PRIMARY VASCULAR TUMOURS OF BONE:
TOWARDS A NEW CLASSIFICATION BASED ON PATHOLOGY AND GENETICS

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Wijsheid vindt men in de boeken
Wijs zijn zal men verder zoeken

Guido Gezelle (1830 - 1899)

Aan Ricky, Lysander en Aeneas

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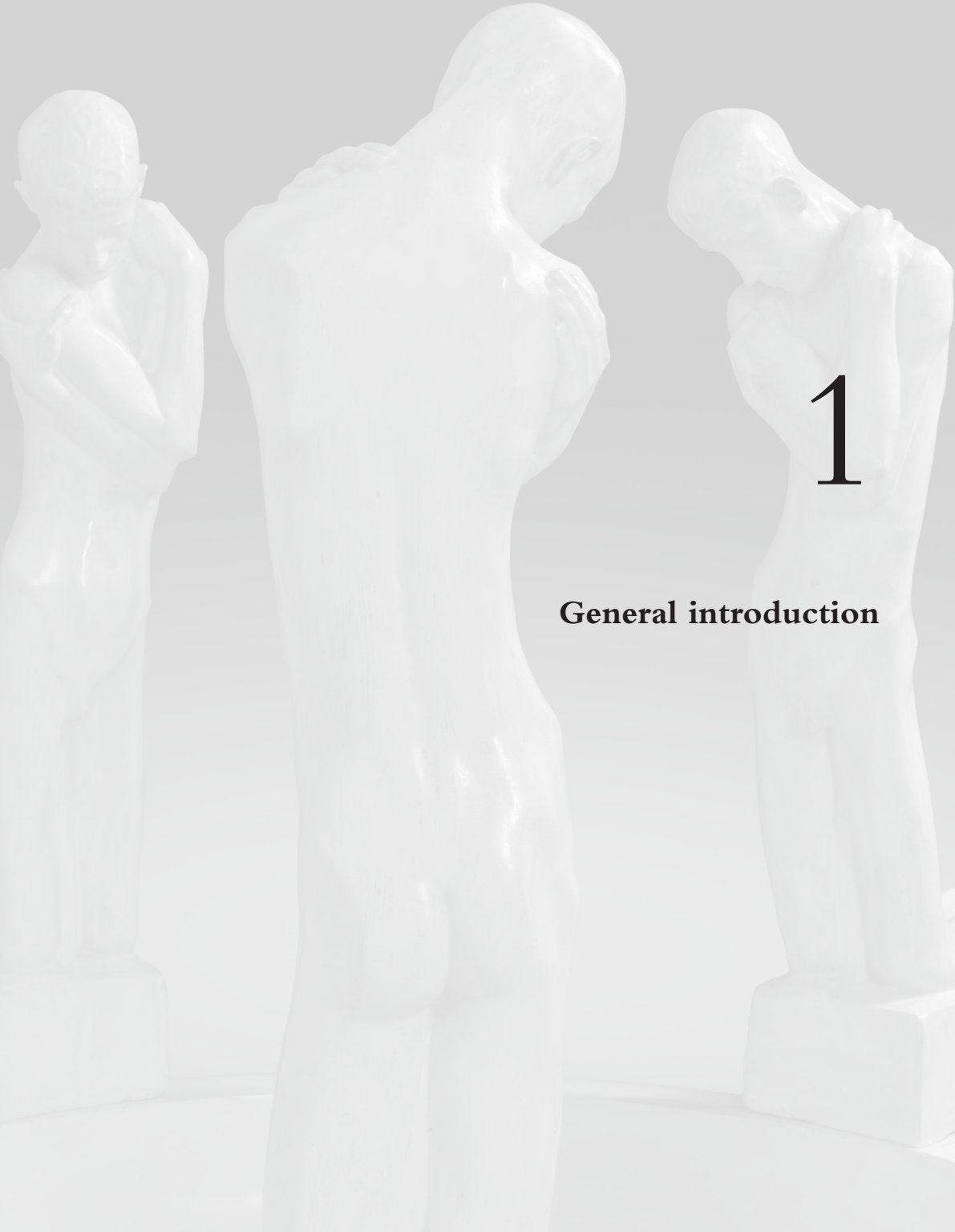

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General introduction

1

I. Embryology: the vascular system and endothelial cells

The vertebrate circulatory system is formed by two distinct processes during embryogenesis. At first, there is vasculogenesis, defined as de novo blood vessel formation by differentiation of mesoderm-derived endothelial precursor cells [(haem)angioblasts][1, 2]. This is followed by angiogenesis, which includes the proliferation of endothelial cells and formation of new blood vessels from pre-existing vessels[1,2].

The vascular system, consisting of blood vessels and the heart, is the first identifiable structure in the developing embryo[3,4]. The first signs of blood vessel formation occur in the gastrulation stage at day 7.5 in the blood islands of the yolk sac[1,5]. Subsequently, the blood island fuses and forms an immature vascular network, known as the primary plexus. In a next phase, vascular remodelling in the yolk sac leads to the formation of the complex yolk sac vasculature[1,2]. At the same time, angioblasts aggregate into solid endothelial strands along both sides of the neural tube in the embryo[2,5]. They differentiate into the dorsal aorta, vitelline vessels and primary plexuses of lungs, spleen and heart[1]. Although the exact embryogenesis is not fully elucidated, it is clear that these two processes are driven by different signalling pathways and transcriptional regulators. Fibroblast growth factor 2 (FGF2), bone morphogenetic protein 4 (BMP4), its downstream target Indian Hedgehog (IHH), vascular endothelial growth factor A(VEGF-A) and E-twenty six (ETS) transcription factors are key players in vasculogenesis and are important for the differentiation of endothelial and hematopoietic cells[6]. VEGF-A, retinoic acid, transforming growth factor beta (TGF-b), Notch and VEGF signalling pathways are more important during endothelial cell proliferation and angiogenic sprouting, which is also known as angiogenesis[6].

II. Endothelial markers

The starting point of an adequate diagnosis is conventional histological examination using haematoxylin and eosin stained slides, in combination with clinical and/ or radiographical information. In addition, immunohistochemistry is a very useful and sometimes even an essential diagnostic tool to evaluate or confirm the line of differentiation in tumours. Because the classification of vascular tumours can be difficult, different antibodies have been described and used to demonstrate endothelial differentiation. The most commonly used markers are described below.

CD31, also known as platelet endothelial cell adhesion molecule 1 (PECAM-1), is a single-chain type I transmembrane protein member of the immunoglobulin (Ig) superfamily and has a molecular weight of ~83kDa. The extracellular domain contains six Ig-like homology units of the C2 subclass, similar to cell-cell adhesion molecules. CD31 plays a role in angiogenesis[7,8] and diapedesis of leukocytes[9]. It is abundantly expressed on the surface of embryonic vascular and adult endothelial cells[10,11]. Therefore, it has been regarded over the past years as the most sensitive immunohistochemical marker for endothelial cell differentiation. However, it is not an

absolutely specific marker because it is also expressed in macrophages, dendritic cells, platelets, monocytes, neutrophils and a subset of T- en B-lymphocytes and natural killer cells[10,11]. In cultured endothelial cells it is diffusely distributed in the plasma membrane, but when cell-cell contacts are formed it is concentrated at the regions of cell-cell contact[12] where it plays a role in endothelial cell contact[10].

CD34, also known as hematopoietic progenitor cell antigen CD34, is a heavily glycosylated type I transmembrane protein with a molecular weight of ~41kDa. The function of CD34 is still not completely elucidated. To date, it is accepted that CD34 plays a role in cell adhesion and cell transduction[10,13]. CD34 is expressed on the most primitive pluripotential stem cells and hematopoietic progenitor cells of all lineages. The expression is eventually gradually lost when lineage committed progenitors differentiate. It is also present in dendritic interstitial cells, dermal dendrocytes, endometrial stroma, some lymphatic endothelial cells, interstitial cells of Cajal and a subset of fibroblasts[4,10,14]. Because of the expression on endothelial cells and vascular tumours, CD34 is used as a vascular marker. However, compared to CD31, CD34 is less sensitive and less specific[10].

von Willebrand Factor (vWF), also known as Factor VIII-related antigen, is a multimeric plasma glycoprotein with a molecular weight of ~250 kDa. It is synthesized by endothelial cells, megakaryocytes and platelets. In endothelial cells it is located within the Weibel-Palade bodies. vWF has a dual key role in both primary and secondary hemostasis by mediating the adhesion of platelets towards the wound site and chaperoning clotting factor VIII[10, 15]. Mutations of the vWF gene are known to cause von Willebrand disease and are characterized by ecchymoses, hemorrhage and a prolonged bleeding time. vWF is a highly specific endothelial marker. However, it is much less sensitive compared to CD31 and CD34[4,10].

FLI1 (Friend leukaemia integration 1 transcription factor) is a member of the ETS family of DNA binding transcription factors and has a molecular weight of ~51kDa[16]. FLI1 is involved in cellular proliferation and tumourigenesis. It was first discovered in Ewing sarcoma, since approximately 90% of these tumours have a specific translocation, t(11;22)(q24;q12), resulting in the EWSR1-FLI1 fusion protein[17]. However, a study of small blue round cell tumours revealed also nuclear expression of FLI1 in normal endothelial cells and small lymphocytes[16] and therefore FLI1 was reported as the first nuclear marker of endothelial differentiation. However, it is also expressed in a small subset of melanomas, Merkel cell carcinomas, synovial sarcomas, breast carcinomas[18], and nearly all epithelioid sarcomas show positive staining for FLI1[3].

