraganglia of the head and neck region, but they can also arise in the parasympathetic paraganglia of the mediastinum or in the orthosympathetic para-aortic and retroperitoneal paraganglia. They are highly vascular and usually characterized by an indolent, non-invasive growth pattern. Most cases are sporadic, but a significant number (10-50%) have been shown to be familial. Mutations in 3 of the 4 genes encoding subunits of succinate dehydrogenase (SDH, complex II in the mitochondrial respiratory chain) have been implicated in the familial forms of the disease: *SDHB*, *SDHC*, and *SDHD*[1-3]. In our population, the majority of hereditary paraganglioma cases are associated with two founder mutations in the *SDHD* gene on 11q23[4]. In addition to these SDH related cases, another hereditary paraganglioma family has been identified with linkage to a region on 11q13, the *PGL2* locus[5]. No mitochondrial complex II genes, including *SDHA*, are located in the 11q13 region, and the identity and function of the *PGL2* gene are yet unknown. Mutations in *SDHB*, *SDHC* and *SDHD* are also implicated in the formation of phaeochromocytomas, tumors arising in cells derived from the neural crest in the adrenal medulla[6-8]. In PGL2-linked cases no association with phaeochromocytoma formation has been found to date.

A recent genome-wide expression study of phaeochromocytomas identified two distinct clusters: one containing SDH- and VHL-associated phaeochromocytomas and another containing MEN2- and NF1-associated phaeochromocytomas, while both clusters contained sporadic cases[9]. The cluster containing SDH- and VHL-associated phaeochromocytomas was characterized by a transcription signature of reduced oxidoreductase activity and increased angiogenesis and hypoxia[9]. In order to gain further insight into PGL2 function and identity, we have performed a gene expression study evaluating gene expression in head and neck paragangliomas of different genetic backgrounds: SDHD-linked, PGL2-linked and sporadic cases without a mutation in the *SDHB*, *SDHC* or *SDHD* gene. In addition to a supervised gene-based analysis, a supervised pathway-based analysis was performed, evaluating differences in gene expression for predefined pathways and functional gene groups. We evaluated in more detail gene groups that are known to be associated with SDH function and paraganglioma-or phaeochromocytoma formation, i.e. metabolism, cell cycle, hypoxia, and angiogenesis related pathways. In addition, we evaluated the gene sets that differentiate the SDH/VHL- from the NF1/MEN2-associated phaeochromocytoma cluster in the aforementioned phaeochromocytoma gene expression study, using our dataset[9]. Finally, gene clusters located within or close to the *PGL2* locus on 11q13, the

SDHD locus on 11q23, and the imprinted 11p15 region were assessed. The latter region has previously been implicated in SDHD-linked paraganglioma formation[10]. The results of both gene- and pathway-based analyses show remarkable similarity in the gene-expression profiles of SDHD-linked , PGL2-linked and sporadic paragangliomas, suggesting that paraganglioma formation involves the same mechanisms and pathways in these paraganglioma subgroups.

Methods

Tumor specimens

Samples from head and neck paragangliomas were obtained from the tissue banks of the department of Pathology at the Leiden University Medical Center (LUMC) (all sporadic and SDHD-related cases and one PGL2-linked case) or the University Medical Center (UMC) St. Radboud (all but one PGL2-linked cases). All specimens were handled according to the ethical guidelines, as described in the *Code for Proper Secondary Use of Human Tissue in the Netherlands* of the Dutch Federation of Medical Scientific Societies (FEDERA). Diagnosis of paraganglioma was confirmed by histology in all cases. All paragangliomas were carotid body tumors arising in the carotid bifurcation in the neck. No malignant paragangliomas were included in the study. Eighteen paraganglioma cases were selected: 7 cases with a known D92Y founder mutation in the *SDHD* gene, 6 cases from the family with significant linkage tot the *PGL2* locus on 11q13, and 5 sporadic cases[5]. The latter were defined as 'sporadic' because mutation scanning of *SDHB*, *SDHC*, and *SDHD* was negative, while the family histories of these cases were negative for HN-paraganglioma or any of the other clinical stigmata that would suggest the involvement of *VHL*, *NF1* or the *RET* gene.

Mutation scanning

SDHB, *SDHC*, and *SDHD* genes were scanned for the presence of mutations at the laboratory for DNA diagnostics at the LUMC. All exonic regions of these genes were tested by direct sequencing using the Sanger method on an ABI 3177 Genetic Analyzer, starting with the exon containing the known Dutch founder mutations in *SDHD* followed by exons that had previously been found to contain pathogenic mutations in *SDHD*, *SDHB*, and *SDHC* (in that order) in the Dutch population[4,11]. If that remained negative, scanning was completed by analyzing the remainder of exons of these genes. More recently, the sporadic, mutation-negative cases were also examined by MLPA for the presence of large deletions in *SDHB*, *SDHC*, and *SDHD*[12]. MLPA was carried out with the P226 MLPA kit,

containing probes for all exons and the promoter of each of these genes (27 different probes), according to the MRC Holland protocol[13].

RNA isolation and microarray hybridization

Tissue samples were snap frozen in liquid nitrogen and stored at -70°C. An experienced pathologist (PCWH) estimated the tumor percentage of the samples. Only samples with a tumor percentage of more than 70% were included in the study. Sample preparation was performed according to the Affymetrix protocol (Affymetrix Inc., Santa Clara, CA) [14]. In brief, 30 5 µm sections were taken from each frozen tissue sample and total RNA was extracted using Trizol (Life Technologies, Inc., Rockville, MD), and purified using RNeasy columns according to the manufacturers protocol (Qiagen, Valencia, CA). A minimum of 10 µg of total RNA was used to synthesize cDNA with the Superscript Choice system (Life Technologies, Rockville, MD). First strand cDNA synthesis was performed with T7-(dT)24 oligomer primer, followed by second strand synthesis using T4 DNA polymerase. The resultant was purified using Phase Lock Gel and precipitated in ethanol. Synthesis of biotine labeled cRNA was performed using the BioArray HighYield Transcript Labeling Kit (Enzo Diagnostics, Inc., Farmingdale, NY) according to the protocol of the manufacturer. In vitro transcription (IVT) reactions took place at 37°C for 4,5 hours. The labeled cRNA was purified using RNeasy columns (Qiagen, Valencia, CA) and fragmented in fragmentation buffer at 94°C for 35 minutes. Fragmented cRNA prepared from each individual sample was then transferred to a specialized Affymetrix hybridization centre (Leiden Genome Technology Centre, LGTC). Here the samples were hybridized according to the manufacturers' protocol in a concentration of 0,5 µg/µl to a human GeneChip U95A-v2 (Affymetrix), containing approximately 8500 probe sets. The data discussed in this publication have been deposited in NCBIs Gene Expression Omnibus (GEO), and are accessible through GEO Series accession number GSE12921[15].

Sample size calculation

Sample size calculations were performed according to the method described by Pounds and Cheng[16].

Normalization and expression analysis

Acquisition and quantification of array images was performed using the MAS software package (Affymetrix). All arrays were normalized with gcrma normalization using the R statistical software package available from Bioconductor[17-19].

Unsupervised clustering analysis

Unsupervised two-way hierarchical clustering was performed with complete linkage and Euclidian distance metrics, using the R statistical software package available from Bioconductor[18,19].

Supervised analysis

The R package 'Linear Models for Microarray Data' (LIMMA) was used for the assessment of differential expression of individual genes between paraganglioma subgroups[20]. Overall gene expression differences between paraganglioma subgroups were evaluated with the 'global test' designed by J.J. Goeman using the R package 'global test' available on Bioconductor[18,19,21]. In order to evaluate subtle differences between paraganglioma subgroups, we analyzed all pathways in the Catalog of Human Gene Sets v2.0, containing 1687 gene sets, available from the Broad Institute as part of their publicly accessible Gene Set Enrichment Analysis (GSEA) software package[22,23]. Instead of the statistical method used in the GSEA software, we used the global test developed by Goeman et al., because the latter tends to have more power to detect gene sets with small effect sizes[24-26]. Specific attention was paid to the gene sets that were significantly represented in SDH-linked phaeochromocytomas in a recent gene expression study by Dahia et al.[9]. Next, we applied the gene set that differentiated SDH- from MEN2-associated phaeochromocytomas in the aforementioned study to our data using the global test[9,21]. Furthermore, we performed a pathway-based analysis using the global test on manually curated gene sets, focusing specifically on pathways involved in processes or conditions that are known or assumed to play a role in paraganglioma formation, i.e. proliferation, survival, apoptosis, cell cycle regulation, metabolism and hypoxia, based on pathways described in literature and the publicly available pathway databases KEGG and Biocarta[27-29]. In addition to the evaluation of functionally related genes we also performed the global test on some topographically related gene groups on chromosome 11, i.e. the *PGL2* minimal haplotype on 11q13, the *SDHD* region on 11q23, and 11p15, an imprinted region that has been implicated in SDHD-linked paraganglioma and phaeochromocytoma formation[10,21]. In all, 264 manually curated pathways and functionally related gene sets were tested. All tests, both for genes and pathways, were corrected for multiple testing based on the false discovery rate (FDR) criterion, using the method of Benjamini and Hochberg[30].

Table 1. Clinicopathological characteristics and mutation status.

Sample	Tumor	Location	Family history	Mutation	Sex	Age at onset (yrs)	Multiple paragangliomas
1	PGL04	CBT	*PGL2*	–	f	28	yes
2	PGL01	CBT	*PGL2*	–	f	28	yes
3	PGL02	CBT	*PGL2*	–	m	37	yes
4	PGL19	CBT	*PGL2*	–	f	32	yes
5	PGL05	CBT	*SDHD*	D92Y	m	43	yes
6	PGL06	CBT	*SDHD*	D92Y	m	47	yes
7	PGL13	CBT	*SDHD*	D92Y	f	29	yes
8	PGL14	CBT	*SDHD*	D92Y	f	45	no
9	PGL16	CBT	*SDHD*	D92Y	f	47	yes
10	PGL17	CBT	*SDHD*	D92Y	f	74	no
11	PGL10	CBT	SPOR	–	f	44	no
13	PGL12	CBT	SPOR	–	f	49	no
14	PGL15	CBT	SPOR	–	f	38	no
15	PGL23	CBT	SPOR	–	f	70	no
16	PGL20	CBT	SPOR	–	m	27	no

CBT = carotid body tumor; *PGL2* = positive family history for *PGL2*-linked paragangliomas; *SDHD* = positive family history for *SDHD*-linked paragangliomas; SPOR = sporadic sample, negative family history of paraganglioma or phaeochromocytoma and no mutation in the *SDHB*, *SDHC* or *SDHD* gene; D92Y = p.Asp92Tyr, a Dutch founder mutation in the *SDHD* gene; m = male patient, f = female patient.

Results

Due to the rarity of PGL2-linked paragangliomas, sample sizes in this study are inevitably limited. In all, 21 samples were hybridized including 3 duplicates. Four samples (1 SDHD-linked sample, 2 PGL2-linked samples and 1 duplicate experiment) were excluded because of poor RNA or hybridization quality, leaving 15 different tumors in the analysis (5 sporadic, 6 SDHD-linked and 4 PGL2-linked samples) (Table 1).

Sample size calculation

Calculations showed that with this sample set and assuming that at least 30 to 35 genes are truly differentially expressed between subgroups with a fold change of 2.0 or more, at least 10 differentially expressed genes would be detected with a false discovery rate of 0.1.

Unsupervised analysis

Two-way hierarchical clustering of SDHD-linked, PGL2-linked and sporadic paragangliomas revealed no clear clusters. No grouping according to genetic background was found (Figure 1). In fact, overall gene expression was very similar in all paraganglioma samples, with high correlation coefficients for overall gene expression between all tumors irrespective of genetic background.

Supervised analysis

Using the LIMMA analysis, we did not find individual genes that are significantly differentially expressed between sporadic, SDHD- and PGL2-linked paragangliomas. The global test did not reveal significant differences in overall gene expression between paraganglioma subgroups. Using all 1687 functional gene sets from the Catalog of Human Gene Sets incorporated in the GSEA software, analysis with the global test revealed no significant differences in gene expression between SDHD- and PGL2-linked tumors, SDHD-linked and sporadic tumors, or PGL2-linked and sporadic tumors for any gene set when corrected for multiple testing. In a recent phaeochromocytoma gene expression study, several gene sets from the Catalog of Human Gene Sets were found to be significantly represented in SDH-associated phaeochromocytomas[9]. These gene sets comprise microtubule activity, oxidoreductase activity, HIF1α, angiogenesis, proteasome degradation, electron transport chain, CCR3, collagen and glutathione metabolism[9]. In our study, no significant differential expression between sporadic, SDHD- and PGL2-linked paragangliomas was found for these gene sets. Dahia et al. also identified a gene set differentiating SDH- from MEN2-associated phaeochromocytomas[9]. This gene set contained 400 probes, encoding 288 different annotated genes. 212 of these 288 genes were also represented on the Affymetrix U95A chip used in this study. No significant differential expression between sporadic, SDHD- and PGL2-linked head and neck paragangliomas was observed for this gene set (data not shown). Next, we performed the global test on manually selected pathways assumed to play a role in paraganglioma formation, i.e. proliferation-, survival-, apoptosis-, cell cycle regulation-, metabolism- and hypoxia-related pathways. In all, 264 pathways and functional gene sets were tested. No significant differential expression was observed for any of these gene sets between the paraganglioma subgroups (data partially shown in Figure 2). Last, we performed a more detailed evaluation of genes located on chromosome 11 loci of interest (11q23, 11q13 and 11p15). This analysis also did not reveal significant differences between paraganglioma subgroups (data partially shown in Figure 3).