ERG, also known as avian v-ets erytroblastosis virus E26 oncogene homolog, is a member of the ETS family of transcription factors and has a molecular weight of ~55 kDa. It is constitutively expressed by endothelial cells and it has been shown to play a role in the regulation of angiogenesis and apoptosis of endothelial cells[4,10,19]. Recent immunohistochemical studies have demonstrated that ERG is a highly specific endothelial marker. However, it has been shown that

it also plays an important role in carcinogenesis of a subset of prostate carcinomas containing the TMPRSS2-ERG fusion[4,20-22]. Moreover, some myeloid precursor cells[4,23] and rare cases of Ewing sarcomas, containing the EWSR1-ERG fusion, are positive as well[4]. Recent publications have shown that ERG expression is also present in one and up to two third of the epithelioid sarcomas, especially when an antibody directed against the ERG N-terminus was used[3,4].

D2-40 is a commercially available monoclonal antibody directed against human podoplanin. Podoplanin, also known as type I alveolar cell marker hT1alpha-2 and Aggrus, is a type I transmembrane glycoprotein with extensive O-glycosylation and a molecular weight of ~40kDa. Some studies have demonstrated that it is regulated by the lymphatic-specific homeobox gene *Prox 1*, a transcription factor responsible for the development of lymphatic progenitors from embryonic veins[24]. Podoplanin promotes platelet aggregation, and possesses a platelet aggregation-stimulating (PLAG) domain[25]. Podoplanin is expressed in a large number of normal tissue cells, such as mesothelial cells, osteocytes and osteoblasts, follicular dendritic cells of lymphoid tissue, etc…, but also in a variety of different tumour types, in example a subset of vascular tumours, epithelioid mesothelioma, adrenal cortical carcinoma, seminoma/dysgerminoma[26].

Ulex Europaeus Agglutinin 1 (UEA-1) is one of the oldest endothelial markers and has been used for some time. UEA-1 binds to glycoproteins and glycolipids in endothelial cells. However, these glycoproteins and glycolipids are also found in red blood cells and epithelial cells of ABH blood group secretors[27]. Because UEA-1 also reacts with all kinds of epithelial cells, it has no longer any diagnostic value since the discovery of more sensitive and specific endothelial markers such as CD34 and CD31.

Other endothelial markers

Over the years, many other markers such as LYVE-1, Prox1, claudin 5, WT1, VEGF and GLUT1 have been used or are still used as endothelial markers, sometimes to emphasize specific differentiation of the endothelium. However, these markers are not exclusively positive on endothelial cells and therefore not widely used and studied.

III. Vascular tumours

It is generally accepted that vascular tissue or tissue with the immunohistochemical repertoire of endothelial cells as discussed above can give rise to tumours and tumour-like malformations in the skin, soft tissue and viscera. These lesions are part of a wide spectrum of entities, and for clinical purposes classification schemes have been proposed and evolved over the years. Benign haemangiomas are one of the most common soft tissue tumours, whereas angiosarcomas are their malignant counterpart and are extremely rare, highly aggressive and comprise less than 1% of all sarcomas[28]. Other vascular lesions, originally grouped as haemangioendotheliomas, are

more aggressive as compared to benign haemangiomas while they are not full blown malignant angiosarcomas. To overcome problems with ambiguous terms such as "intermediate malignancy" or "borderline malignant potential", the 2002 World Health Organization classification endeavoured to classify soft tissue tumours into four categories: overtly benign lesions, locally aggressive not metastasizing lesions, locally aggressive rarely metastasizing lesions and frankly malignant lesions. In 2013, also vascular tumours of bone were classified as such[29] (Table 1)

Table 1. Overview of all vascular tumours of soft tissue and bone as listed in the 2013 WHO Classification of Tumours of Soft Tissue and Bone.

	Vascular Tumours of Soft Tissue	Vascular Tumours of Bone
Benign	Haemangioma synovial venous arteriovenous haemangioma/ malformation epithelioid haemangioma angiomatosis lymphangioma	Haemangioma
Intermediate (locally aggressive)	Kaposiform haemangioendothelioma	Epithelioid haemangioma
Intermediate (rarely metastasizing)	Retiform haemangioendothelioma Papillary intralymphatic angioendothelioma Composite haemangioendothelioma Kaposi sarcoma Pseudomyogenic (epithelioid sarcoma-like) haemangioendothelioma	
Malignant	Epithelioid haemangioendothelioma Angiosarcoma of soft tissue	Epithelioid haemangioendothelioma Angiosarcoma

IV. Vascular tumours of bone

In bone, the nutrient arteries penetrate the cortex and branch into an abundant network of small arteries and capillaries. The terminology of vascular tumours of bone has been a matter of debate over the years, which has led to the use of different terminology and different classification systems used simultaneously over the years (Table 2). None of these classification systems have been generally accepted due to the lack of consistent terminology, accepted histological criteria and only a limited correlation with clinical outcome. In particular, the classification of vascular tumours of bone belonging to the intermediate category, or representing low-grade vascular tumours of bone, previously simply grouped as "haemangioendotheliomas", has been extremely difficult. Solitary haemangiomas are relatively common in bone. Autopsy studies have reported that haemangiomas are present in up to 10%[30]. These lesions occur most frequent as

an asymptomatic incidental finding in the skull or spine, although extraspinal locations are also reported. However, despite the rich vascularity of bone primary malignant vascular tumours of bone are extremely rare[31]. They represent less than 1% of primary malignant bone tumours reported by the Netherlands Committee on Bone tumours[32] and 0,5% of those registered at the Mayo Clinic[33]. Because of their rareness, little systematic knowledge is available. Clinically they seem to be extremely aggressive and have a very poor prognosis. Currently, epithelioid haemangioma, previously also known as haemangioendothelioma of bone, has been recognized as a locally aggressive vascular neoplasm with distinct histological criteria and an excellent prognosis[29,34]. The term "haemangiopericytoma" of soft tissue was already abandoned in the 2002 World Health Organisation (WHO) Classification of Tumours of Soft Tissue and Bone.
It has been accepted that it merely represents a nonspecific growth pattern exhibited by a large number of tumours, and "haemangiopericytoma of soft tissue" could be reclassified as solitary fibrous tumours, monophasic synovial sarcoma, and (infantile) myofibromatosis or myofibroblastic laesions[29,35,36]. Similar lesions occur in bone, which led us to question whether also "haemangiopericytoma of bone" is a true entity (see Chapter 7). In the 2013 WHO classification "haemangiopericytoma of bone" is also no longer recognized as a separate entity.
The characteristics of the different histological types of vascular lesions of bone are described in more detail in Chapter 2 of this thesis.

V. Multifocality

Both benign and malignant vascular tumours of bone can occur multifocally, and multifocal disease in bone is even more common as compared to the same tumours occurring in soft tissue. The distribution of these lesions can be either contiguous, involving adjacent bones, or non-contiguous, involving two or more distant sites[37]. Multiple benign vascular lesions, known as haemangiomatosis, are considered a developmental disorder (vascular hamartomas)[37]. Up to one third of the angiosarcoma of bone and nearly two third of the epithelioid haemangioendotheliomas of bone occur multifocally[29]. Although the exact mechanism remains unclear, one could speculate i) they could be metastatic lesions (skip metastasis in bone), ii) there could be a genetic disorder (somatic mosaicism) facilitating tumour formation at multiple locations; iii) the production of multiple circulating growth factors in the bone marrow could lead to vascular proliferation resulting in multifocal malignancy. However, Pansuriya and co-workers identified IDH1 and IDH2 mutations in a large proportion of echondromas and spindle cell haemangiomas[38]. These mutations occurred also in the majority of multiple lesions within one patient[38]. More recently, Antonescu et al. demonstrated the presence of the characteristic WWTR1-CAMTA1 fusion transcript product with the presence of an identical breakpoint in WWTR1 and CAMTA1 in all different tumour nodules of two patients with multicentric epithelioid haemangioendothelioma of the liver[39]. These findings support the hypothesis of clonal disease and suggest that the tumour nodules are metastatic implants, rather than synchronous multiple neoplastic clones[39].

Table 2. Schematic overview of the different classification schemes proposed over the years.

Entities	Wenger et al. 2000 All lesions	O'Connell et al. 2001 Epithelioid lesions	WHO 2002 All lesions	Maclean et al. 2006 Epithelioid and spindle cell shaped lesions	WHO 2013 All lesions
Haemangioma	Haemangioma	n.r	Haemangiomas and related lesions	n.r.	Haemangioma
Epithelioid haemangioma	Epithelioid haemangioma	Epithelioid haemangioma		n.r.	Epithelioid haemangioma
Spindle cell haemangioma	n.e.	n.e		Spindle cell haemangioma	n.e.
Haemorrhagic epithelioid and spindle cell haemangioma	n.e.	n.e.		Haemorrhagic epithelioid and spindle cell haemangioma	n.e.
Haemangioendothelioma	n.e.	n.e.		Haemangioendothelioma	n.e.
Haemangiomatosis	Haemangiomatosis	n.e		n.r.	n.e.
Hobnail haemangioendothelioma	n.e.	n.e	n.e	Hobnail haemangioendothelioma	n.e.
Kaposiform haemangioendothelioma	n.e.	n.e.	n.e	Kaposiform haemangioendothelioma	n.e.
Epithelioid haemangioendothelioma	Epithelioid haemangioendothelioma	Epithelioid haemangioendothelioma	Angiosarcoma	Epithelioid haemangioendothelioma	Epithelioid haemangioendothelioma
Haemangioendothelioma, low grade	Haemangioendothelioma	n.r		n.r.	Angiosarcoma
Haemangioendothelial sarcoma, low grade		n.r		n.r.	
Angiosarcoma, low grade		n.r		n.r.	
Haemangioendothelioma, high grade	Angiosarcoma	n.r		n.r.	
Haemangioendothelial sarcoma, high grade		n.r		n.r.	
Angiosarcoma, high grade		n.r		n.r.	
Epithelioid angiosarcoma	n.e.	Epithelioid angiosarcoma	n.e.	Epithelioid angiosarcoma	
Kaposi sarcoma	n.e.	n.e.	n.e.	Kaposi sarcoma	n.e.

n.e. non-exsisting entity in this classification
n.r. not relevant in this classification

VI. Molecular alterations in vascular tumours

So far, only few studies focussed on molecular genetic changes in vascular tumours of bone. The identification of the molecular genetic background could help to better classify these vascular tumours of bone and provide new therapeutic options to improve survival and prognosis. For its soft tissue counterpart, few of the molecular genetic changes are known. Over the past years, researchers have shown a great interest in vascular tumours, mostly of soft tissue, resulting into new insights in molecular changes and a possible role in tumourigenesis. Genetic analysis of angiosarcomas of soft tissue, which has been mostly single case reports or publications of very small tumour groups, showed complex genomic aberrations suggesting angiosarcoma belongs to the groups of sarcomas with a complex genetic profile. In this context the retinoblastoma (Rb) pathway or TP53 pathway is most often involved. Therefore, some authors have suggested p53 gene mutations[40,41] and chromosomal anomalies to be essential in their development. Although nearly 50% of the angiosarcomas showed p53 overexpression by immunohistochemistry, only in 4% a p53 mutation was detected[42]. Moreover, Antonescu and colleagues identified a subset (10%) of angiosarcomas to contain *KDR* (*kinase insert domain receptor* encoding for VEGFR2) mutations which correlated with a strong KDR protein expression and breast localisation[42,43]. Furthermore, high level MYC amplification was shown in radiation-induced and lymphedema-associated angiosarcoma and not in primary angiosarcomas or radiation-induced atypical vascular lesions[42-45], suggesting a possible role in tumourigenesis specifically in secondary angiosarcomas. In 25% of the secondary angiosarcomas FLT4 co-amplification was also present[42, 45]. However, recent publications have shown that MYC amplification can also occur in a small subset of primary angiosarcomas[42,46]. A recent whole-genome sequencing study of both spontaneous (primary) and secondary angiosarcomas demonstrated that not all secondary angiosarcomas harbour a MYC amplification, in this study only 11 of 19 well documented secondary angiosarcomas (58%) showed a MYC amplification[47] (Table 3). Moreover, they described two new mutations in secondary angiosarcomas: 45% of the secondary angiosarcomas harboured an inactivating mutation in the *PTPRB* (*VE-PTP*) gene, a negative regulator of angiogenesis by inhibition of vascular endothelial growth factor receptor 2 (VEGFR2), vascular endothelial (VE)-cadherin and angiopoietin signalling. Moreover, three of these secondary angiosarcomas also demonstrated a mutation in *PLCG1*[47]. The *PLCG1* gene encodes for phospholipase Cγ1 (PLCγ1), a tyrosine kinase signal transducer in the PIK3CA signalling pathway. In contrast, only a minority of the spontaneous (primary) angiosarcomas showed genetic alterations in well-known cancer genes, such as PIK3CA, CDKN2A, NRAS, KRAS, and none of these alterations were recurrent[47]. Thus, a significant subset of angiosarcomas, especially breast (KDR) and secondary (FLT4, PTPRB) angiosarcomas demonstrate mutations in genes involved in the regulation of angiogenesis.

Table 3. Overview of the reported genetic aberrations in vascular tumours of soft tissue.

Tumour type	Reported genetic aberrations	Number (%)	Localisation	References
Epithelioid haemangioma	ZFP36-FOSB fusion	20%	bone, soft tissue, penis, lymph node	Antonescu 2014
Pseudomyogenic haemangioendothelioma	SERPINE1-FOSB fusion	1/1 (100%), 2/12 (17%)	soft tissue	Trombetta 2011; Walther 2014
Epithelioid haemangioendothelioma	t(1;3)(p36.3;q25) with fusion gene CAMTA1-WWTR1			Errani 2011
	fusion gene YAP1-TFE3			Antonescu 2013
Angiosarcoma	KDR mutation	4/39 (10%)	breast	Antonescu 2009, Guo 2011
	TP53 mutation	4%	liver	Anonescu 2012
Secundary angiosarcoma	High-level amplification of MYC	11/19 (58%) and 20/20 (100%)		Behjati 2014, Italiano 2012
	Co-amplification of FTL4	1/21 (5%)		Behjati 2014, Italiano 2012
	PTPRB mutation	10/22 (45%)		Behjati 2014
	PLCG1 mutation	3/22 (7%)		Behjati 2014

Over the years, in two epithelioid haemangioendothelioma of soft tissue a translocation t(1;3)(p36.3;q25) had been described, suggesting a recurrent genetic aberration[29,48]. However, only recently the genes involved in this translocation have been identified: *WWTR1* (also known as *TAZ*) on 3q25 fuses with *CAMTA1* on 1p36, resulting in a fusion gene and eventually activating a novel transcriptional program[39]. Moreover, a *YAP1-TFE3* gene fusion has been identified in epithelioid haemangioendothelioma lacking the *WWTR1-CAMTA1* fusion[49]. The latter subgroup seems to have a more distinct morphology including well-formed vessels as compared to classic epithelioid hemangioendothelioma. Although most of these tumours are located within the soft tissue also one of the reported cases was located within the bone (vertebral body)[42,49].

Pseudomyogenic haemangioendothelioma is a more recently described intermediate malignant vascular tumour affecting children and young adults. It is characterized by loose fascicles or sheets of round or oval shaped cells with vesicular nuclei and prominent nucleoli, surrounded by abundant homogeneous eosinophilic cytoplasm[50]. These lesions also have a tendency to occur multifocally. A recent genetic study has identified a balances translocation t(7;19)(q22;q13) in two patient (one lesion of one patient and three lesions of the same patient) and a unbalanced der(7)t(7;19) translocation in another case, resulting in fusion of SERPINE1 and FOSB genes. This entity has been originally described in soft tissue[51,52]. However currently, there have been some reports of primary bone lesions as well[53,54]. Also in a subset (20%) of epithelioid haemangioma, a benign vascular tumour, a ZFP36-FOSB fusion has been detected[55].

VII. Technological challenges/research pitfalls

Since malignant vascular tumours of bone are extremely rare, the collaboration of multiple pathology laboratories- mostly with a special interest in bone and soft tissue pathology – is needed to collect a substantial number of tumours. Many of those laboratories, including our department, had to search within their archives with the consequence that over the years different decalcification techniques had been used which possibly could have an impact on immunohistochemistry or the performed molecular tests. Since the decalcification method never has been specified within the pathology report, it hampers or complicates additional testing, such as reliable immunohistochemical evaluation.

Decalcification of bone is needed in order to be able to cut and evaluate tissue material containing or coming from bone. Decalcification is a chemical process by which calcium hydroxyapatite crystals and other minerals present in bone (or pathological calcifications) dissolve in a decalcification solution, and therefore bone or bony tissue gets the physical characteristics of dense connective tissue[56]. In general, there are three main groups of decalcifying agents:

a. strong mineral acids (e.g. hydrochloric or nitric acid at concentrations up to 10%)
b. weak organic acids (e.g. formic acid, Kristensen buffer)
c. chelating agent (e.g. ethylenediaminetetracetic acid (EDTA))

Strong acids, such as hydrochlorid or nitric acid at concentrations up to 10%, have the advantage of being the most rapid method of decalcification. However, they cause tissue swelling and cellular damage after the usage of 24 to 48 hours and therefore negatively affect the cellular morphology and immunoreactivity of the tissue.
Weak organic acids, such as formic acid, are the most frequently and widely used decalcification agents. Although these acids decalcify more slowly compared to the strong acids, they are gentler and therefore suitable for most routine specimens enabling additional immunohistochemical staining. Since formic acid can damage tissue, and therefore hamper antigens and enzyme histochemical staining, sodium formate (buffered formic acid by Kristensen) can be added as a buffer to counteract the injurious effects of the acid. Moreover, formic acid affects the DNA and leads to defragmentation of the DNA. Ethylene-diamine-tetracid acid (EDTA), a chelating agent, binds metallic ions, such as calcium, on the surface of the apatite crystal thereby slowly reducing its size. This implicates that it is a very slow process, so not suitable for routine diagnostics. However it is a very gentle technique with little tissue and DNA damage and little effect on tissue stainability. Moreover, this method is highly suitable for techniques such as *Fluorescent In Situ Hybridization* (FISH) and Polymerase Chain Reactions (PCR)[56].
Because of the increasing importance of molecular testing, especially within bone and soft tissue tumours and the knowledge of effects of decalcification, EDTA decalcification should be, whenever possible, the preferred method. Although the pathology reports of the material used in this project do not specify the decalcification method, we can assume that most tissue samples are decalcified by either weak organic acids (formic acid) or strong mineral acids.

VIII. Aim of this study and outline of the thesis

The aim of the research described in this thesis is to investigate whether we could determine certain histomorphological characteristics and molecular genetic features which could be helpful in the classification of primary vascular tumours of bone. We compared our dataset of vascular tumours of bone with a small group of angiosarcomas of soft tissue in order to see whether these are truly different tumours or whether they should be regarded as one entity with a different localization.
In **Chapter 2** a detailed summary of the literature and the history of the classification of the different types of primary vascular tumours of bone are presented.
In **Chapter 3** we collected a multi-institute retrospective series of 42 angiosarcomas of bone and investigated their clinicopathological characteristics in relation to outcome.
In **Chapters 4-6** the underlying molecular and genetic changes in primary vascular tumours of bone are investigated. We analysed the well characterized series that we described in chapter 3 using immunohistochemistry to investigate expression of proteins involved in angiogenesis, the cell cycle, or in tumourigenesis in angiosarcoma of soft tissue (**Chapter 4**). For comparison we included a series of angiosarcomas of soft tissue. Because the use of acids during decalcification degrades the DNA and thereby hampers the analysis of genetic aberrations, as described above,

we next optimized the array-Comparative Genomic Hybridization (array-CGH) technique for use on decalcified formalin fixed paraffin embedded tissue in **Chapter 5**. Subsequently, in **Chapter 6** we applied this technique to detect genomic aberrations in angiosarcomas of bone in comparison to a small group of angiosarcoma of soft tissue.