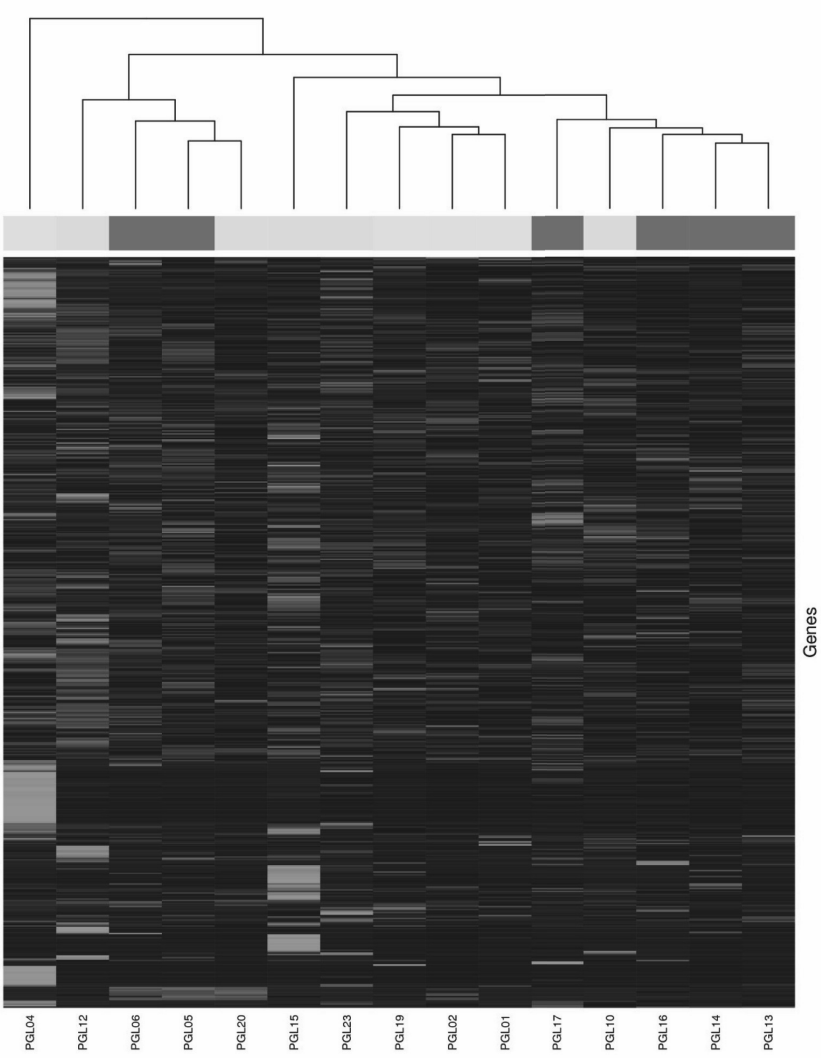

Figure 1. Two way hierarchical clustering analysis of genetically defined paraganglioma subgroups. Two way hierarchical clustering of PGL2-linked (yellow squares in the top row), SDHD-linked (blue squares in the top row), and sporadic (grey squares in the top row) head and neck paragangliomas. Samples are represented as columns and genes as rows. Expression levels are normalized for each gene. The mean is zero, and the color scale indicates the expression of the gene relative to the mean. Red indicates high expression, black indicates mean expression, and green indicates low expression levels. Overall gene expression is very similar for all samples, no well defined sample clusters can be found.

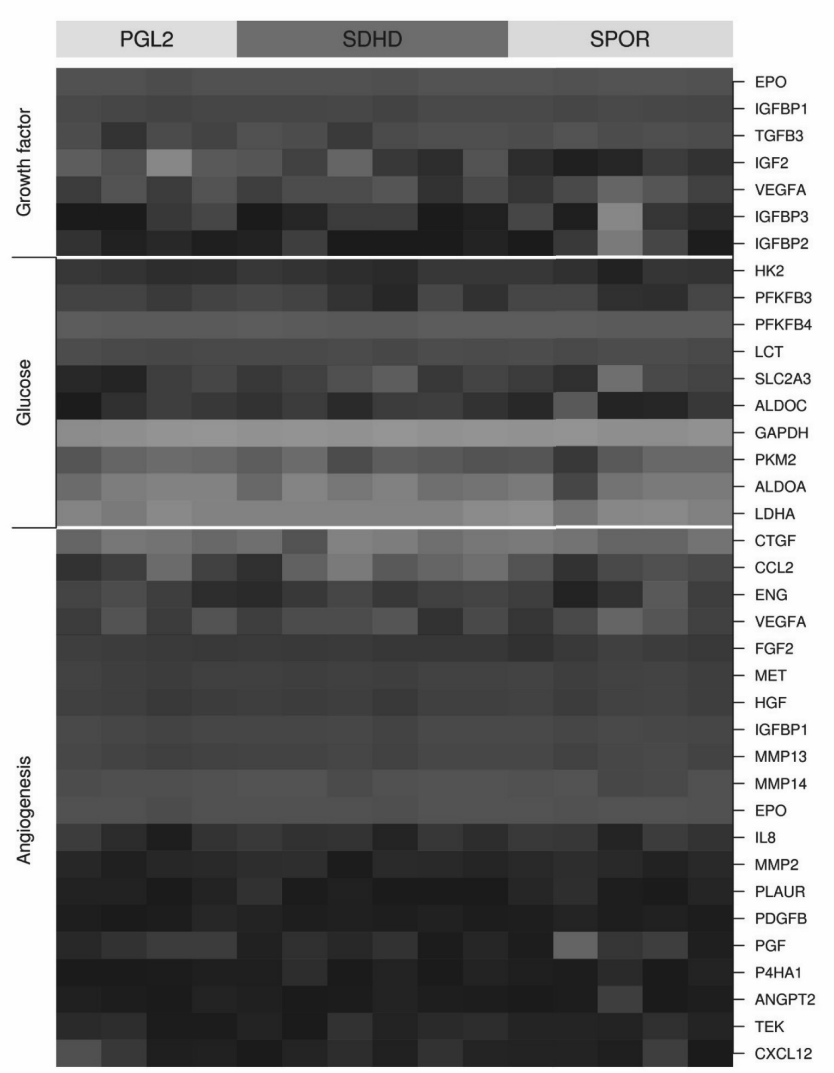

Figure 2. Heatmap of HIF1α target genes. Samples are represented as columns and genes as rows. Samples are ordered from left to right: PGL2-linked paragangliomas (yellow), SDHD-linked paragangliomas (blue), and sporadic paragangliomas (grey). In all, 264 pathways and functional gene sets related to processes that are assumed to play a role in paraganglioma formation (i.e. proliferation, survival, apoptosis, cell cycle regulation, metabolism and hypoxia) were tested (data not shown). None of them showed significant differential gene expression between SDHD-linked, PGL2-linked and sporadic paragangliomas, including the gene sets encoding SDH and HIF1α target genes involved in the processes of angiogenesis, glucose metabolism and proliferation.

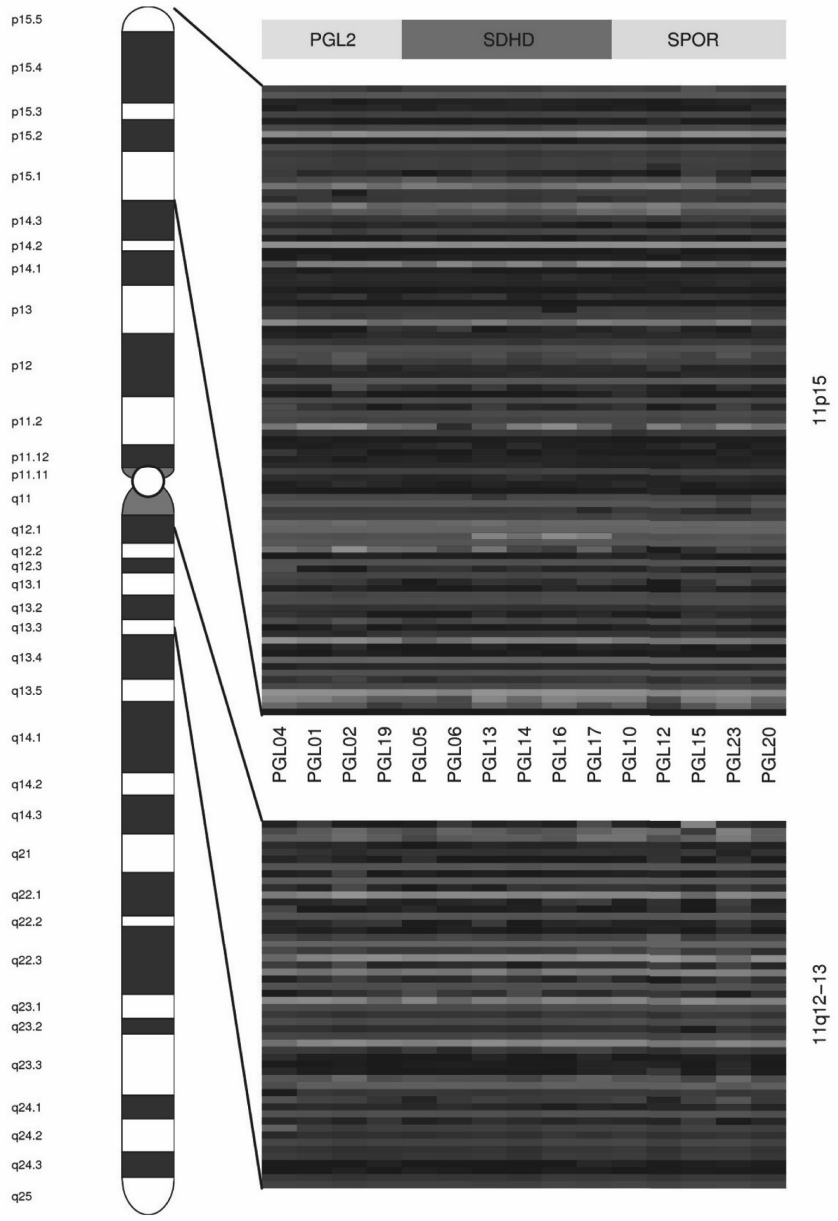

Figure 3. Heatmap of chromosome 11 genes located on 11p15 and the PGL2 minimal haplotype on 11q13. The upper heatmap represents genes located on chromosome 11 region 11p15, and the lower heatmap the PGL2 minimal haplotype located on 11q13. Samples are represented as columns and genes as rows. Samples are ordered from left to right: PGL2-linked paragangliomas (yellow), SDHD-linked paragangliomas (blue), and sporadic paragangliomas (grey). No significant differences in gene expression can be observed for genes located on the 11p15 region, which has been implicated in SDHD-linked paraganglioma formation, or for genes within the PGL2 minimal haplotype located on 11q13.

Discussion

In our gene expression analysis of sporadic, SDHD- and PGL2-linked paragangliomas of the head and neck, no significant differences in gene expression profile were observed between these genetically defined paraganglioma subgroups. Instead, we found considerable similarity between PGL2-linked, SDHD-linked and sporadic tumor samples in both unsupervised and supervised analyses (Figures 1, 2 and 3). This correlates well with the observation that sporadic as well as SDHD-linked and PGL2-linked paragangliomas of the head and neck share important clinical characteristics like the age of onset of symptoms, the indolent growth pattern, and a usually benign behavior of the tumor, although multiple paragangliomas are less often observed in sporadic cases[31-34]. Furthermore, all head and neck paraganglioma subtypes share the typical histological architecture of the 'zellballen', groups of neoplastic chief cells surrounded by sustentacular cells[35,36].