Finally, in **Chapter 7** we question whether the rare vascular tumour previously designated as "haemangiopericytoma of bone" is a true entity or not. We collected a number of primary haemangiopericytoma of bone and re-analysed these lesions in **Chapter 7** using histology, immunohistochemistry and *Fluorescent In Situ Hybridization* (FISH) analysis.

Finally, results are summarized in **Chapter 8** and it is discussed how the results of our studies, integrating morphology and genetics, have contributed to the 2013 WHO classification of vascular tumours of bone.

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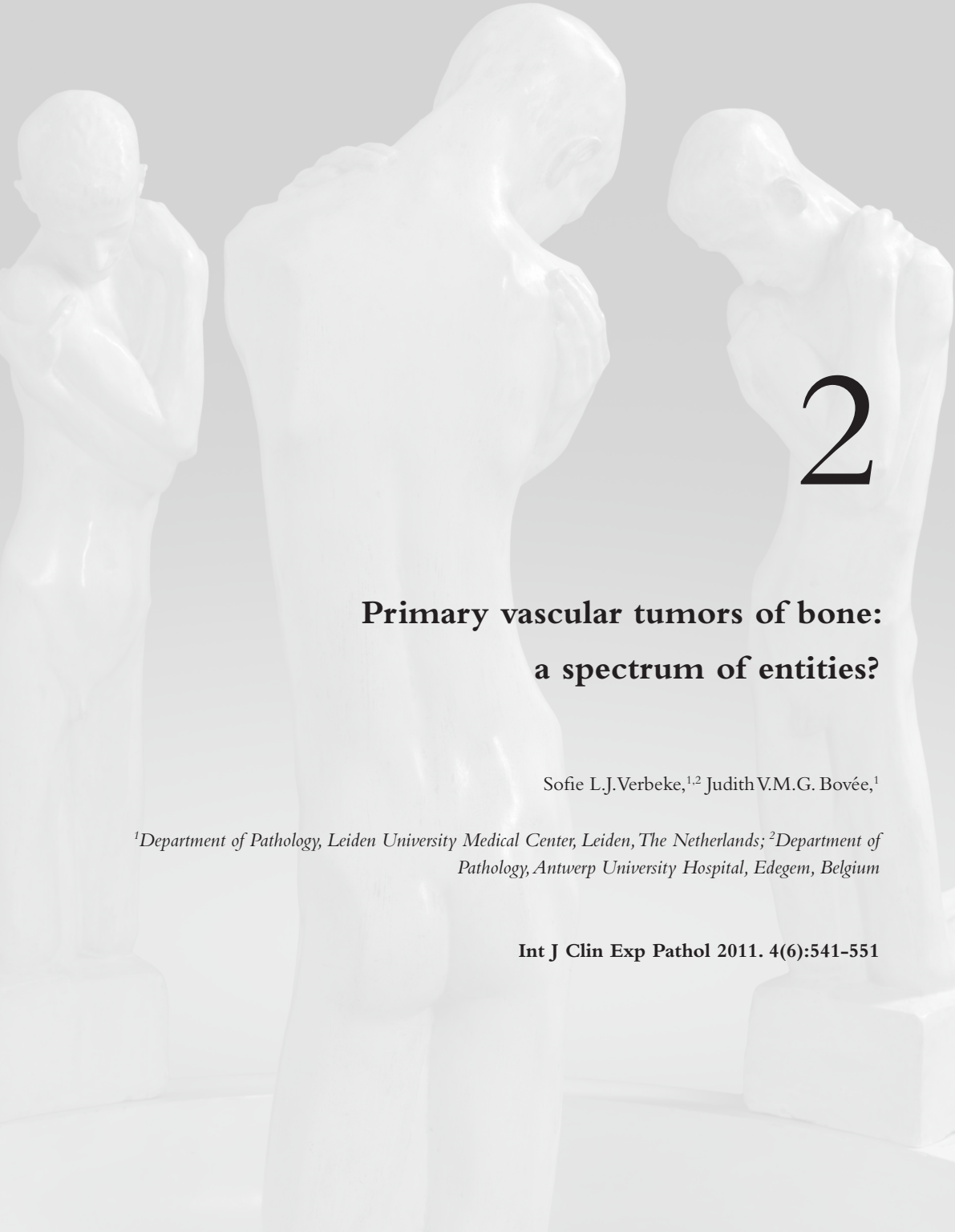

2

Primary vascular tumors of bone: a spectrum of entities?

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Abstract

Vascular tumors of bone are a heterogeneous group. Numerous terms have been introduced as well as different classification systems. None of the classification schemes have been accepted due to lack of consistent terminology, accepted histologic criteria, and limited correlation with clinical outcome. It is acknowledged that vascular tumors of bone originate from endothelial cells, resulting in variable expression of endothelial markers. None of these markers are useful to discriminate between benign and malignant lesions. Although radiologic appearance is not specific, radiologic multifocality should trigger to include a vascular neoplasm in the differential diagnosis. This review gives an overview of current literature by describing all different histologic subtypes in correspondence with clinical, radiologic and genetic data. We propose the classification of vascular tumors of bone according to the three-tiered World Health Organization classification scheme for soft tissue tumors dividing entities into a benign, intermediate and malignant category. Hemangioma is the most often and commonly recognized benign lesion. Epithelioid hemangioma has been better defined over the past few years. Based on its locally aggressive behavior and occurrence of lymph node metastases, classification within the intermediate category could be considered. Angiosarcoma is the only accepted term for high-grade malignant vascular tumor of bone and so far, epithelioid hemangioendothelioma is the only accepted low-grade malignant vascular tumor of bone. It is still unclear whether other low-grade malignant vascular tumors of bone (e.g. hemangioendothelioma) truly exist. Unfortunately, molecular / genetic studies of vascular tumors of bone which might support the proposed classification are very sparse.

Introduction

Today, vascular tumors of bone consist of a wide variety of different clinicopathologic entities, ranging from benign lesions on one hand and frankly malignant tumors at the other hand. Since the first report on malignant vascular tumors of bone in 1921 by Wells [1], various entities have been described and many different terms have been proposed. Over the years, terms such as angiosarcoma, hemangiosarcoma and hemangioendothelioma have been used sometimes as synonyms or to stress different histologic entities, confusing numerous medical experts [2-6]. Also the classification of vascular tumors of bone is highly controversial, especially considering the lack of consistent terminology, accepted histological criteria, and the limited correlation with clinical outcome. As a consequence, so far none of the suggested classification schemes have been generally accepted [3, 4, 6, 7]. Wenger and Wold acknowledged the confusing terminology and proposed in 2000 a new classification system for benign and malignant vascular tumors and stated that these lesions should be regarded as a spectrum [6, 8]. However, this is still controversial since a spectrum implicates the possibility of progression of a benign lesion towards a malignancy over time and only single case reports have described this phenomenon [9-16].

Since 1942, it is generally accepted that vascular tumors of bone originate from endothelial cells [17]. The exact mechanism or possible genetic aberrations resulting in tumorigenesis still remains unknown. In this review we want to give an overview and update of the current classification of vascular tumors of bone (Table 1) by describing all different histologic subtypes in correspondence with clinical, radiologic and when available genetic or biologic data. Vascular tumors, for which a primary bone origin is extremely rare, are not discussed within this review. Since molecular studies on vascular tumors of bone are sparse, their value in the classification of these lesions is limited.

Radiographic Imaging

Because of the heterogeneity of vascular tumors of bone, imaging is not very specific. However, some radiographic alterations can indicate the probability of a benign or malignant osseous vascular tumor (Figure 1, 2, 4 and 5). By conventional radiographs, the majority of the hemangiomas show a well demarcated, lucent lesion with frequent coarse trabeculations [8, 18, 19]. Although cortical expansion can be seen in hemangiomas, cortical disruption and invasion into the surrounding soft tissue is most often characteristic of malignancy. Moreover, malignant vascular tumors of bone are most often characterized by an ill-defined, osteolytic lesion with cortical disruption and endosteal scalloping. Up to one third of the malignant vascular tumors of bone presents with synchronic multiple osseous lesions which can be either contiguous (adjacent bones affected) or disseminated [20]. Although the radiographic features of malignant vascular tumors of bone are non-specific, multifocal lesions in one anatomic region should trigger the radiologist to include a vascular neoplasm in the differential diagnosis [20, 21].

Table 1. Proposed classification of vascular tumors of bone

Benign vascular tumors of bone
Hemangioma
Cavernous
Capillary
(Hem)angiomatosis
Non-aggressive, regional
Non-aggressive, disseminated (cystic angiomatosis)
Aggressive or massive osteolysis or Gorham Stout's Disease
Intermediate (locally aggressive, rarely metastasizing) vascular tumors of bone
Epithelioid hemangioma
Malignant vascular tumors of bone
Epithelioid hemangioendothelioma
Angiosarcoma
Primary
Irradiation-induced
Bone infarction associated

Immunohistochemistry

It is generally accepted that vascular tumors, both of soft tissue and bone, originate from endothelial cells resulting in a variable expression of endothelial markers such as CD31, CD34, Fli-1 and von Willebrand Factor (Factor VIII) [22-24]. Although it has been reported that CD31 and von Willebrand Factor are the best diagnostic markers for malignant vascular tumors of bone, the use of a panel of endothelial markers is essential to confirm the diagnosis because a minority of the malignant tumors only express CD34 [25]. Based on the expression of the endothelial markers it is impossible to discriminate between benign and malignant vascular tumors. Vascular tumors variably express D2-40 (31%) [25], a presumed lymph-endothelial marker, and its expression in angiosarcoma is associated with a worse prognosis, suggesting lymphangiosarcoma of bone may exist [26, 25]. Cytokeratin (69%) [25] and/ or epithelial membrane antigen (4-35%) [27, 28], are also expressed, in particular but not exclusively in neoplasms with an epithelioid morphology [20, 25]. Since these lesions have a tendency to occur multifocal (contiguous or disseminated), the epithelioid morphology and keratin positivity may easily lead to an erroneous diagnosis of metastatic carcinoma.