In a recent gene expression study by Dahia et al. of sporadic, SDHB-, SDHD-, VHL-, MEN2- and NF1-associated phaeochromocytomas, two phaeochromocytoma clusters were identified: a cluster containing VHL- and SDH-linked tumors and another containing MEN2- and NF1-linked tumors[9]. Gene set enrichment analysis showed that microtubule activity, oxidoreductase activity, HIF1α, angiogenesis, proteasome degeneration, electron transport chain, chemokine CCR3, collagen and glutathione metabolism gene sets were significantly represented in the gene expression signature of SDH-linked phaeochromocytomas[9]. In our study, the GSEA pathway-based supervised analysis of sporadic, PGL2- and SDHD-linked paragangliomas did not reveal significant differences between the subgroups for all GSEA gene sets, including the gene sets that characterized SDH tumors in the study by Dahia et al.[9]. The authors also identified a gene set that differentiated SDH-linked tumors from MEN2-linked phaeochromocytomas[9]. When applying this differentiating gene set to our dataset, significant differences in gene expression could not be found. These findings suggest that all paraganglioma subgroups in our study share the characteristics that defined the SDH-linked tumors in the study by Dahia et al., i.e. a signature of hypoxia, reduced oxidoreductase, and increased angiogenesis[9]. Further characterization of the gene expression profiles of head and neck paragangliomas would require comparison with normal paraganglionic tissue. However, due to the microscopic size of normal paraganglia and their close anatomical relations with essential nerves and blood vessels it is not feasible to acquire this in sufficient quantity and quality to reliably perform RNA-based tests such gene expression microarrays.

In the present study, more detailed analysis of manually selected pathways and functional gene sets that are assumed to play a role in paraganglioma formation, i.e. processes of

metabolism, angiogenesis and hypoxia as well as proliferation, survival, apoptosis and cell cycle related pathways also did not reveal significant differential expression between sporadic, SDHD-linked and PGL2-linked paragangliomas. A striking finding is that there is no significant differential expression of SDH genes between paraganglioma subgroups. This is in agreement with prior observations of SDHB suppression and enhanced expression of SDHA in sporadic, SDHD- and PGL2-associated tumors[9,37]. Of further interest is the observed similar gene expression between all paraganglioma subgroups for HIF1α and HIF1α downstream target genes (Figure 2). HIF1α and HIF1α downstream target genes have been shown to be upregulated in SDH-linked tumors[9,38-40]. The mechanism of HIF1α induction in tumors with SDH mutations has recently been shown to be succinate accumulation resulting from loss of SDH function, leading to inhibition of HIF-α-prolyl hydroxylases and thus to elevated HIF1α activity[39,41]. The transcription factor HIF1α regulates a host of genes that are involved in proliferation and survival, angiogenesis and glucose metabolism, and the elevated HIF1α activity or pseudo hypoxic drive is thought to be the basic mechanism of tumorigenesis in SDH-linked paragangliomas[39,42,43]. It has been demonstrated that in PGL2-linked tumors SDH function is disrupted, as it is in SDHD-linked paragangliomas[37]. PGL2- and SDHD-linked tumors also appear to share the features of increased HIF1α activity and upregulation of HIF1α targets that results from SDH inactivity[9,37,41]. These findings may hold important clues for the function of the yet unidentified *PGL2* gene on 11q13, as a defect in the yet unidentified *PGL2* gene seems to have consequences similar to a mutation in the *SDHD* gene. No mitochondrial complex II genes are known to be located in the 11q13 region, but the *PGL2* gene could affect SDH function by interfering with SDH assembly, transport or insertion into the mitochondrial membrane, or encode a cofactor that is essential for proper SDH function. Alternatively, *PGL2* gene function could be more directly associated with HIF1α stability and thus constitute the pseudohypoxic drive that leads to paraganglioma formation. We did not find significant differences in expression between paraganglioma subgroups for the *PGL2* minimal haplotype on 11q13, and further research to clarify the exact *PGL2* identity is currently ongoing.

Another important clinical feature shared by both SDHD- and PGL2-linked tumors is the remarkable parent-of-origin-dependent inheritance of disease. Inheritance of paraganglioma occurs in an autosomal dominant way only when paternally transmitted, while no phenotype develops after maternal transmission[44,45]. Previously, we demonstrated that in SDHD-linked head and neck paragangliomas and phaeochromocytomas this exclusive paternal transmission of the disease is caused by consistent loss of the entire maternal chromosome 11[10]. We hypothesized that selective loss of an as yet unidentified, imprinted gene on the 11p15 region drives this selective

chromosome loss, and may also be important in the formation of non-SDHD-linked paraganglioma[10]. In line with this hypothesis, recently *H19*, a paternally imprinted gene on 11p15, has been put forward as the tumor suppressor gene responsible for the parent-of-origin-dependent inheritance in SDHD-linked head and neck paragangliomas[46]. In the present study, supervised analysis of all chromosome 11 probe sets on the array, as well as more detailed analysis of genes on chromosome 11p15, 11q23 (location of the *SDHD* gene) and 11q13 (location of the *PGL2* locus), did not show significant expression differences between sporadic, PGL2- and SDHD-linked tumors (Figure 3). It is possible that this result reflects the loss of chromosome 11 in all these paraganglioma subgroups. As the relation between chromosome loss and gene expression alterations is complex, we must interpret the observed lack of gene expression differences between these groups cautiously in this context. It has been shown previously that all SDHD-linked HN-paragangliomas show loss of the entire copy of the wildtype maternal chromosome 11, and the same applies to PGL2-linked paragangliomas[10]. Partial or entire chromosome 11 loss has also been observed in sporadic paragangliomas, although only in 2 out of 9 cases[47]. Chromosome 11 loss could thus be an important step in paraganglioma formation irrespective of the genetic background.

Conclusion

In this study of sporadic, SDHD- and PGL2-linked paragangliomas of the head and neck, we have found very similar gene expression profiles for all three genetic subgroups. This correlates well with observations of comparable histopathology and clinical behavior. More detailed analysis of gene sets that have previously been shown to characterize SDH-linked tumors, as well as pathways known to be implicated in SDH-linked paraganglioma formation, show no differential gene expression for these paraganglioma subgroups. This suggests that a defect in the yet unidentified *PGL2* gene, like a mutation in the *SDHD* gene, disrupts normal SDH function. Further gene expression analysis of the *PGL2* locus on 11q13 in this study did not reveal the *PGL2* identity. The lack of differential gene expression of chromosome 11 genes between the paraganglioma subgroups might further indicate that chromosome 11 loss, as demonstrated in SDHD-linked paragangliomas, is an important feature in the formation of a paraganglioma regardless of the genetic background.

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Chapter 7

Parent-of-origin-dependent inheritance in SDHD-linked paragangliomas

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Abstract

Germline mutations in succinate dehydrogenase subunits B, C and D (*SDHB*, *SDHC* and *SDHD*), genes encoding subunits of mitochondrial complex II, cause hereditary paragangliomas and phaeochromocytomas. In *SDHB* (1p36)- and *SDHC* (1q21)-linked families, disease inheritance is autosomal dominant. In *SDHD* (11q23)-linked families, the disease phenotype is expressed only upon paternal transmission of the mutation, consistent with maternal imprinting. However, *SDHD* shows biallelic expression in brain, kidney and lymphoid tissues. Moreover, consistent loss of the wild-type (wt) maternal allele in SDHD-linked tumors suggests expression of the maternal *SDHD* allele in normal paraganglia. Here we demonstrate exclusive loss of the entire maternal chromosome 11 in *SDHD*-linked paragangliomas and phaeochromocytomas, suggesting that combined loss of the wt *SDHD* allele and maternal 11p region is essential for tumorigenesis. We hypothesize that this is driven by selective loss of one or more imprinted genes in the 11p15 region. In paternally, but not in maternally derived *SDHD* mutation carriers, this can be achieved by a single event, that is, non-disjunctional loss of the maternal chromosome 11. Thus, the exclusive paternal transmission of the disease can be explained by a somatic genetic mechanism targeting both the *SDHD* gene on 11q23 and a paternally imprinted gene on 11p15.5, rather than imprinting of *SDHD*.

Introduction

Paragangliomas (PGL) of the head and neck are neuroendocrine tumors arising in branchiomeric and intravagal paraganglia. They are rare, highly vascular, mostly benign tumors usually characterized by an indolent growth pattern. Paragangliomas, like normal paraganglia, consist of two cell types: the type I or chief cells, which represent the neoplastic population in paragangliomas, and the type II or sustentacular cells[1]. The most common site is the carotid body, a chemoreceptive organ in the bifurcation of the carotid artery that senses oxygen levels in peripheral blood in a way that is not yet fully understood. Most paragangliomas appear to be sporadic, but a significant minority of the cases (10-50%) has been shown to be familial. Recently, several genes have been implicated in these familial forms of the disease. Analysis of families carrying the PGL1 gene revealed germline mutations in the succinate dehydrogenase complex-subunit D (SDHD) gene on 11q23[2]. This gene encodes a mitochondrial protein, an anchoring subunit of the mitochondrial respiratory chain complex II.

Subsequently, mutations in other subunits of the same mitochondrial complex II were also found to be associated with hereditary paraganglioma. The SDHB gene (1p36.1-p35) encodes a catalytic subunit of mitochondrial complex II and has been implicated in familial paraganglioma of the head and neck as well as in familial paraganglioma of the adrenal medulla, better known as pheochromocytoma[3]. Both *SDHD* and *SDHB* appear to act as tumor suppressor genes in hereditary paraganglioma. The *SDHC* gene (1q21) encodes the second anchoring subunit of the mitochondrial complex II and mutations in this gene have recently been shown to cause hereditary paraganglioma as well[4]. Furthermore, a hereditary paraganglioma family with linkage to a region on 11q13.1, the PGL2 locus, has been described[5]. However, no mitochondrial complex II genes are known to be located in this region.

Interestingly, strikingly different inheritance patterns have been found for paragangliomas of different genetic background. Whereas *SDHB*- and *SDHC*-linked pedigrees show autosomal dominant inheritance, *SDHD*- and PGL2-linked pedigrees exhibit a clear parent-of-origin effect: inheritance of paraganglioma occurs in an autosomal dominant way only when paternally transmitted, while no phenotype develops after maternal transmission. This pattern is consistent in all *SDHD*-linked pedigrees, and suggests sex-specific epigenetic modification of the maternal *SDHD* allele, consistent with genomic imprinting[6]. However, no evidence of a physical imprint, for example, methylation of the 11q22.1-23 region, has been found. Furthermore, the *SDHD* gene is biallelicly expressed in human brain, kidney and lymphoid tissue[2]. It has been suggested that the imprinting of *SDHD* is restricted

to the paraganglia cells, but loss of the maternal *SDHD* allele is frequently observed in paraganglioma from *SDHD*-mutation carriers, an event that is unlikely to promote tumor growth when the maternal allele is already silenced by an imprint[2,7,8]. We hypothesized that somatic, selective loss of the whole maternal chromosome 11 could explain the exclusive paternal inheritance of the disease, mimicking maternal imprinting of the *SDHD* gene. We performed fluorescent in situ hybridization (FISH) studies on 23 *SDHD*-linked tumors using different probe sets in order to test for loss of chromosome 11, and loss of heterozygosity (LOH) analysis using several microsatellite markers to determine the parental origin of the lost chromosome. Complete loss of a chromosome 11 copy was found in all tumors, and LOH analysis on a subset of seven tumors from patients for whom parental DNA samples were available revealed the exclusive maternal origin of the lost chromosome. We propose that the selective loss of the maternal chromosome 11 copy is driven by the allelic phasing of the *SDHD* germline mutation and a paternally imprinted tumor suppressor gene on 11p15.

Materials and methods

Patients and families

Diagnosis of paraganglioma was based on medical history, physical and otolaryngological examination, radiological imaging and histopathology of the excised tumor. After obtaining informed consent, peripheral blood was obtained from patients and their parents for genomic DNA isolation. Routinely processed archival paraffin-embedded carotid body paraganglioma or phaeochromocytoma tissue from patients with the D92Y Dutch founder mutation in the *SDHD* gene were obtained from the archives of the Department of Pathology of the Leiden University Medical Center[2,29].

Mutation detection

The D92Y mutation in the *SDHD* gene was detected by direct sequencing of PCR products obtained from peripheral blood lymphocyte (PBL) DNA as described previously[2].

Interphase FISH on paraffin-embedded tissue sections

We performed interphase FISH on paraffin-embedded sections as previously described[30]. The pLC11A probe and the PUC1.77 probe for the centromeric alphoid repeat DNA of chromosomes 11 and 1, respectively, were kindly provided by Dr. J. Wiegant (Department of Molecular Cell Biology, LUMC, Leiden, The Netherlands)[31,32]. We have chosen the PUC1.77 probe as a reference because of our extensive experience with the interpretation of the signals given by this probe and a previous LOH study did not indicate involvement

of chromosome 1 in PGL1/SDHD-linked paragangliomas[33]. The probes were labeled by standard nick translation with biotin-16-aUTP or digoxigenin-11-dUTP (Roche, Basel, Switzerland). A total of 200 nuclei were analyzed for each sample by two independent investigators (EFH and ESJ).

Triple color interphase FISH on nuclei isolated from paraffin-embedded tissue

Isolation of intact nuclei, hybridization and immunodetection were performed as previously described, with slight modifications[34]. The hybridization mix contained 50% formamide, 3 ng/µl of each of the three probes (either PUC1.77, pLC11A and 3F7 or PUC1.77, 371C18 and 469N6) and a 50-fold excess of human Cot-1 DNA (Invitrogen Life tech., Paisley, UK). A volume of 5 µl of the mix was applied directly onto the slides and covered with an 18 x 18mm^2 coverslip. After a denaturation step of 8 min at 80°C, the slides were incubated overnight at 37°C in a moisture chamber. The BAC probes 371C18 (telomere 11p), 469N6 (telomere 11q) and 3F7 (11q23, containing the *SDHD* gene) were obtained from the Children's Hospital Oakland Research Institute (Peter de Jong BAC library RP11). All probes were labeled by standard nick translation with biotin-16-aUTP, digoxigenin-11-dUTP or fluorescein-12-dUTP (Roche). A total of 200 nuclei were analyzed for each sample and probe combination by two independent investigators (EFH and ESJ).

Flow cytometry analysis and flow sorting

Cell preparation and staining procedures were performed as described elsewhere[35]. Pepsin digestion was used to isolate whole nuclei from 45 mm thick paraffin sections. Nuclei were subsequently stained with propidium iodide. DNA content was determined with a FACscan flow cytometer (Becton & Dickson, Immunocytometry Systems, San Jose, CA, USA). On average, 100.000 nuclei were measured in each sample. If the DNA histogram showed a single $G_{0,1}$ and G_2 peak both populations were subsequently sorted on a FACsorter (FACSVantage SE, Becton & Dickson, Immunocytometry Systems, San Jose, CA, USA). Owing to the G_2 arrest often detected in paraganglioma cells, the $G_{2,M}$ population was considered enriched for tumor cells[12]. If the DNA histogram showed $G_{0,1}$ peaks, the left peak was considered to represent the diploid and the right peak the aneuploid population. Cells were sorted directly into 1.5 ml microfuge tubes and DNA was subsequently isolated as previously described[36].

LOH analysis

LOH analysis was performed as previously described[1]. Genotypes of patients and their parents were established for the markers D11S1984 and D11S2362 (11p15), D11S4183 (11p11), D11S1335, D11S1765 and D11S4075 (11q13) and D11S1647, D11S3178 and pDJ159Ogt1R (11q23). Markers were informative if they were heterozygous in the patient,

and the parental origin of the alleles could be unambiguously derived. Subsequently, in informative cases both diploid and aneuploid or diploid and the $G_{2,M}$ fractions were tested.

Results

We started with FISH experiments on tissue sections from five paragangliomas from D92Y carriers. The rationale for initially choosing sections rather than cell suspensions was the expectation that this would facilitate the visual selection of nuclei of the type I (chief) cells. The sections were hybridized with centromere probes for chromosomes 11 and 1, the latter chromosome serving as a ploidy reference. Loss of centromere 11 relative to centromere 1 was found in all tumors, in 45-65% of nuclei (Figure 1). Of the nuclei with three signals for chromosome 1, 13-54% had two signals for chromosome 11, 5-54% had one signal for chromosome 11 and 5-32% had no signals for chromosome 11. Of the nuclei with two signals for chromosome 1, 44-66% had one signal for chromosome 11 and 8–31% had no signals for chromosome 11, whereas of the nuclei with only one signal for chromosome 1, 0-7% had no signals for chromosome 11.

To exclude the possibility that loss of signals due to tissue sectioning could have interfered with the results, we next hybridized isolated whole nuclei of 10 paragangliomas, three of which were also studied in the first study. Whereas the use of suspensions precluded the selection of type I cells, evaluation of an unselected sample of 200 nuclei still demonstrated the relative loss of centromere 11 in all samples in 35-63% of nuclei (Figure 2).

To discriminate between loss of the entire chromosome and subchromosomal loss due to complex rearrangements, we next analyzed isolated whole nuclei of nine paragangliomas and two pheochromocytomas from D92Y mutation carriers that were not used in the previous studies, using a triple color FISH technique. This allows simultaneous detection of two probes on chromosome 11 and one probe on centromere 1 (Figure 3). First, we studied the centromere 1 and 11 probes in combination with a BAC probe that covers the *SDHD* gene on 11q23 (Figure 3a and c). Concomitant loss of both probes located on chromosome 11 relative to centromere 1 was observed in all samples, in 24-65% of paraganglioma and 31-62% of pheochromocytoma nuclei (Figure 4a).

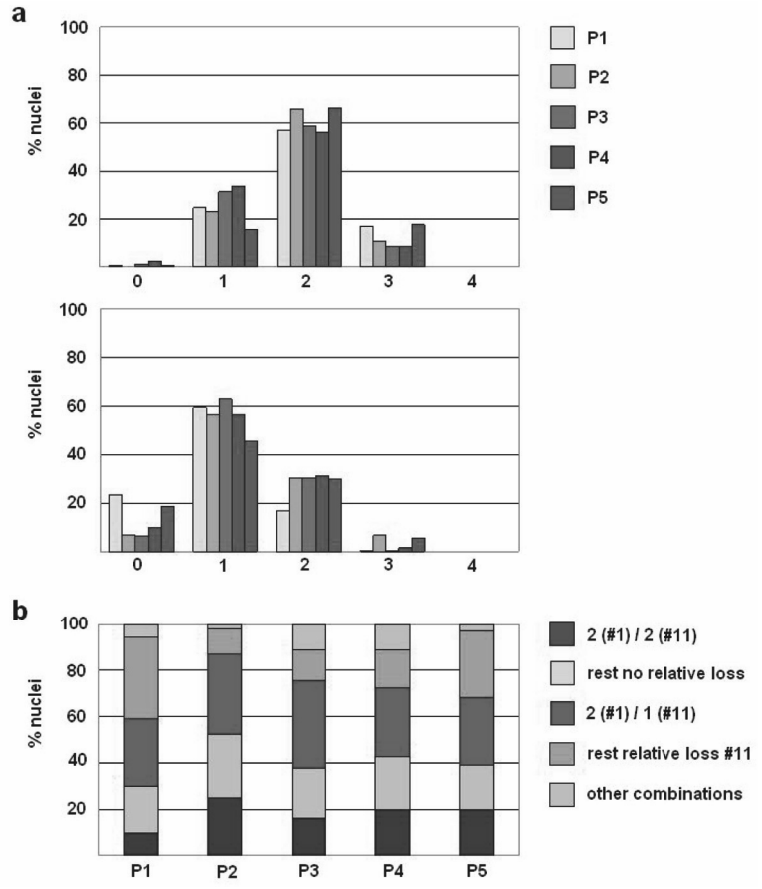

Figure 1. Results obtained from interphase FISH analysis of paraffin-embedded sections of five SDHD-linked paragangliomas (P1- P5). (**a**) Frequency distribution of signals obtained with the centromere 1 (PUC1.77) probe (upper panel) and the centromere 11 (pLC11A) (lower panel). Compared to chromosome 1, there is a clear loss of chromosome 11 centromere signals. More than two chromosome 1 signals are observed in 9-17% of the nuclei, indicating aneuploidy or tetraploidy. (**b**) Loss of centromere 11 relative to centromere 1 signals (red and orange) is observed in 46-65% of the nuclei. Loss of centromere 1 signals relative to centromere 11 ('other combinations') is 2-11%.

Next, we used BAC probes for the subtelomeric regions of 11p and 11q, with the centromere 1 probe as a reference (Figure 3b and d). Concomitant loss of both probes located on chromosome 11 relative to centromere 1 was found in 26-70% of paraganglioma and 23-54% of pheochromocytoma nuclei (Figure 4b). In both triple-color experiments, loss of one of the two probes located on chromosome 11 relative to the other was observed in only a small minority of nuclei (1-7% and 2-4%, respectively), demonstrating that the

observed relative loss of chromosome 11 involves the entire copy. Thus, relative loss of chromosome 11 signals was observed in all 23 tumors, ranging from 23 to 70% (mean = 40%).

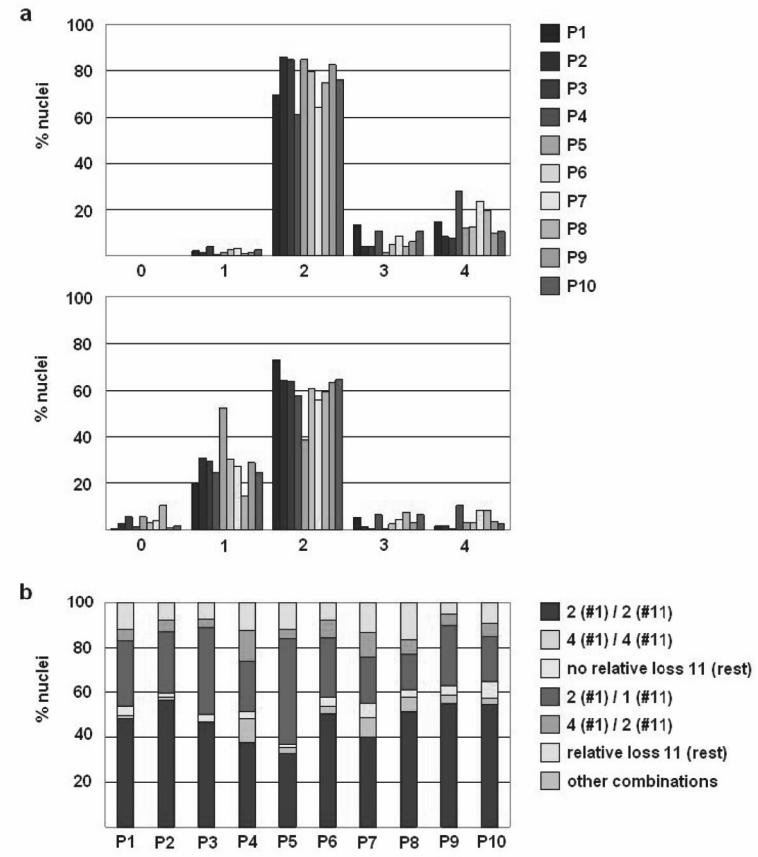

Figure 2. Interphase FISH results from isolated whole nuclei of 10 SDHD-linked paragangliomas (P1- P10). (**a**) Frequency distribution of signals obtained with the centromere 1 (PUC1.77) probe (upper panel) and the centromere 11 (pLC11A) probe (lower panel). Compared to chromosome 1, there is a clear loss of chromosome 11 centromere signals. More than two chromosome 1 signals are observed in 12-40% of the nuclei, indicating aneuploidy or tetraploidy. (**b**) Loss of centromere 11 relative to centromere 1 signals (red and orange) is observed in 35-63% of the nuclei. Loss of centromere 1 signals relative to centromere 11 ('other combinations') is negligible (0-1%).

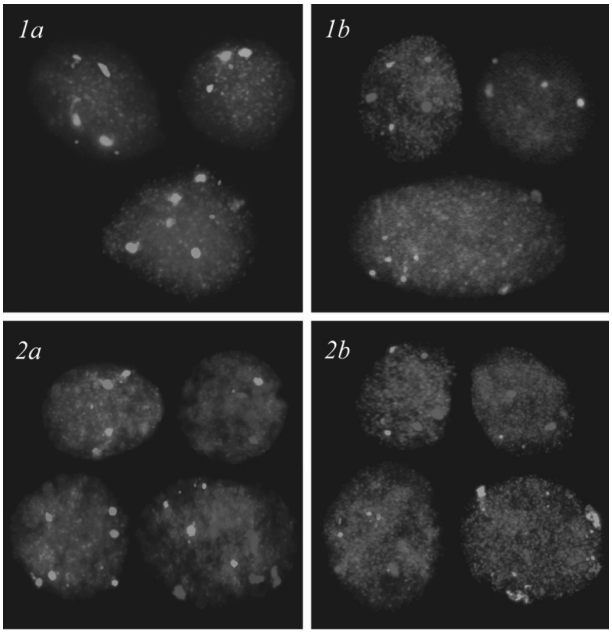

Figure 3. Triple colour FISH on whole nuclei isolated from paraffin-embedded tissue. Probe/colour combinations are centromere 11 (pLC11A, green), centromere 1 (PUC1.77, blue) and 11q23 (RP11-3F7, red) **(1a, 2a)**, and subtelomere 11p (RP11-645I8, green), subtelomere 11q (RP11-469N6, red) and centromere 1 (blue) **(1b, 2b)**. Each panel is a composite of individually captured nuclei. **(1a)** Paraganglioma cell nuclei. Top left : diploid nucleus with two signals for each probe, top right: monosomy for chromosome 11, bottom: tetraploidy for centromere 1 and diploidy for each chromosome 11 probe. **(1b)** Paraganglioma cell nuclei. Top left : diploid nucleus, top right: chromosome 11 monosomy and relative chromosome 11 loss in a tetraploid nucleus (bottom). **(2a)** Phaeochromocytoma cell nuclei. Top left : diploid nucleus, top right: chromosome 11 monosomy, bottom left : relative chromosome 11 loss in a tetraploid nucleus, bottom right: tetraploid nucleus without relative chromosome 11 loss. **(2b)** Phaeochromocytoma cell nuclei. Top left : diploid nucleus, top right: chromosome 11 monosomy, bottom left : relative chromosome 11 loss in a tetraploid nucleus, bottom right: a tetraploid nucleus without relative chromosome 11 loss.