Benign Vascular Tumors of Bone

Hemangioma

Hemangioma of bone (Figure 1, Table 2) is the most common benign vascular tumor of bone [8]. Its etiology is still unknown. Moreover, it is still unclear whether these lesions are true neoplasms or should be regarded as hamartomas [18, 29]. Despite the lack of appropriate data regarding the incidence or prevalence of hemangioma, autopsy reports have demonstrated that vertebral hemangioma occurs in approximately 10% of adults [18]. Hemangiomas occur in both men and women, with a wide age range and are mostly located in skull and vertebra [8, 18].

Although these lesions can cause various signs and symptoms, the majority of patients present with an asymptomatic and incidental radiographic finding [8, 18]. Several different histologic subtypes are described, such as cavernous, capillary and sclerotic hemangioma [18]. Malignant transformation is only described in a few cases [9-15]. In general, hemangiomas have a very good prognosis and a low recurrence rate.

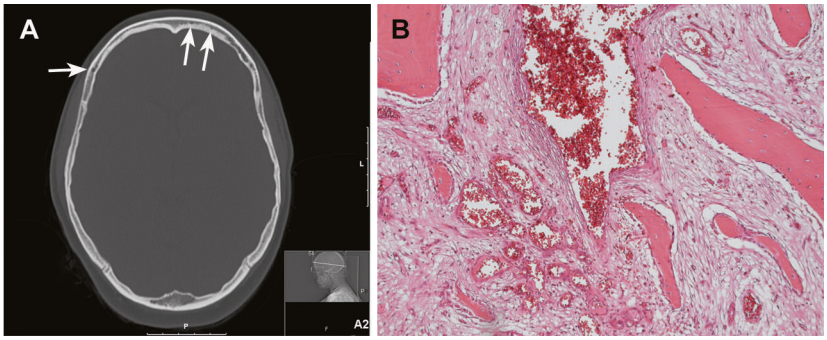

Figure 1. Hemangioma of bone: **A.** conventional X-ray of the scalp showing multiple small well-demarcated osteolytic lesions (arrows); **B.** multiple vascular spaces lined by non atypical endothelial cells filled with erythrocytes.

Table 2. Overview of epidemiologic, clinical and histologic characteristics of the different subtypes of vascular tumors of bone

	Hemangioma	Angiomatosis	Epithelioid hemangioma	Epithelioid hemangioendothelioma	Angiosarcoma
M vs F ratio	2:3	males>females	1.4:1	males>females	males>females
Age range (d)	1^{th}-8^{th}	1^{th}-7^{th}	1^{th}-8^{th}	1^{th}-8^{th}	2^{nd}-8^{th}
Peak age (d)	4^{th}-5^{th}	within first 3 decades of life	4^{th}	2^{nd}	6^{th}-8^{th}
Location	skull, vertebrae	shoulder, hip	long tubular bones	long tubular bones of extremities	long tubular bones of extremities, spine
Multifocality (%)	5-18%	100%	18%	50-64%	33%
Histology					
Growth pattern	vascular spaces	vascular spaces	lobular growth pattern periphery: arteriolar-like vessels central: epithelioid cells	strands/ cords of solid nests epithelioid endothelial cells Intracytoplasmatic vacuoles myxoid/ hyalinized stroma	heterogeneous: vasoformative to solid macronucleolus <5 eosinophils/ 10 HPF
Atypia	no	no	No	variable degree	yes
Mitoses/ 10 HPF	no	no	<5	no or little	≥3
Atypical mitoses	never	never	Never	no or little	yes
Survival	100%	dependent on visceral involvement	100%	dependent on visceral involvement	33% 5-year survival
Recurrence rate	low	n.k.	8%	n.k.	n.k.
Metastatic rate	0%	0%	2%	n.k.	high
Treatement	conservative	dependent on the extent	curettage, marginal en bloc resection	en bloc resection	dependent on tumor stage, multimodality treatment

d = decades; n.k. = not known.

Angiomatosis

Skeletal angiomatosis (Figure 2, Table 2) is a rare disorder and is defined as multiple cystic bone lesions with or without soft tissue involvement. Soft tissue involvement is present in 60-70%, and in general the spleen is affected [30]. Clinical presentation is dependent on localization, the size and the number of lesions and can vary from an incidental finding to local pain, swelling and/ or pathological fracture [31-34]. These lesions are classified based on their clinical behavior (aggressive or nonaggressive) and pattern of skeletal involvement (regional or disseminated) [20, 31, 35]. Regional involvement is defined as corrosion of one or more bones of one anatomic region, whereas disseminated involvement is characterized by multifocal disease with typically involvement of the trunk bones [35]. Gorham's Disease, also known as massive osteolysis or disappearing bone disease, is an aggressive form of regional skeletal angiomatosis. Although the etiology still remains unknown, half of the cases are associated with trauma [36, 37]. This disease results in progressive destruction of one bone and sometimes also adjacent bones. It is merely a clinicoradiologic diagnosis, since the histology is reminiscent of hemangioma [35, 20]. Malignant transformation to angiosarcoma is highly unusual, but has been described [16]. In extraordinary cases, angiomatosis is associated with syndromes such as von Hippel-Lindau syndrome, Maffucci's syndrome, Klippel-Trenaunay syndrome, Kasabach Merritt syndrome, Parkers-Weber syndrome and Osler-Weber-Rendu disease [35, 38]. In the majority of these syndromes the etiology and pathogenic mechanisms are unknown. However, von Hippel-Lindau syndrome and Osler-Weber-Rendu disease are caused by genetic aberrations in the VHL gene and HHT genes, respectively [39,40]. Prognosis of angiomatosis is dependent on the extent and localization of the disease [35, 38, 41]. Extended visceral involvement bears a more aggressive course, especially due to massive hemorrhaging [35, 38, 41].

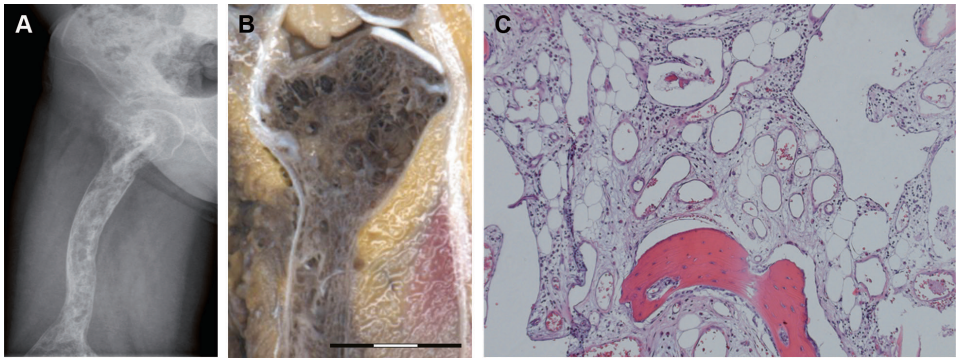

Figure 2. Angiomatosis of bone (Gorham- Stout disease, vanishing bone disease): **A.** Radiology: conventional x-ray of the femur showing bone deformation with multiple osteolytic lesions; **B.** Gross examination of an amputation specimen showing multiple cystic lesions within the bone marrow of the femur (scale in centimeters); **C.** Histology demonstrating the morphology of a hemangioma consistent of multiple vascular spaces lined by non atypical endothelial cells filled with erythrocytes (haematoxilin-eosin staining, 20x).

Benign or intermediate?

Epithelioid Hemangioma

Currently, epithelioid hemangioma (Figure 3, Table 2) (previously known as angiolymphoid hyperplasia with eosinophilia or histiocytoid hemangioma) is a recently described and accepted clinicopathologic entity in bone [7, 42]. The majority of epithelioid hemangiomas present as solitary lesions. Rarely, local cortical destruction and extension into the surrounding soft tissue have been reported. Also, small foci of necrosis can be present. The precise classification of this newly described entity is still controversial. Some authors such as Wenger and Wold have considered this a benign lesion [8]. Although Nielsen and colleagues have demonstrated that epithelioid hemangioma is a locally aggressive, rarely metastasizing tumor, they argue about the true malignant potential of this neoplasm [42]. In the 2002 World Health Organisation Classification of Tumours of Soft Tissue and Bone, all soft tissue tumors are categorized into a four-tiered classification system: benign, intermediate locally aggressive, intermediate rarely metastasizing and malignant. The intermediate category is defined by an infiltrative and locally destructive growth pattern, often recurring and occasionally (< 2%) metastasizing [20]. If these criteria are applied to epithelioid hemangioma of bone, recurring in 11% and metastasizing in 2.7% [42], this entity fits best within this intermediate category, in between hemangioma (benign) and angiosarcoma (malignant) of bone [20]. Curettage or limited local surgery (marginal en bloc resection) is considered to be an adequate therapy and has an excellent prognosis [42]. Although an allergic reaction, trauma, and an auto-immune process have been implicated as possible causes in the soft tissue counterpart [43, 44], no data regarding genetic alterations or pathophysiologic mechanisms have been reported so far for bone.