To determine the parental origin of the lost chromosome 11, we performed LOH analysis on seven paragangliomas and two pheochromocytomas that were also analyzed by triple-color FISH. For these cases, patient- as well as parental PBL-derived DNA samples were available. LOH analysis was performed after tumor cell populations were enriched by fluorescence activated cell sorting (FACS) of the aneuploid $G_{0,1}$ fraction, or the often increased $G_{2,M}$ fraction of diploid tumors, with the diploid $G_{0,1}$ fraction as a reference[1,9]. We used three markers on 11p and five on 11q. In five paragangliomas and two pheochromocytomas, LOH analysis was informative for at least one marker on both chromosome arms. For two paragangliomas, the analysis was informative for only

one marker, either on 11p or 11q. In aneuploid- or $G_{2,M}$-cell populations, all evaluable LOH experiments showed loss of maternal alleles. As expected, retention of heterozygosity was not observed (Figure 5). In the diploid cell populations and patient PBL DNA samples, no LOH was found (data not shown).

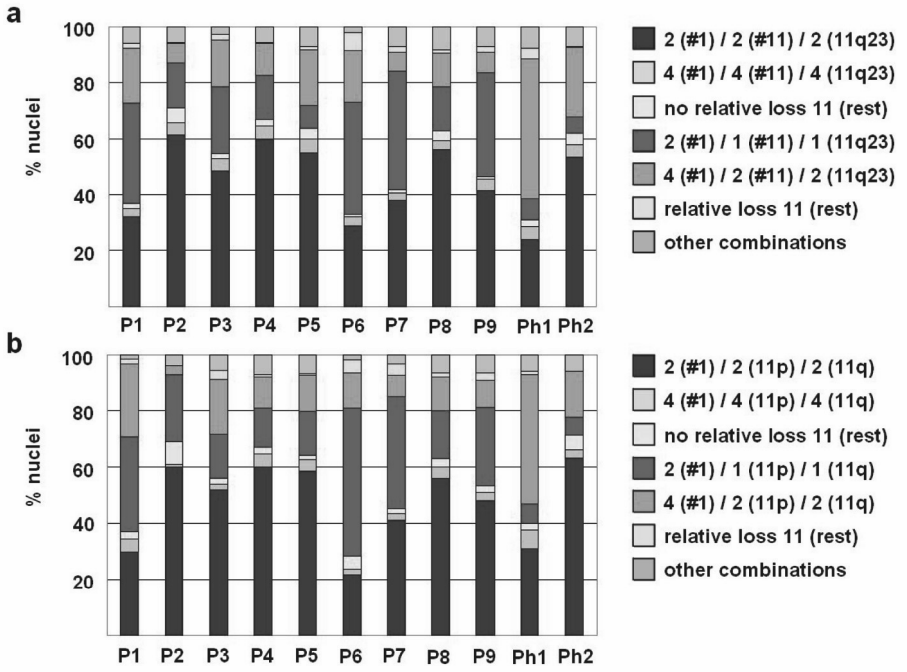

Figure 4. Counts of whole nuclei isolated from paraffin-embedded material of paragangliomas (P6-P14) and phaeochromocytomas (Ph1- Ph2), analysed by triple colour interphase FISH. (**a**) Results for centromere 11 (pLC11A), centromere 1 (PUC1.77) and 11q23 (RP11-3F7) probes. Simultaneous loss of both chromosome 11 probes relative to centromere 1 (red and orange) was observed in 24-65% of paragangliomas and 31-62% of phaeochromocytomas. (**b**) Results for centromere 1, subtelomeric 11p (RP11-645I8) and 11q (RP11-469N6) probes. Simultaneous loss of both chromosome 11 probes relative to centromere 1 (red and orange) was observed in 26-70% of paragangliomas and 23-54% of phaeochromocytomas. For each tumor, distributions are very similar in (a) and (b) indicating high reproducibility of the technique. Note that in both (a) and (b) nonsimultaneous loss of chromosome 11 probes or loss of centromere 1 signals relative to chromosome 11 signals (white) is infrequent (3-8% and 2-7%, respectively).

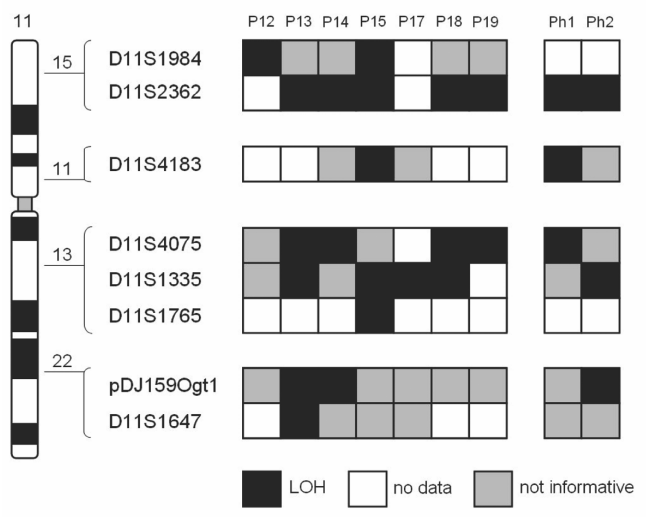

Figure 5. LOH analysis of sorted aneuploid $G_{0,1}$ or diploid $G_{2,M}$ fractions of isolated nuclei of paraffin-embedded paragangliomas (P12- P19) and phaeochromocytomas (Ph1- Ph2). LOH involved the maternal allele in all cases in which the parental origin of the lost allele could be assessed (black). Retention of heterozygosity was not found for any of the informative markers.

Discussion

The results obtained in this study demonstrate the loss of an entire copy of chromosome 11 in all investigated *SDHD*-linked paragangliomas. By LOH analysis, we were able to unequivocally demonstrate the maternal origin of the lost chromosome copy in a subset of seven paraganglioma and two phaeochromocytoma cases from which parental blood DNA samples were available. However, even without this direct proof, Knudson's two-hit model predicts that in case of paternal transmission of the germline mutation, loss of the wildtype maternal allele should have occurred in the tumor.

Although loss of a centromere 11 already indicates loss of the entire chromosome 11, we obtained additional evidence by the triple color FISH experiments with telomeric probes and the 3F7 probe containing the *SDHD* gene. Since it was not possible to accurately discriminate type I cells in the FISH experiments on isolated nuclei, the evaluation of an unselected sample of 200 nuclei unavoidably included non-neoplastic cells as well. This explains most of the variation in loss of chromosome 11 between the different cases and the concordance of the results obtained with different probe sets for the individual tumors (Figure 4). FISH on tissue sections, while permitting selection of type I cell nuclei, did not

yield significantly higher percentages of nuclei with relative chromosome 11 loss because of loss of signals from sliced nuclei. The latter problem would have seriously complicated, if not precluded, the interpretation of triple color FISH experiments on tissue sections and thus nuclear suspensions were used in all further experiments.

The selective loss of the entire maternal chromosome 11 explains why *SDHD*-linked tumors appear to arise only upon paternal transmission of the mutation, even though the *SDHD* gene itself is not imprinted. The latter is supported by the observed biallelic expression of *SDHD* in several human tissues[2]. Although it is not uncommon for the somatic 'second hit' in the Knudson model of tumorigenesis to involve a gross chromosomal mechanism such as non-disjunctional chromosome loss, it is intriguing that in *SDHD*-linked paragangliomas this appears to be the preferred mechanism for the second hit. We hypothesize that a second target gene on chromosome 11, which is subject to genomic imprinting, is involved in tumor formation. A growth advantage is gained when the wild-type maternal *SDHD* allele on 11q23 and the active maternal copy of this second, paternally imprinted gene are lost simultaneously. As the only region known to harbor an imprinted gene cluster on chromosome 11 is 11p15, we further hypothesize that this second gene is located here. Within that model, the most parsimonious mechanism would be a single event, viz. the loss of the entire maternal chromosome 11 copy in case of a maternal wt *SDHD* allele and paternal inheritance of the *SDHD* mutation (Figure 6a). Loss of the maternal wt *SDHD* allele only, for example, by loss of a part of 11q, would not target the second tumor-suppressor gene on 11p15 and therefore not lead to tumor formation (Figure 6b). In case of maternal inheritance of the *SDHD* mutation, loss of paternal alleles would not lead to tumor formation for the same reason (Figure 6c and d). At least two events caused by different chromosomal mechanisms will be required to inactivate both *SDHD* and the imprinted gene on 11p15 when *SDHD* is maternally transmitted. These are successive loss of the paternal wt *SDHD* allele by, for example, mitotic recombination, followed by loss of the recombined paternal chromosome containing the paternal 11q23 region and the maternal 11p15 region (Figure 6e). Given the evidence for complex LOH mechanisms in solid tumors, it is somewhat surprising that the probability of this occurring in paraganglioma formation appears to be very low or zero, since no cases of maternal transmission have been reported to date[10,11]. One explanation might be that the number of cell divisions in normal paraganglia is simply too low since in most head and neck paragangliomas the growth fraction is lower than 1%[12]. Selective loss of the whole maternal chromosome 11 would explain the exclusive paternal transmission of disease in paraganglioma linked to the PGL2 locus as well, because it is also located on 11q[5]. It would also explain the absence of generation skipping of tumor susceptibility in *SDHB* (1p36-p35)- and *SDHC* (1q21)-linked families. In the latter two, loss of the maternal 11p15 region is probably

also essential for tumor development, since *SDHB*, *SDHC* and *SDHD* encode subunits from the same mitochondrial complex. Dannenberg et al. detected loss of 11p in two out of nine sporadic paragangliomas by comparative genomic hybridization, but the mutation status of *SDHB*, *SDHC* or *SDHD* was not investigated[8]. Furthermore, loss of 11p has been reported in 45% of 11 sporadic abdominal paragangliomas[13]. Since data on the parental origin of the 11p losses are lacking, a major role for loss of maternal 11p in sporadic paragangliomas, although likely, still remains to be proven.

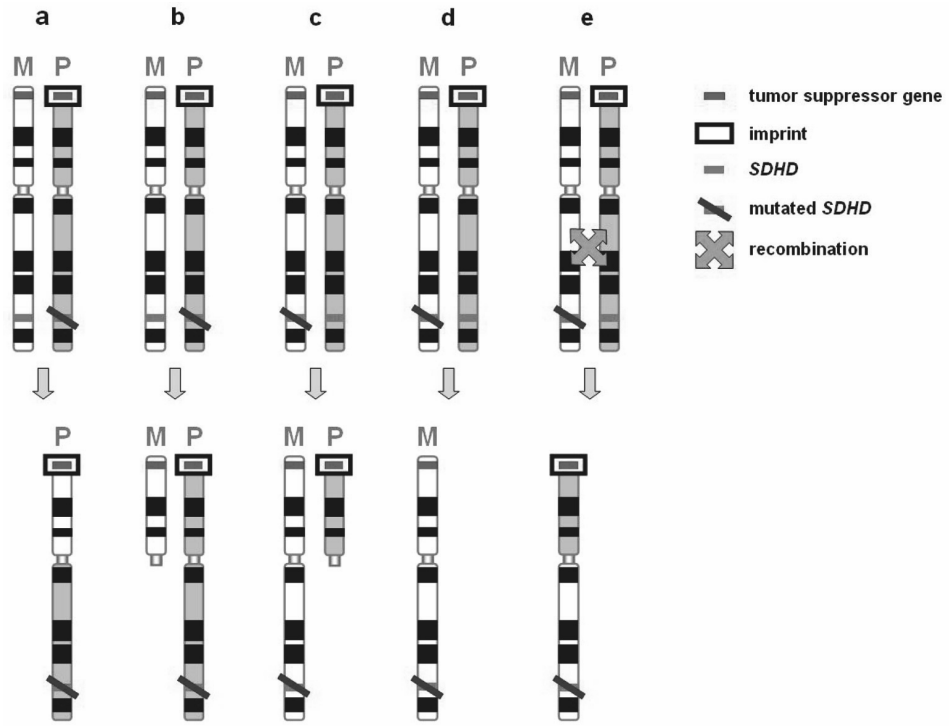

Figure 6. Model for the imprinted transmission of SDHD-linked paraganglioma. Maternal (white) and paternal (grey) chromosomes are depicted. (**a**) Both the maternal 11q region, containing the wt SDHD allele, and the maternal 11p region, containing the active tumor suppressor allele, are targeted. In case of an event targeting only the wt maternal SDHD allele on 11q (**b**), the active maternal tumor suppressor allele on 11p15 is not affected and tumor development is inhibited. In case of maternal inheritance of the SDHD mutation, a second hit targeting the wt paternal allele by, for example, a deletion of the paternal 11q region (**c**) or even the whole paternal chromosome 11 (**d**) will leave the maternal 11p15 region intact and tumor formation is not initiated. When the SDHD mutation is maternally transmitted, at least two events caused by different chromosomal mechanisms will be required to inactivate both the wt SDHD allele and the active maternal allele of the imprinted tumor-suppressor gene on 11p15, namely loss of the paternal wild-type SDHD allele by, for example, mitotic recombination, followed by loss of the recombined paternal chromosome containing the paternal 11q23 region and the maternal11p15 region (**e**). Apparently, this sequence of events is very unlikely in vivo.