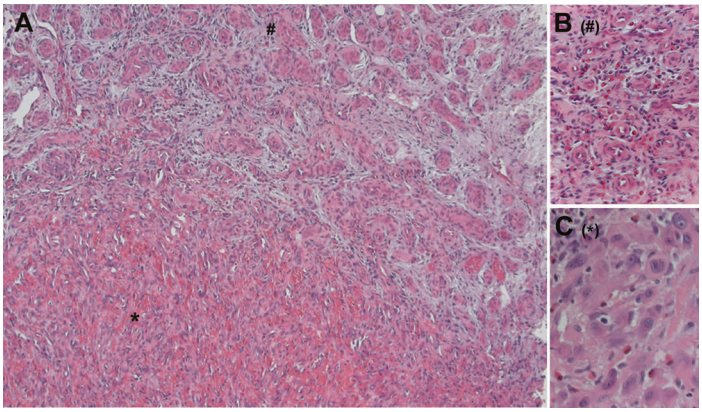

Figure 3. Epithelioid hemangioma of bone. **A.** Histologic overview of an epithelioid hemangioma of bone composed of two distinct areas (marked with * and #) haematoxilin-eosin staining, 10x): **B (#).** the peripheral area with numerous small arteriolar-like vessels and an infiltrate consistent of numerous eosinophilic granulocytes (haematoxylin-eosin staining, 40x) and **C (*).** the cellular central area with large, polyhedral, epithelioid cells with a more solid growth pattern intermixed with eosinophilic granulocytes (haematoxylin-eosin staining, 40x).

Malignant Vascular Tumors of Bone

Epithelioid Hemangioendothelioma

Epithelioid hemangioedothelioma of bone (Figure 4, Table 2) is, similar to its soft tissue counterpart, considered a low grade malignant vascular tumor. All bones can be affected, however approximately 50% of these tumors occur in the long tubular bones of the extremities [4, 6, 45]. Multifocality is more frequently seen - in about 50 to 64% - as compared to angiosarcoma of bone and it is unclear whether this is a synchronous involvement or is caused by metastatic spread [4, 6, 20]. Pain is the most common clinical presentation, however non-specific [6, 20, 36]. Gross examination can vary from a soft, red nodular mass to a firm tan-white mass [4, 20, 36]. So far, no genetic alterations are described within epithelioid hemangioendothelioma of bone. However, in two cases of epithelioid hemangioendothelioma of soft tissue (one arising in the liver and one arising in the soft tissue of an extremity) an identical translocation involving chromosomes 1 and 3 [t(1;3)(p36.3;q25)] have been described, suggesting a tumor-specific translocation [36, 46, 47]. Literature about the behavior and prognosis of this entity is somewhat conflicting [4]. It seems that tumors with a visceral involvement behave worse [6, 45, 48, 49].

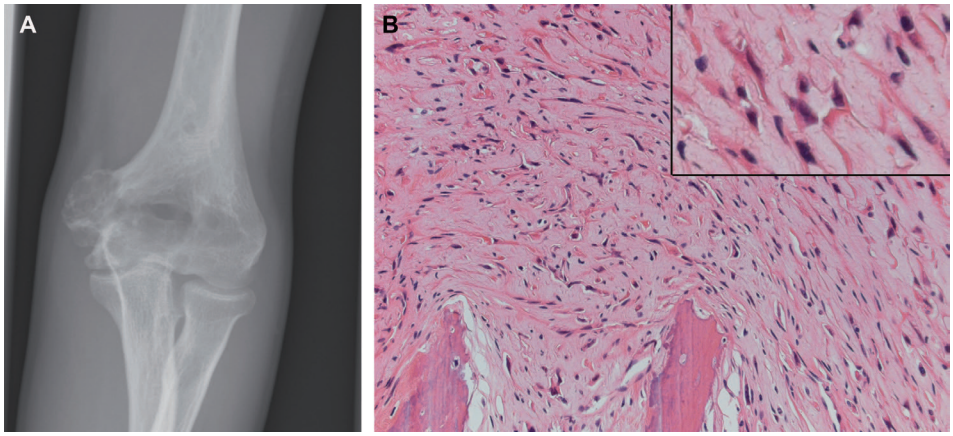

Figure 4. Epithelioid hemangioendothelioma of bone A. Radiology: conventional X-ray of the distal humerus showing an excentric osteolytic lesion (arrow); **B.** Histology: characteristic strands and cords of epithelioid endothelial cells surrounded by a hyalinized stroma (haematoxilin-eosin staining, 20x) with inset: showing the nuclear detail and presence of cytoplasmic vacuoles (haematoxilin-eosin staining, 63x).

Angiosarcoma

Today, angiosarcoma (Figure 5, Table 2) is the most accepted term for high-grade vascular malignancy in bone [20]. These tumors are rare and account for less than 1% of malignant bone tumors. The majority of these tumors arising in bone are primary, however, a very small percentage is either radiation induced or associated with bone infarction. Although there is no specific clinical presentation, the majority of the patients present with a chronic dull pain and/or tumor mass [20, 29]. The latter is more often seen in patients with a solitary lesion [29]. About one third of the tumors are multifocal. It is still unclear whether this is due to synchronous

involvement of different bones by multiple separate foci or is caused by metastatic spread [7, 29, 42]. At the histologic level, angiosarcoma of bone represents a heterogeneous group of lesions, ranging from well-differentiated tumors with a clear vasoformative growth pattern to poorly differentiated tumors with a more solid growth pattern sometimes even mimicking metastatic carcinoma [20, 25]. Due to the heterogeneity of these tumors and the overlap in nomenclature over the past years, there is no full agreement regarding exact histologic criteria defining these tumors, although there is some consensus about the presence of nuclear atypia and mitoses [3, 4, 6]. We recently reported that primary angiosarcoma of bone exhibiting more than 3 mitoses per 10 HPF, with a prominent nucleolus and fewer than five eosinophilic granulocytes per 10 HPF have a more aggressive course and worse outcome, indicating that these histologic criteria have prognostic value [25]. Recently, a novel t(1;14)(p21;q24) translocation has been described in an angiosarcoma of bone [50]. This is the first cytogenetic aberration reported in angiosarcoma of bone. However, small series have shown the involvement of tumor-suppressor genes such as p53 and p16, mainly in angiosarcoma of soft tissue, suggesting a possible role in tumorigenesis in a subset of angiosarcomas. P53 gene mutations are most commonly found in angiosarcoma of the liver associated with toxic vinyl chloride exposure [51-53] and angiosarcoma of the scalp [54, 55]. However, it was sporadically reported in angiosarcoma of the breast, extremities, heart, lung, liver (not toxic induced) and also in one angiosarcoma of bone [52, 54-57]. Moreover, the involvement of c-MYC, K-RAS and KDR (VEGFR2) has been recently described [53, 56, 58-60]. Whereas high levels of c-MYC amplification are found in angiosarcoma secondary to irradiation or chronic lymphedema [59], KDR mutations are present in primary angiosarcoma of the breast [58], suggesting that angiosarcoma (of soft tissue) can be separated in different subtypes each with tumor-specific alterations and as a consequence different therapeutic targets. It is still unclear whether primary angiosarcoma of bone is a true separate entity or is similar to primary angiosarcoma of deep soft tissues. It is generally accepted that angiosarcomas have an aggressive course with a one and 5-year survival rate of 55% and 33%, respectively [25].

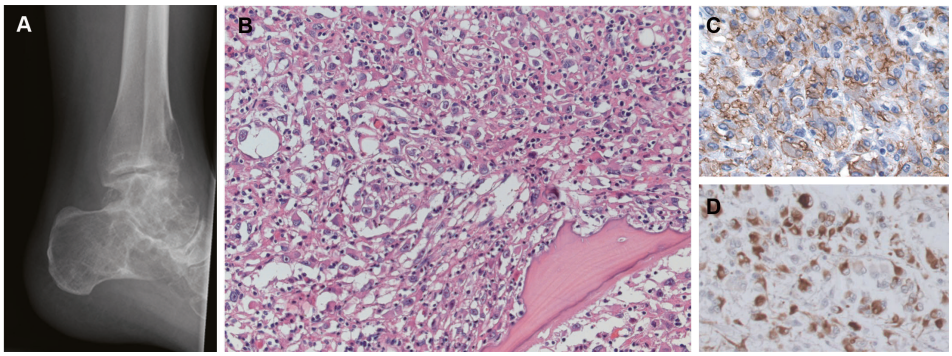

Figure 5. Angiosarcoma of bone. A. Radiology: conventional X-ray of the foot with multiple not sharply demarcated osteolytic lesions; **B.** Histology: epithelioid angiosarcoma of bone with a solid growth pattern, consisting of atypical endothelial cells with an epithelioid morphology (haematoxilin-eosin staining, 20x); **C.** CD31 showing a diffuse positive staining of the tumor cells (20x); **D.** cytokeratin: showing positive staining in a part of the tumor cells (20x).

Controversial/ Disputable entities: do they exist?

Hemangioendothelioma

The existence of hemangioendothelioma of bone as a true, separate entity has been highly controversial in the literature [42, 61]. Some authors and investigators believe that there is a subgroup of vascular tumors of bone representing a low-grade malignancy, preferably called hemangioendothelioma [3, 5, 6, 35, 62]. However, the absence of apparent histologic criteria and restricted correlation with clinical outcome have hampered the general acceptance of this entity. Nielsen and colleagues have demonstrated that over the years many authors have reported vascular tumors of bone labeled as hemangioendothelioma, which demonstrate histologic features that are identical to epithelioid hemangiomas [42]. To date, it is therefore unclear whether a low-grade angiosarcoma other than epithelioid hemangioendothelioma truly exists.