Interestingly, a high percentage (86%) of loss of chromosome 11 was also found in 31/36 (86%) of Von Hippel-Lindau (VHL) related pheochromocytomas, of which 25/31 had loss of both 11p and 11q whereas six had only 11p loss[14]. The investigators suggested that this observation might indicate the involvement of a different but essential and complementary genetic pathway in VHL-linked pheochromocytoma tumorigenesis. The results of our study emphasize a role for loss of 11p, and in particular the maternal copy, in *SDHD*-linked pheochromocytoma formation as well. LOH of maternal 11p15, often with duplication of paternal 11p15, occurs frequently in human pediatric tumors including Wilm's tumors, embryonal rhabdomyosarcomas, hepatoblastoma and adrenocortical carcinomas[15,16].

There is convincing evidence that LOH of 11p15 leads to disruption of the regulation of expression of oppositely imprinted genes, in particular *H19* and *IGF2*, in a variety of tumors[15,16]. The *IGF2* gene product is a survival factor and strong mitogen that is overexpressed in a variety of human tumors including hereditary paragangliomas and pheochromocytomas[17]. *H19* codes for an untranslated RNA that acts a negative trans regulator of *IGF2* expression[18,19].

Disruption of imprinted expression of 11p15 has also been implicated in the Beckwith–Wiedemann syndrome and focal hyperplasia of Langerhans islets causing congenital hyperinsulinism (FoCHI)[20-22]. There is an interesting parallel between our findings of maternal chromosome 11 loss in hereditary paraganglioma and loss of maternal 11p15 in FoCHI[22]. This disease is caused by a paternally inherited, recessive mutation of the *ABCC8*- or *KCNJ11*-gene, which is located on 11p15.4, that is, outside the imprinted region. The lesions show a strongly decreased expression of *H19* and increased expression of *IGF2*. Thus, like in paraganglioma, a single somatic event targets the wild-type allele of a non-imprinted susceptibility gene on the maternal chromosome 11 as well as the maternally imprinted 11p15 region, and in both types of diseases this results in exclusive paternal transmission. Although the development of solid tumors in general is a multi-step genetic evolution process, it is unclear why tumor development in *SDHD* mutation carriers specifically requires loss of a putative maternally expressed tumor-suppressor gene, in addition to loss of wt *SDHD*. It has been speculated that the tumorigenic effects of *SDHD* inactivation might be explained by either mitogenic effects of elevated levels of reactive oxygen species or blocking of apoptosis due to mitochondrial dysfunction[23,24]. On the other hand, oxidative stress may trigger pro-apoptotic signaling and create a selection pressure for mutational activation of anti-apoptotic pathways. Since the IGF pathway has found to be involved in anti-apoptotic signaling, loss of the maternally expressed

H19 gene, a known suppressor of *IGF2*, might be an essential step in paraganglioma development[25,26].

SDHD-linked paraganglioma is a striking, and to our knowledge, first example of the effect of allelic phasing on the penetrance of a hereditary tumor syndrome in man. Recently, allelic phasing of mouse chromosome 11 deficiency was found to influence p53 tumorigenicity[27]. The deletion on chromosome 11 elevated the tumor susceptibility and modified the tumor spectrum when in trans with the p53 mutation. Many genes display differential expression of parental alleles, due to genomic imprinting or genetic regulation[28]. Conceivably, a certain dosage ratio of cancer-related alleles, which are coincidentally located on the same chromosome in cis-configuration, may provide a selective growth advantage. The tumorigenic potential of acquired chromosome aneuploidy, a hallmark of many solid tumors, would then be dependent on the allelic phasing or imprinting status of these genes. Our study provides a clear-cut example of this mechanism, which might also apply to an individual's overall susceptibility to more common forms of cancer.

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Chapter 8

Summary and conclusion

Summary

Conclusion

Future perspectives

Summary

Chapter 1 consists of a review of the present knowledge of the clinical characteristics, the genetics, heredity and tumor biology of paragangliomas and pheochromocytomas.

Chapter 2 is a review of the literature on recent advances in the understanding of the genetics of paragangliomas. Current insights as well as future directions are discussed, showing that major progress has been made in this field since the discovery of mutations in SDH genes as a cause of paraganglioma syndrome.

Chapter 3 presents an overview of the relative frequency of mutations in SDH genes that are associated with paraganglioma-pheochromocytoma syndrome in the Netherlands. In this study, we find that the large majority of mutations in SDH subunits or co-factors involve SDHD, followed by SDHAF2 and SDHB, whereas SDHC mutations are extremely rare. In addition, we found that the overwhelming majority of SDH-mutation carriers in the Netherlands carry one of only 6 Dutch founder mutations in SDHAF2, SDHB and SDHD. Out of these 6 founder mutations, the p.Asp92Tyr founder mutation in SDHD is by far the most prevalent, accounting for 69% of all Dutch SDH mutation carriers. Both the dominance of SDHD founder mutations and the limited genetic heterogeneity among SDH mutation carriers are unique to the Netherlands.

Chapter 4 consists of a study of the mutation status and clinical characteristics of a series of 236 Dutch head and neck paraganglioma patients treated at the Leiden University Medical Center. In line with the findings in chapter 3, this Dutch patient series is characterized by a high prevalence of *SDHD* mutations. Contrasting with studies performed in other European countries, the majority (80%) of the patients in this cohort present with a family history positive for paraganglioma syndrome. Surprisingly, we find that even in patients with a negative family history for paragangliomas, hereditary forms of the paraganglioma syndrome are found in the majority of cases. In this patient group too, the disease is frequently linked to mutations in the *SDHD* gene.

The clinical consequences of SDHD mutations are also evaluated in this chapter: an early mean age at onset of paraganglioma syndrome of 38 years, a high risk of multiple paragangliomas (73%), a risk of concurrent pheochromocytomas (13%) and extra-adrenal paragangliomas (8%), and a small risk of metastatic disease (2%). Carriers of mutations in SDHAF2, SDHB and SDHC are also identified in this patient series, as well as patients without a mutation in any of these genes, but these subgroups constitute a small minority of the Dutch head and neck paraganglioma population. We argue that the high prevalence

of Dutch SDHD founder mutations, as well as the small numbers of SDHB-linked and SDH mutation-negative cases imply that the prevalence of paraganglioma syndrome may be higher in the Netherlands than elsewhere.

In **chapter 5**, a large, multigenerational Dutch paraganglioma family linked to the D92Y (also p.Asp92Tyr or c.274G>T) founder mutation in SDHD is described. The SDHD.D92Y mutation is the dominant cause of head and neck paragangliomas in the Netherlands. As all mutation carriers in this family carry the same mutation, we were able to describe its phenotype in detail, and found that it does not differ much from the phenotypes of other *SDHD* mutations. In addition, by including a large number of asymptomatic family members, we were able to make accurate calculations of the penetrance of this founder mutation, both for the occurrence of paragangliomas as well as for symptomatic disease. We found, in accordance with our expectations, no maternal transmission of SDHD-linked disease. We found that a paternally transmitted mutation confers a high lifetime risk of paragangliomas of 87%, however this is lower than previous estimates. Moreover, we found that the life time risk of developing paraganglioma-associated symptoms is considerably lower (57%).

Chapter 6 comprises of a gene expression study, comparing the expression levels of more than 8.000 genes in SDHD-linked, PGL2-linked and sporadic paragangliomas. At the time of analysis, the exact identity of the *PGL2* gene was unknown, and an attempt was made to define its function and thus clarify its identity on the basis of a distinctive gene expression profile. However, no significant differences could be identified in the gene expression of these genetic subgroups. Even in selected subsets of genes that are known or suspected to play a role in the pathways that lead to paraganglioma tumorigenesis, no differences could be found. We therefore hypothesized that this might be because *SDHD* and *PGL2* mutations exert a similar effect on the functionality of succinate dehydrogenase. We now know, since the identification of *SDHAF2* as the *PGL2* gene and the discovery of its role in SDH activity, that this is indeed the case.

In **chapter 7**, a model is put forward to explain the peculiar inheritance pattern in SDHD-linked paragangliomas and pheochromocytomas. It has been known for some time that tumors almost never occur in *SDHD* mutation carriers that have inherited the mutation via their mother. However, if the same mutation is inherited via the father, the risk of developing paraganglioma syndrome is very high. This mode of inheritance causes paraganglioma syndrome to skip generations and is consistent with maternal imprinting of the *SDHD* gene. However, methylation or imprinting of the SDHD gene itself has never been established, and bi-allelic expression of *SDHD* has been demonstrated in non-

paraganglion tissues. In addition, SDHD acts as a tumor suppressor gene in paraganglioma syndrome, i.e. the loss of the wild-type SDHD allele is a prerequisite for paraganglioma development, which would be counter-intuitive if the wild type allele was already silenced by methylation.

In this study, we observe that in SDHD-linked paragangliomas and pheochromocytomas, the LOH does not only target the wild-type *SDHD* allele, but involves the whole maternal chromosome 11, suggesting that the loss of another gene on chromosome 11 is essential for paraganglioma development. As this somatic loss consistently affects the maternal chromosome 11 copy, it is likely that this other gene is exclusively maternally expressed, thus paternally imprinted. These conjectures all point in the direction of genes located within the 11p15.5 region, a major imprinted gene cluster in the human genome, and we hypothesize that a paternally imprinted gene on 11p15.5 acts as an additional tumor suppressor in SDHD-linked paraganglioma syndrome.

According to this model, loss of the wild-type *SDHD* allele alone is insufficient for tumor formation in *SDHD* mutation carriers. Only upon loss of both the wild-type *SDHD* allele on 11q23 and the active maternal copy of a tumor suppressor gene on 11p15.5, tumor formation will occur. In paternally, but not in maternally derived *SDHD* mutation carriers, this can be achieved by a single event: non-disjunctional loss of the maternal chromosome 11 (chapter 1, figure 6). The virtually exclusive paternal transmission of the disease can be thus explained by a somatic mechanism targeting both the wild type *SDHD* gene on 11q23 and the maternal copy of a paternally imprinted gene on 11p15.5, rather than imprinting of SDHD itself. This model could explain the parent-of-origin-dependent inheritance in SDHAF2-linked paraganglioma as well, as SDHAF2 is also located on the long arm of chromosome 11. It furthermore leaves room for maternal inheritance of disease, as other mechanisms inactivating both the wild type *SDHD* allele and the maternal 11p15.5 region could also cause tumor formation, however, maternal transmission is predicted to occur very rarely as this would require more complex somatic rearrangements.

Conclusion

Since the discovery of mutations in SDH genes as the cause of hereditary head and neck paragangliomas in the year 2000, great progress has been made in the identification of pathogenic mutations, the description of phenotypic differences in between the causative genes, and the understanding of the molecular biology linking SDH defects with neoplastic growth.

In this thesis, the relative importance of the pathogenic mutations in the SDH genes in the Netherlands is elucidated, revealing a remarkable role of founder effects, especially in *SDHD*, but also in *SDHB* and *SDHAF2*. The prevalence of Dutch founder mutations has been recognized before, but their absolute dominance, especially of the SDHD.D92Y mutation, and the relative low numbers of other SDH mutations in the Dutch population represent a new insight. We argue that these findings may underlie an increased prevalence of head and neck paragangliomas in the Netherlands.

A comprehensive understanding of the natural course of the disease and the risk of developing multifocal, adrenal, metastatic, or symptomatic disease is important in the clinical decision making in head and neck paraganglioma patients. As complete eradication of paragangliomas is not always possible or may confer a high risk of morbidity, especially in bilateral disease, the consequences of any treatment must always be weighed against the consequences of no intervention. By studying a large patient cohort and an extended paraganglioma family, we were able to characterize SDHD-linked paraganglioma patients, and thus the majority of the Dutch head and neck paraganglioma population, by an early mean age at diagnosis (26.5-37.9 years), a high rate of multiple tumors (65-74%), an intermediate risk of concurrent pheochromocytomas (8-21%), and a low risk of malignancy (2-3%). In addition, we found that whereas mutations in SDHD confer a high lifetime risk of developing a paraganglioma, not all paraganglioma patients develop tumor-related symptoms. Therefore, bearing in mind the words of Le Compte ("*the greatest danger to these patients is the treatment rather than the disease"*), a conservative treatment strategy seems appropriate in the majority of Dutch head and neck paraganglioma patients.

As the Dutch SDHD-linked phenotype does not differ significantly from the SDHD-linked phenotype found elsewhere in Europe or the United States, we furthermore conclude that the high prevalence of Dutch founder mutations in SDHD is a reflection of specific aspects of the Dutch demography and socio-economic history, rather than a result of environmental factors such as residential altitude.

Another important feature of SDHD-linked paragangliomas is the virtually absent maternal transmission of the disease. We have shown that the 'second hit' in SDHD-linked paragangliomas involves not only the wild type SDHD allele but the whole maternal chromosome 11 copy, suggesting a model that involves the combined loss of the wild type *SDHD* allele and a maternally expressed, paternally imprinted tumor suppressor located on 11p15.5 as an essential step in SDHD-linked paraganglioma formation. The almost exclusive paternal transmission of disease would then be the result of the colocation of this imprinted tumor suppressor and the wild type *SDHD* allele on the maternal copy

of chromosome 11. As of yet, the paraganglioma tumor suppressor on 11p15.5 has not been identified with certainty, but if substantiated, this model explains the parent-of-origin dependent inheritance in the absence of imprinting of the *SDHD* gene itself. The fact that exclusive paternal inheritance of disease is also found in SDHAF2-linked paraganglioma families supports this model, as *SDHAF2*, like *SDHD*, is located on the long arm of chromosome 11 (11q13).

The proposed model furthermore explains the observation that true maternal transmission of SDHD-linked disease is possible, but rare. Simultaneous loss of the wild type *SDHD* allele and the active tumor suppressor allele can be achieved in a single event in case of a paternally inherited *SDHD* mutation (by loss of the whole maternal chromosome 11 copy), whereas it would require at least 2 separate hits targeting separate regions and/or separate copies of chromosome 11 in case of a maternally inherited mutation, a sequence of events that is almost certainly less likely to occur in vivo. In support of this model, additional events targeting the maternal 11p15.5 region have indeed been identified in the recently reported rare occurrences of true maternal inheritance of SDHD-linked disease.

The model could also explain the higher penetrance of SDHAF2 and SDHD-linked disease as opposed to SDHB- and SDHC-linked disease. As explained above, SDHD- and SDHAF2-linked tumorigenesis may be initiated by a single event targeting the whole maternal chromosome 11 copy. Assuming that loss of the maternal tumor suppressor allele on 11p15.5 is a prerequisite for the development of all SDH-linked paragangliomas, paraganglioma formation would require at least 2 separate hits, targeting the maternal 11p15.5 region and the *SDHB* or *SDHC* wild type allele on chromosome 1 in SDHB- and SDHC-linked disease.

In broader terms, the model for the parent-of-origin-dependent inheritance of SDHD-linked paragangliomas illustrates the importance of the location of disease genes on the genome, and demonstrates that even in alleged monogenetic diseases, multiple genes may be involved as essential initiators of disease or modifiers of disease risk.

Future perspectives

In order to further clarify the problem of the parent-of-origin-dependent inheritance in SDHD- and SDHAF2-linked paragangliomas, future research is needed into the role of the 11p15.5 region both in SDHD- and SDHAF2-linked paragangliomas as well as in SDHB-, SDHC- and VHL-linked cases. The identification of the additional tumor suppressor gene or genes responsible for this phenomenon will almost certainly shed more light on the molecular mechanisms that underlie paraganglioma formation and probably help explain aspects of tumor behavior. In general, it will broaden our understanding of the significance of modifier genes for the occurrence and form of disease.

Paraganglioma research has improved our insight into the link between hypoxia regulation, metabolic disruptions and tumor formation. However, in spite of the progress made, some tantalizing questions still remain unanswered. It is presently unknown why germ line mutations in genes encoding SDH, a complex that is so vital to the energy supply of cells, preferably produce tumors in the paraganglion system, and do not (with the exception of SDHA mutations) cause a more generalized or severe disease phenotype. It is furthermore surprising that mutations in different subunits of the same complex (SDH), all resulting in SDH deficiency, give rise to quite distinct paraganglioma syndromes. On the other hand, it is equally surprising that mutations in genes with such different functions as the SDH genes and *TMEM127* or *MAX*, all cause the same tumor type. These unresolved issues illustrate the long way to go before the different, interacting molecular mechanisms that cause paraganglioma are unraveled. As hypoxia pathway signaling and the switch to aerobic glycolysis are characteristics of a large variety of neoplasms, elucidating these pathways may have ramifications beyond the field of paragangliomas and pheochromocytomas. Already, several agents have been identified that exert a possible anti-cancer effect through interaction with key components of the hypoxia pathway. By contributing to the expanding knowledge in this field, paraganglioma research will almost certainly continue to be a powerful example of the way in which the study of a rare condition illuminates basic principles in biological and pathogenic processes, and facilitates the discovery of causes and remedies of more common forms of disease.

Addendum

Samenvatting en conclusie

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Samenvatting en conclusie

Samenvatting

Hoofdstuk 1 bestaat uit een overzicht van de huidige stand van kennis met betrekking tot de klinische kenmerken, de genetica en de tumorbiologie van paragangliomen en feochromocytomen.

Hoofdstuk 2 is een overzicht van recente ontwikkelingen op het gebied van de genetica van paragangliomen. Zowel de huidige inzichten als de toekomstperspectieven worden besproken, waarbij de grote progressie in de kennis van de genetica van paragangliomen wordt belicht die is geboekt na de ontdekking van de succinaat-dehydrogenase (SDH) genen als veroorzakers van paragangliomen.

In **Hoofdstuk 3** wordt de relatieve frequentie van mutaties in de SDH genen die geassocieerd zijn met paragangliomen en feochromocytomen in Nederland beschreven. Uit deze studie blijkt dat in Nederland de overgrote meerderheid van deze mutaties het *SDHD* gen betreffen, gevolgd door mutaties in *SDHAF2* en *SDHB*, terwijl *SDHC* mutaties zeer zeldzaam zijn. Voorts wordt aangetoond dat het merendeel van de mutatiedragers één van slechts 6 Nederlandse foundermutaties dragen, en dat de Asp92Tyr foundermutatie in SDHD alleen al 69% van alle mutaties in SDH genen vertegenwoordigt. Zowel de dominantie van de foundermutaties in de Nederlandse populatie als ook het beperkte aantal verschillende mutaties dat hier wordt aangetroffen in SDH genen maken de Nederlandse situatie uniek.

Hoofdstuk 4 betreft een studie naar de genetische en klinische karakteristieken van een groot cohort van patiënten met een hoofdhals paraganglioom, behandeld in het Leids Universitair Medisch Centrum (LUMC). Het patiëntencohort wordt gekenmerkt door een hoge prevalentie van mutaties in het *SDHD* gen, hetgeen in lijn is met de bevindingen in hoofdstuk 3. In tegenstelling tot onderzoek uitgevoerd elders in Europa heeft een hoog percentage van de patiënten in dit cohort een positieve familie anamnese. Een opmerkelijke bevinding is dat in Nederland ook de meerderheid van de paraganglioom patiënten die zich presenteren met een negatieve familie anamnese een erfelijke vorm van de aandoening blijkt te hebben. Vaak is de onderliggende oorzaak ook dan een mutatie in het *SDHD* gen.

In dit hoofdstuk worden vervolgens de klinische consequenties van mutaties in het *SDHD* gen geëvalueerd: patiënten met een *SDHD* mutatie hebben bij het stellen van de diagnose een gemiddelde leeftijd van 38 jaar, een hoog risico op het ontwikkelen van meerdere

paragangliomen (73%), een risico op het ontwikkelen van een feochromocytoom (in 13%) of een extra-adrenaal paraganglioom (in 8%), en een klein risico op gemetastaseerde ziekte (2%). Naast patiënten met een SDHD mutatie worden in dit cohort ook SDHAF2-, SDHB- en SDHC- mutatiedragers en patiënten zonder mutatie in een SDH gen geïdentificeerd, maar zij vormen slechts kleine minderheden. Er wordt gesteld dat de hoge prevalentie van *SDHD* founder mutaties, alsmede de kleine aantallen *SDHB* mutatiedragers en paraganglioom patiënten zonder aantoonbare mutatie in een SDH gen, kunnen wijzen op een hogere prevalentie van hoofdhals paragangliomen in Nederland ten opzichte van wat globaal als gemiddelde prevalentie wordt aangenomen.

In **Hoofdstuk 5** worden meerdere generaties van een grote familie van dragers van de D92Y (ook wel aangeduid met p.Asp92Tyr of c.274G>T) mutatie in SDHD beschreven. Deze Nederlandse foundermutatie is de meest frequent voorkomende mutatie onder Nederlandse patiënten met een hoofdhals paraganglioom. Daar in deze studie alle aangedane familieleden dragers zijn van dezelfde mutatie, kan een goed beeld verkregen worden van het fenotype van deze mutatie. Het blijkt dat dit fenotype niet erg verschilt van het fenotype van andere SDHD mutaties of van SDHD mutaties in andere Europese landen. Tevens kan een accurate schatting van de penetrantie van de p.Asp92Tyr mutatie gemaakt worden, zowel voor het vóórkomen van paragangliomen alsook voor het optreden van gerelateerde symptomen, omdat in deze familie ook een groot aantal asymptomatische familieleden zijn getest op dragerschap. In deze familie zijn geen paragangliomen ontstaan na maternale overerving van de mutatie, conform de verwachting. Indien de mutatie van de vader wordt geërfd, blijkt de kans op het ontstaan van paragangliomen gedurende het leven echter zeer groot (87%), hoewel dit risico toch lager is dan in andere studies wordt gevonden. Het risico op het ontstaan van symptomatische paragangliomen is aanmerkelijk lager (57%).

Hoofdstuk 6 behandelt een genexpressie studie, waarin de genexpressie van 8.000 genen wordt onderzocht in sporadische, SDHD- en PGL2-geassocieerde paragangliomen. Op het moment dat deze studie werd uitgevoerd was de identiteit van het *PGL2* gen nog niet bekend, en in deze studie is getracht om aan de hand van verschillen in genexpressie de functie van dit gen af te leiden en zo het *PGL2* gen te identificeren. Er konden echter geen significante verschillen in het genexpressie patroon worden ontdekt tussen de bovengenoemde subgroepen. Zelfs in geselecteerde genensets, waarvan bekend is of wordt vermoed dat ze een rol spelen in de vorming van paragangliomen, konden geen verschillen worden aangetoond. Wij stelden dat dit veroorzaakt zou kunnen worden doordat mutaties in het *SDHD* gen en het *PGL2* gen een vergelijkbaar effect hebben op de activiteit van succinaat-dehydrogenase. Met de ontdekking van de identiteit van het

PGL2 gen als zijnde het *SDHAF2* gen, en het ophelderen van de rol die dit gen heeft in het functioneren van het succinaat-dehydrogenase complex, is inmiddels duidelijk geworden dat dit inderdaad het geval is.

In **Hoofdstuk 7** wordt een model voorgesteld dat het bijzondere overervingspatroon van SDHD-geassocieerde paragangliomen en feochromocytomen verklaart. Het is al enige tijd bekend dat paragangliomen niet of slechts zeer zelden ontstaan indien de verantwoordelijke mutatie in het *SDHD* gen wordt geërfd via de moeder, terwijl er wel een grote kans is op het ontstaan van paragangliomen als dezelfde mutatie wordt doorgegeven via de vader. Hierdoor kan het vóórkomen dat de aandoening generaties 'overslaat', terwijl de *SDHD* mutatie wel wordt doorgegeven. Deze wijze van overerving zou kunnen worden verklaard door maternale inprenting van het *SDHD* gen. Er zijn echter belangrijke bevindingen die pleiten tegen inprenting van het *SDHD* gen zelf: ten eerste is er geen methylatie (en dus geen daadwerkelijke transcriptieblokkade) van het *SDHD* locus op chromosoom 11 (11q23) aangetoond. Ten tweede komen elders in het lichaam (in andere weefsels dan de paraganglions) beide *SDHD* allelen tot expressie. Voorts wordt frequent het verlies van het wild type *SDHD* allel geobserveerd (ook wel 'loss of heterozygosity', of LOH genoemd), hetgeen tegen inprenting van het gen pleit omdat LOH geen selectief voordeel voor tumorgroei oplevert als het wild type allel door inprenting toch al niet tot expressie zou komen.

Uit het onderzoek dat wordt behandeld in dit hoofdstuk blijkt dat in SDHD-geassocieerde paragangliomen niet alleen het wild type *SDHD* allel verloren gaat, maar dat er sprake is van selectief verlies van de gehele maternale kopie van chromosoom 11. Dit somatische verlies van het gehele chromosoom suggereert dat een ander gen, gelegen elders op chromosoom 11, een essentiële rol speelt bij de tumorvorming. Omdat het verlies steeds het gehele chromosoom betreft, en niet alleen de 11q23 regio waar het *SDHD* gen is gelegen, lijkt het waarschijnlijk dat dit gen zich aan de andere zijde van het centromeer op de korte arm van chromosoom 11 (11p) bevindt. Voorts is het aannemelijk dat alleen de maternale kopie van dit gen tot expressie komt, omdat juiste de maternale kopie selectief verloren gaat. De hypothese stelt derhalve dat dit tweede gen functioneert als additioneel tumor suppressor gen en aan paternale inprenting onderhevig is. Dit wijst erop dat het gen gelokaliseerd moet worden in de 11p15.5 regio, een gebied dat bekend staat vanwege het uitgebreide cluster van ingeprente genen dat er gelegen is.

Het model voorspelt dat het verlies van het wild type *SDHD* allel op 11q23 op zich onvoldoende is voor de initiatie van tumorgroei, en dat tumoren zich uitsluitend vormen als ook het actieve maternale allel van de tumor suppressor op 11p15 verloren gaat.