Hemangiopericytoma

This tumor was first described by Stout and Murray in 1942 as a vascular soft tissue neoplasm, composed of a proliferation of endothelial sprouts and tubules surrounded by rounded or spindle-shaped cells typically supported by a meshwork of reticulin fibers [63]. Occasional solitary bone lesions have been reported ever since. In the early nineties it became clear that many different tumor types could mimic a hemangiopericytoma-like growth pattern. Therefore, it was stated by several authors that this is most likely a non-specific histologic growth pattern, rather than a true diagnosis [64-66]. Today, the 2002 WHO Classification of Tumours of Soft tissue and Bone Tumors does not recognize this entity any longer [67] and in soft tissue it is accepted that most of these lesions can be classified as solitary fibrous tumors, monophasic synovial sarcomas or myofibromatoses [64,65,67,68]. Also in bone it has recently been demonstrated that these tumors are most probably solitary fibrous tumors or synovial sarcoma of bone [25]. Positive immunohistochemical reaction for epithelial membrane antigen and/ or cytokeratin as well as the detection of the tumor-specific translocation t(X;18)(p11.2;q11.2) is helpful for the diagnosis of synovial sarcoma of bone, whereas diffuse CD34 reactivity is seen in the majority of solitary fibrous tumors of bone [25]. Although heterogeneous cytogenetic aberrations have been reported for larger solitary fibrous tumors of soft tissue [69], this has not been confirmed in solitary fibrous tumors of bone.

Summary/ Conclusion

Vascular tumors of bone consist of a heterogeneous group of entities, which over the past decade have been better delineated, especially regarding the entity epithelioid hemangioma. Based on its locally aggressive behavior as well as the occurrence of lymph node metastases (in 2%) classification within the intermediate category, in between hemangioma (benign) and angiosarcoma (malignant), could be considered (table 1). Epithelioid hemangioendothelioma is a separate entity morphologically identical to its soft tissue counterpart and is the only accepted low-grade malignant vascular tumor of bone. It is still debatable whether other

low-grade malignant vascular tumors of bone exist. Therefore, it is recommended to avoid the term hemangioendothelioma of bone because it could confuse clinicians and radiologists. Unfortunately no molecular genetic data are available to support the proposed classification. Future molecular studies might reveal whether there is indeed a continuum between hemangioma and angiosarcoma i.e. according to a multistep genetic progression model as is also known for instance for chondrosarcoma [70], liposarcoma [71], or colorectal cancer [72]. Also, molecular studies may shed light on whether angiosarcoma of bone is comparable to angiosarcoma of deep soft tissue or whether it represents a separate entity within the heterogeneous group of angiosarcomas.

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3

Distinct histological features characterize primary angiosarcoma of bone

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Abstract

Aims: To define the histological criteria of primary angiosarcoma of bone.

Methods and results: Forty-two angiosarcomas of bone in 23 males and 15 females were studied. Histological criteria were related to patients' outcome. Eleven patients had multifocal lesions. Lesions were located in the long and short tubular bones followed by the pelvis, spine and trunk. Tumour cells were positive for CD31 in 38 of 40, von Willebrand Factor in 21 of 35, CD34 in 15 of 38, smooth muscle actin in 22 of 36, D2-40 in 11 of 35, and keratinAE1/AE3 in 27 of 39. Thirty-nine tumours showed an epithelioid phenotype. One- and 5-year survival rates were 55% and 33%, respectively. Survival analysis showed that a macronucleolus, three or more mitoses per 10 high power field (HPF) and less than five eosinophilic granulocytes per 10 HPF within a tumour was associated with an even worse survival compared to the overall group.

Conclusions: Because keratin positivity is seen in the majority of cases, pathologists should avoid misinterpretation as metastatic carcinoma. A macronucleolus, three or more mitoses per 10 HPF and fewer than five eosinophilic granulocytes per 10 HPF can be used to further define angiosarcoma of bone.

Introduction

Vascular tumours of bone, other than ordinary hemangiomas, are uncommon[1] and represent less than 1% of primary malignant bone tumours reported by large registries such as the Netherlands Committee on Bone Tumours[2] or the Mayo Clinic.[3] Clinically, these tumours present with widely varying signs and symptoms, and also at microscopic level a wide diversity of histological features are seen, varying from an easily recognizable vasoformative proliferation to an undifferentiated tumour and, occasionally, even mimicking metastatic carcinoma.[4-11] Their behaviour is unpredictable although, clinically, they can be extremely aggressive.[3, 11-13]

Over the years, the terminology and classification of vascular tumours of bone has been a matter of debate due to the lack of uniform terminology, accepted histological criteria, and the lack of good correlation with clinical outcome of the proposed entities. Although several classification schemes have been suggested, so far none has been generally accepted.[4-6] Various entities are described in the literature, sometimes used as synonyms or to stress different histological entities, confusing not only pathologists, but also radiologists, oncologists, and orthopaedic surgeons.[4-6,8,9] In 2000 Wenger and Wold[4] acknowledged the confusing terminology, and suggested that endothelial malignancies of bone should be regarded as a spectrum. This spectrum is formed with, on one end, the overtly benign lesions, and at the other end the frankly malignant lesions (Figure 1). In general, ordinary haemangioma is often easy to diagnose, being characterized by mature blood-filled vessels lined by flattened endothelial cells without marked cytonuclear atypia and lacking mitotic figures.[11] At the other end of the spectrum, angiosarcoma is the most widely accepted term for high-grade malignant vascular tumour of bone. Overt malignant features, such as high number of mitoses and frank cytonuclear atypia, facilitate this diagnosis, although there is no consensus in the literature regarding the exact criteria.[4-6] Another vascular tumour that is considered malignant is epithelioid haemangioendothelioma of bone, with a distinct well-characterized morphology, similar to its soft tissue counterpart, that can be differentiated most often from other endothelial lesions.[11]

There is no consensus regarding the nomenclature of tumours intermediate between ordinary haemangioma and angiosarcoma. Epithelioid haemangioma is a well-described entity (Figure 1).[14] Epithelioid haemangioma can be multifocal, can destroy the cortex and extend into the soft tissue, has a local recurrence rate of 8% and metastasizes to lymph nodes in 2%.[14] One could therefore consider epithelioid haemangioma as belonging to the intermediate category as recognized by the World Health Organization (WHO) 2002 classification for soft tissue tumours, which is either locally aggressive or rarely (<2%) metastasizing.[11] Epithelioid haemangioma[14] overlaps greatly with the entity described as 'haemangioendothelioma of bone' by Evans *et al.*,[5] which is a controversial and confusing entity that is presumed to be of the intermediate category or of low-grade malignancy, although consistent histological criteria defining this group of lesions are lacking. Criteria to distinguish bone epithelioid haemangioma and bone haemangioendothelioma, except perhaps for a lobular architecture, can not be extracted from the available literature. The WHO 2002 classification recognizes neither of them as distinct entities, but does recognize epithelioid haemangioma as a subtype of 'haemangioma of bone'.[11]

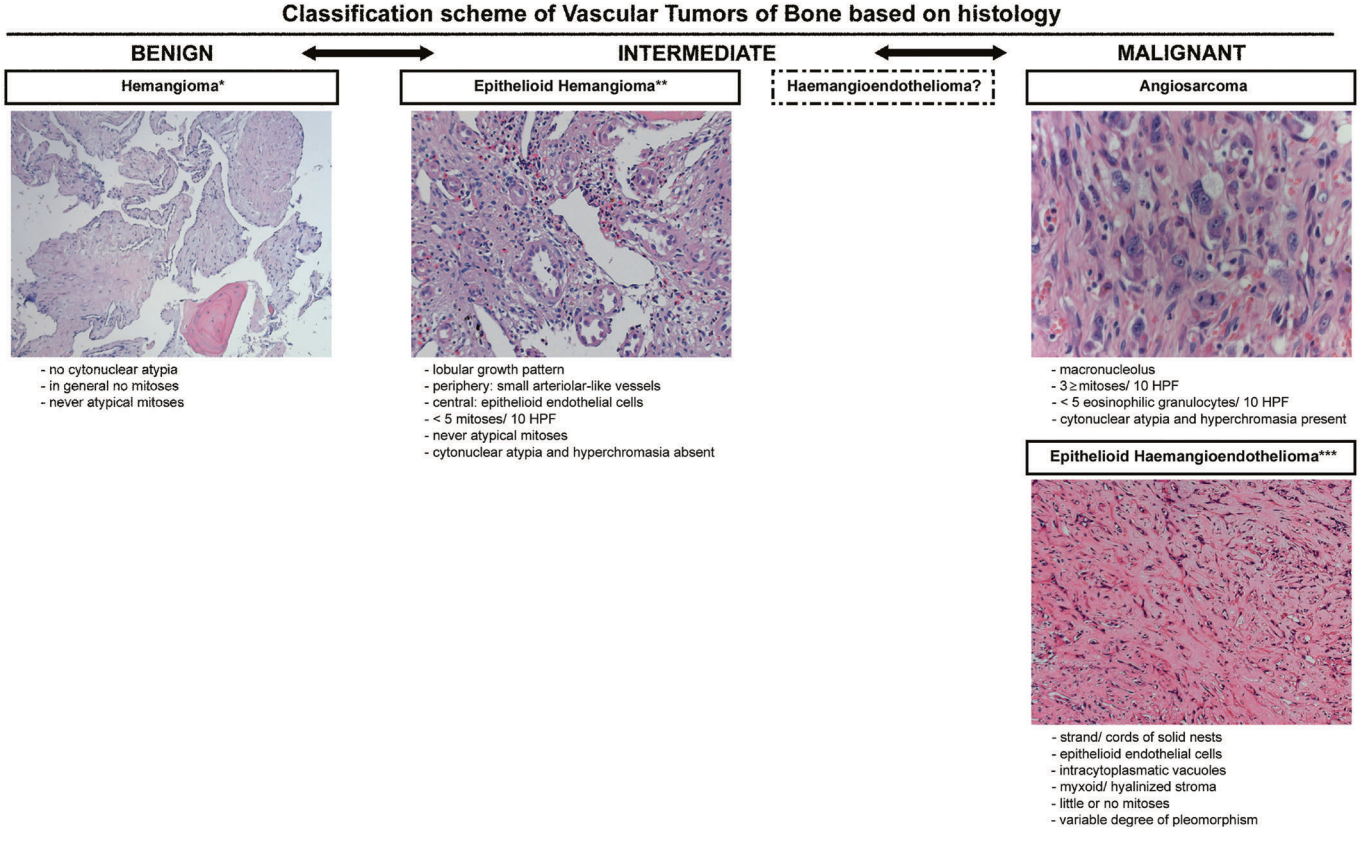

Figure 1. Classification scheme of vascular tumours of bone based on histological criteria described for ⋆haemangioma[11,12], ⋆⋆epithelioid haemangioma[6,14,46], angiosarcoma and ⋆⋆⋆epithelioid haemangioendothelioma[11]. Since the recognition of epithelioid haemangioma, it is unclear whether haemangioendothelioma is still an existent entity (dashed line). Today, it is accepted that epithelioid haemangioendothelioma, comparable to its soft tissue counterpart, is a malignant lesion.