Alleen wanneer de mutatie in het *SDHD* gen via de vader wordt geërfd, kan dit simultane verlies van deze twee genen gerealiseerd worden in één enkele stap, namelijk het verlies van het gehele maternale chromosoom 11, bijvoorbeeld ten gevolge van non-disjunctie tijdens celdeling (zie ook hoofdstuk 1, figuur 6). De vrijwel exclusief paternale overerving van de ziekte wordt met dit model dus niet verklaard door inprenting van het *SDHD* gen zelf, maar door een somatisch mechanisme dat zowel het *SDHD* gen op 11q23 als een paternaal ingeprent tumor suppressor gen op 11p15 treft. Met dit model kan tevens het gelijkende overervingspatroon in SDHAF2-geassocieerde paragangliomen verklaard worden, daar *SDHAF2* ook op de lange arm van chromosoom 11 (11q13) is gelegen. Het model laat voorts ruimte voor maternale overerving van SDHD-geaccocieerde ziekte, omdat andere mechanismen volgens dit model ook tot tumorvorming kunnen leiden, mits zij zowel het wild type SDHD allel als het maternale allel van de additionele tumor suppressor op 11p15 treffen. Aangezien er voor deze weg naar tumorvorming volgens het model meerdere complexe stappen nodig zijn, maakt het model daarbij inzichtelijk dat ware maternale overving van paragangliomen zeldzaam is.

Conclusie

Sinds de ontdekking in het jaar 2000 van mutaties in SDH genen die hereditaire hoofdhals paragangliomen veroorzaken, is er veel vooruitgang geboekt in het identificeren van pathogene genmutaties, het beschrijven van verschillende fenotypen, en het begrip van de moleculair biologische mechanismen die tumorgroei als gevolg van defecten in SDH kunnen verklaren.

In dit proefschrift wordt het relatieve aandeel van de verschillende pathogene SDH mutaties in Nederland onderzocht, waarbij een grote invloed van Nederlandse foundermutaties wordt gevonden, met name in het *SDHD* gen. Hoewel reeds eerder gewag werd gemaakt van een hoge prevalentie van foundermutaties, vertegenwoordigen de absolute dominantie van met name de SDHD.D92Y mutatie, en het relatief geringe aantal andere mutaties in SDH genen nieuwe inzichten met betrekking tot de genetica van hoofdhals paragangliomen in Nederland. Er wordt gesteld dat deze beide bevindingen kunnen duiden op een verhoogde prevalentie van hoofdhals paragangliomen in Nederland.

Een goed begrip van het natuurlijk beloop van de aandoening en het risico op het ontstaan van symptomen, multipele tumoren, feochromocytomen en metastasen is van essentieel belang voor het maken van gedegen klinische afwegingen bij patiënten met een hoofdhals paraganglioom. Daar complete eradicatie van een paraganglioom niet altijd mogelijk is en

gepaard kan gaan met een hoog risico op therapiegerelateerde morbiditeit, zeker in het geval van bilaterale tumoren, moeten de consequenties van een behandeling altijd zorgvuldig gewogen worden tegen de consequenties van een expectatief beleid. Door het bestuderen van een groot cohort van paraganglioom patiënten en een grote paragangliomen familie, hebben wij de karakteristieken kunnen evalueren van SDHD-geassocieerde ziekte, en dus van de meest voorkomende vorm van erfelijke paragangliomen in Nederland. De SDHD-geassocieerde patiënten populatie wordt gekenmerkt door een gemiddelde leeftijd bij diagnose van 26.5-37.9 jaar, het veelvuldig vóórkomen van multipele paragangliomen (in 65-74%), een risico op het ontstaan van feochromocytomen van 8-21%, en een klein risico van 2-3% op het ontstaan van gemetastaseerde ziekte. Tevens kwam uit deze studies naar voren dat hoewel het risico op het ontstaan van paragangliomen voor dragers van een paternaal overerfde SDHD mutatie zeer hoog is, het risico op paraganglioom-geassocieerde symptomen aanmerkelijk lager ligt. In de Nederlandse situatie lijkt daarom, de woorden van Le Compte indachtig ("*the greatest danger to these patients is the treatment rather than the disease"*), een conservatieve behandelingstrategie voor het merendeel van de patiënten gerechtvaardigd.

Daar het Nederlandse fenotype niet wezenlijk verschilt van het SDHD-geassocieerde fenotype dat gevonden wordt elders in Europa of de Verenigde Staten, is het onwaarschijnlijk dat de hoge prevalentie van foundermutaties in Nederland te maken heeft met omgevingsfactoren zoals de ligging op zeeniveau, maar lijkt dit veeleer het gevolg van specifieke aspecten van de Nederlandse demografie en sociaaleconomische geschiedenis.

Een ander opvallend kenmerk van SDHD-geassocieerde paragangliomen is de in hoofdstuk 7 beschreven exclusieve paternale overerving van de ziekte. In dit proefschrift wordt aangetoond dat in SDHD-geassocieerde tumoren niet alleen het wild type *SDHD* allel maar het gehele maternale chromosoom 11 verdwijnt, hetgeen suggereert dat tumorvorming alleen optreedt bij het gecombineerde verlies van het wild type *SDHD* allel en een exclusief maternaal tot expressie komend tumor suppressor gen. Dit model verklaart het opvallende overervingspatroon door het tezamen vóórkomen van zowel de actieve kopie van deze ingeprente tumor suppressor, alsook het wild type *SDHD* allel op het maternale chromosoom 11. Hoewel tot op heden deze tumor suppressor nog niet met zekerheid is geïdentificeerd zou dit model het overervingspatroon kunnen verklaren in afwezigheid van inprenting van het *SDHD* gen zelf. Het feit dat hetzelfde overervingspatroon wordt aangetroffen in families paragangliomen geassocieerd met het SDHAF2 gen (eveneens gelegen op de lange arm van chromosoom 11) ondersteunt de hypothese.

Het model laat daarnaast ook ruimte voor maternale overerving van SDHD-geassocieerde ziekte in zeldzame gevallen. Het gelijktijdige verlies van zowel het wild type SDHD allel als de maternale, actieve kopie van een tumor suppressor gen kan worden gerealiseerd in 1 enkele stap in geval van een paternaal overerfde *SDHD* mutatie (namelijk door het verlies van het gehele maternale chromosoom 11), terwijl er tenminste 2 separate stappen voor nodig zijn in geval van een maternaal overerfde mutatie, stappen die bovendien verschillende regio's van chromosoom 11 en/of verschillende kopieën van chromosoom 11 zouden moeten treffen. Het is zeer aannemelijk dat dit laatste scenario in vivo veel minder frequent vóórkomt. Ondersteuning voor dit model wordt gevonden in de recent gerapporteerde gevallen van maternale overerving van SDHD-geassocieerde ziekte, omdat steeds, naast het verdwijnen van het wild type allel, een tweede verandering wordt aangetroffen in het 11p15.5 gebied, daar waar het geïmprinte genencluster op chromosoom 11 is gelegen.

Het model zou ook duidelijk kunnen maken waarom de penetrantie van mutaties in *SDHD* en *SDHAF2* (beiden gelegen op chromosoom 11) zo veel hoger is dan de penetrantie van mutaties in *SDHB* en *SDHC* (beiden gelegen op chromosoom 1). Zoals boven beschreven is in het geval van paternaal overerfde *SDHD* en *SDHAF2* mutaties slechts één gebeurtenis, namelijk het wegvallen van het maternale chromosoom 11, voldoende voor de initiatie van tumorgroei. Aangenomen dat het ook voor SDHB- en SDHC-geassocieerde tumorgroei van essentieel belang is dat een maternaal tumor suppressor allel op chromosoom 11 (11p15.5) wordt uitgeschakeld, zouden in SDHB- en SDHC-geassocieerde gevallen ook 2 stappen nodig zijn om dit te bewerkstelligen: één die het wild type *SDHB* of *SDHC* allel op chromosoom 1 uitschakelt, en een tweede die het maternale 11p15.5 gebied treft.
In meer algemene zin illustreert dit model het belang van de lokalisatie van pathogene genen op het genoom en toont het aan dat zelfs in aandoeningen waarvan wordt aangenomen dat ze monogenetisch zijn, meerdere genen betrokken kunnen zijn bij het bepalen van het risico op het ontstaan van ziekte.

Toekomstperspectieven

Teneinde het vraagstuk met betrekking tot de overerving van SDH geassocieerde paragangliomen verder op te helderen, is meer onderzoek nodig naar de rol van het 11p15.5 gebied, zowel bij het ontstaan van SDHD- en SDHAF2-, als bij SDHB-, SDHC- en VHL-geassocieerde paragangliomen. De identificatie van een gen in het 11p15 gebied dat als een aanvullende tumor suppressor functioneert bij de formatie van paragangliomen, zou vrijwel zeker ook meer licht werpen op de onderliggende moleculaire mechanismen in

het ontstaan en het gedrag van paragangliomen. Meer in het algemeen zou het veel inzicht kunnen geven in het belang van de locatie van een gen binnen het genoom en de invloed die andere genen kunnen hebben als modificatoren van ziekterisico en verschijningsvorm.

Het paragangliomen onderzoek heeft het inzicht doen toenemen in de moleculaire wegen die de regulatie bij hypoxie en het cellulaire metabolisme kunnen verbinden met tumorgroei. Ondanks deze progressie blijven er echter nog steeds veel prangende vragen onbeantwoord. Zo is het heden nog onbekend waarom mutaties in genen die onderdelen coderen van het succinaat-dehydrogenase, een complex dat zo cruciaal is in de energievoorziening van de cel, leiden tot tumoren in het paraganglion systeem, en niet (met uitzondering van SDHA) tot een veel ernstiger of meer gegeneraliseerd ziektebeeld. Ook is het verrassend dat mutaties in genen die allen onderdelen van hetzelfde succinaat-dehydrogenase complex coderen, en allen leiden tot een verstoorde functie van dit enzym, toch duidelijk verschillende fenotypen veroorzaken. Anderzijds is het opvallend dat mutaties in genen die zulke verschillende functies hebben als de SDH genen en bijvoorbeeld *TMEM127* of *MAX*, allemaal kunnen leiden tot de vorming van hetzelfde type tumor.

Deze onbeantwoorde vragen illustreren de lange weg die nog te gaan is in het ophelderen van de verschillende, onderling verbonden moleculaire mechanismen die aan de basis liggen van het ontstaan van paragangliomen en feochromocytomen. Daar hypoxische signalen en het inschakelen van de anaerobe glycolyse kenmerken zijn van een breed gamma van neoplasmen, kan het ophelderen van deze signaaltransductie routes ook consequenties hebben buiten het veld van paragangliomen en feochromocytomen. Op dit moment worden verschillende middelen geïdentificeerd die een mogelijk anticarcinogeen effect hebben juist door hun interactie met de hypoxische signaaltransductie route. Door bij te dragen aan de groeiende kennis op dit gebied zal het paragangliomen onderzoek vrijwel zeker een mooi voorbeeld blijven van de manier waarop juist het onderzoek naar een zeldzame conditie opheldering kan verschaffen in basale, algemeen geldende biologische en pathogene mechanismen, en de ontdekking van oorzaken en remedies tegen veel prevalentere vormen van ziekte mogelijk kan maken.

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Curriculum Vitae

Erik Frans Hensen was born in 1974 in the city of Arnhem, The Netherlands. In 1992, he graduated at the athenaeum (VWO) of the Lorentz Scholen Gemeenschap, and in the same year started his studies in medicine at the University of Amsterdam (UvA), The Netherlands. From 1994-1996 he was active as a student coach for the Faculty of Medicine of the UvA. In 1996, he was also appointed research coordinator by the Department of Gastroenterology of the Academic Medical Center (AMC), Amsterdam, The Netherlands. In 1999, he graduated Doctor of Medicine *cum laude,* and started as a resident at the Department of Surgery of the Onze Lieve Vrouwe Gasthuis (OLVG) in Amsterdam. In 2001, he started the research for this thesis as a research-resident at the Department of Otolaryngology and Head and Neck Surgery of the Leiden University Medical Center (LUMC). In 2002, he was awarded a scholarship by the Dutch Cancer Society (KWF) for the extent of a year, during which he performed a study into the gene expression of head and neck carcinomas. He trained as a resident at the Department of Otolaryngology and Head and Neck Surgery of the LUMC from 2003-2008, under the supervision of Prof. Dr. J.J. Grote, Prof. Dr. R.J. Baatenburg de Jong, and Prof. Dr. ir. J.H.M. Frijns, consecutively. Part of the training was completed at the Medisch Centrum Haaglanden (MCH) Westeinde, The Hague, under supervision of Dr. J.P. Verschuur, and at the Rijnland Ziekenhuis, Leiderdorp, under supervision of Dr. J.H. Hulshof. From 2008, he is appointed as an otolaryngologist with a special interest in otology at the Department of Otolaryngology and Head and Neck Surgery of the VU Academic Medical Center (VUmc), Amsterdam, The Netherlands.

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