Because there is no consistency in the literature on the exact histological criteria to diagnose angiosarcoma of bone,[4-6] and because it is uncertain if there still remains a category between epithelioid haemangioma/haemangioendothelioma and angiosarcoma, our aim was to delineate further the malignant end of vascular tumours of bone. We collected a large series of cases with follow-up and investigated whether based on histological and/or clinical criteria we could better define high-grade angiosarcoma of bone.

Material and methods

Clinico-pathologic data and histologic review

Eighty tumours, from 74 patients, diagnosed as angiosarcoma of bone were collected from the archives of the departments of Pathology of the Rizzoli Institute, Bologna, Italy (58 tumours from 53 patients), the Netherlands Committee on Bone Tumours (18 tumours in 18 patients), and University Hospitals, Leuven, Belgium (four tumours in three patients). The cases were originally diagnosed between 1964 and 2006. All clinical, radiological and pathological data were reviewed. All tumour samples were reviewed by three pathologists (J.V.M.G.B., P.C.W.H. and C.D.M.F.) and included in this study when the tumour showed histological features of malignancy, defined by atypia, hyperchromasia, mitoses and/or atypical mitoses,[4-6] and stained for at least for one endothelial marker, leaving 42 tumours (14 biopsies and 28 resection specimens) from 38 patients in which the diagnosis angiosarcoma of bone could be confirmed. Based upon histology [epithelioid haemangioma (n = 12), epithelioid haemangioendothelioma, or other diagnosis (n = 20) such as epithelioid sarcoma] and/or absence of at least one positive endothelial marker (n = 6), 38 tumours (34 patients) were excluded. All 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. As accepted in the literature, epithelioid haemangioendotheliomas and so-called haemangiopericytomas of bone are considered to be separate entities and therefore excluded from this study.[3-6, 8, 9, 11, 15-20]

Histology

Histological slides [haematoxylin and eosin (H&E)] were available for all 42 tumours. Various histological parameters as outlined in Table 1 were assessed systematically.

Tissue Microarray

Tissue microarrays (TMAs) were assembled from formalin-fixed, paraffin embedded tissue using standard procedures[21, 22] using a 0.6-mm-diameter punch (Beecher Instruments, Silver Spring, MD, USA). As formalin-fixed, paraffin-embedded tissue was available from 38 patients with vascular tumours of bone, two tissue blocks of different tumours were available in four patients, the arrays contained 42 tumours with four cores of each lesion whenever possible. Also, six tissue cores of tonsil and seven tissue cores of bowel were included for orientation and control purposes. Using a tape-transfer system (Instrumedics, Hackensack, NJ, USA), 4-μm sections were transferred to glass slides.

Table 1. Histological features of angiosarcoma of bone assessed in the total group (42 tumors of 38 patients) and scored in the group of patients with follow-up (FU) data available (33 tumors from 31 patients) associated with clinical outcome.

	Angiosarcoma of bone Total group (n=42)		Angiosarcoma of bone with FU (n=33)		
	No.	%	No.	%	P-value
Solid growth pattern					
Present	36	85.7	27	81.8	0.443
Absent	6	14.3	6	18.2	
Mature bloodvessels					
Present	24	57.1	20	60.6	**0.043***
Absent	18	42.9	13	39.4	
Epithelioid morphology					
Present	39	7.1	31	93.9	0.054
Absent	3	92.9	2	6.1	
Intracytoplasmatic vacuoles					
no	1	2.4	1	3	0.399
Filled	1	2.4	1	3	
Empty	6	14.3	5	15.2	
Filled and empty	34	81.0	26	78.8	
Extravasation of erythrocytes					
Present	40	4.8	32	97	0.443
Absent	2	95.2	1	3	
Iron deposition					
Present	41	2.4	32	97	0.443
Absent	1	97.6	1	3	
Atypia					
Mild	2	4.8	2	6.1	0.900
Moderate	26	61.9	19	57.6	
Severe	14	33.3	12	36.4	
Nucleolus					
1-3 small	27	64.3	221	63.6	**0.011***
macronucleolus	15	35.7	11	36.4	
Mitotic figures					
< 3/10 HPF	22	52.4	16	48.5	**0.002***
≥ 3/10 HPF	20	47.6	17	51.5	
Atypical mitotic figures					
Present	13	31.0	11	33.3	0.443
Absent	29	69.0	22	66.7	
Necrosis					
Present	21	50.0	17	51.5	0.895
Absent	21	50.0	16	48.5	
Stromal component					
Loose connective tissue	12	28.6	11	33.3	0.258
Dens	8	19.0	7	21.2	
Myxoid	1	2.4	1	3	
No stroma	4	9.5	4	12.1	
Admixture	11	26.2	10	30.4	
Not evaluable	6	14.3	0	0	
Lymphocytic infiltrate					
Present	41	97.6	32	97	0.443
Absent	1	2.4	1	3	
Eosinophilic infiltrate					
< 5/10 HPF	23	54.8	17	51.5	**0.021***
≥ 5/10 HPF	19	45.2	16	48.5	

Chi-square test.
* Statistically significant.
HPF, high-power field.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on the TMAs. TMA slides were stained with antibodies against CD31, CD34, von Willebrand Factor (vWF), smooth muscle actin (SMA), D2-40 (podoplanin) and keratinAE1/AE3. Immunohistochemical reactions were performed according to standard laboratory methods.[23] For each antibody a positive and negative external control was included. The antibodies, their sources, antigen retrieval methods, dilutions, positive and negative external control used are documented in Table 2. Staining was evaluated as positive, defined as 25% of individual tumour cells or more being positive, or negative.

Table 2. Antibodies used for immunohistochemical analysis

Antibody	Clone	Dilution	Antigen retrieval	Source
CD31	JC70A	1:200	citrate	Dakocytomation
CD34	QBEnd/10	1:200	-	Neomarkers
vWF	-	1:400	citrate	Dako
SMA	ASM-1	1:2000	-	Progen
D2-40	D2-40	1:100	citrate	Covance
Cytokeratin AE1/AE3	-	1:200	citrate	Neomarkers

Statistical analysis

Overall survival could be calculated for 31 patients for whom follow-up data were available from the date of diagnosis to the date of death or last information. Of these 31 patients, nine biopsies and 22 resection specimens were evaluated for different histological parameters as described previously. Univariate analysis of the histological parameters was performed using the chi-square test for dichotomous variables. Survival was evaluated by Kaplan-Meier analysis and the log-rank test. Values of $P \leq 0.05$ were considered statistically significant. Variables that achieved significance ($P \leq 0.05$) were entered subsequently into a multivariate analysis using the Cox-regression model [described as odds ratios (OR) with 95% confidence intervals (95% CI), together with the *P*–values]. Cox-regression analysis was carried out with clinical outcome (alive or dead) as the independent variable. The data were analyzed using SPSS 16.0 software (Chicago, IL, USA).

Results

Patient data

Patient characteristics

Angiosarcoma of bone has a wide age distribution, ranging from the second to the eighth decade, with two small peaks at the sixth and eighth decade (range 12-78 years; median: 51; Figure 2A). There is a slight preference for males (23 males versus 15 females). The majority of patients presented with local pain and/or swelling. One patient already presented with metastasis at the time of diagnosis. Five patients developed metastasis later in the course of their disease. In three patients, the metastatic status is unknown. Of all 38 patients, 33 (86.8%) were treated: 14 (36.8%) had only surgery and 12 (31.5%) had surgery combined with chemotherapy and/or

radiotherapy. One patient (2.6%) received only chemotherapy and one (2.6%) only radiotherapy. Three patients (7.9%) did not receive any therapy, two of these patients (5.3%) refused therapy. Therapy data of two patients (5.3%) are lacking.

Radiological appearance and localization

Radiological data were available for 26 patients. The overall appearance of the tumours was a destructive osteolytic mass with irregular borders. Only 16 tumours (61.5%) showed cortical disruption, and of those patients only five (31.3%) tumours showed extension into the surrounding soft tissue.

Eleven patients (29%) had multifocal lesions. In seven of them (64%) contiguous bones were affected mostly located in the lower extremities (n = 6), followed by spine (n = 1). Four of them (36%) had disseminated disease, involving distant bones. None of the 11 patients with multifocal lesions had lymph node involvement. Twenty-seven patients (71%) had solitary lesions. Of all patients with solitary tumours the long and short tubular bones (74%) were mostly affected, followed by pelvis (15%), axial location (7%) and trunk (4%; Figure 2B).

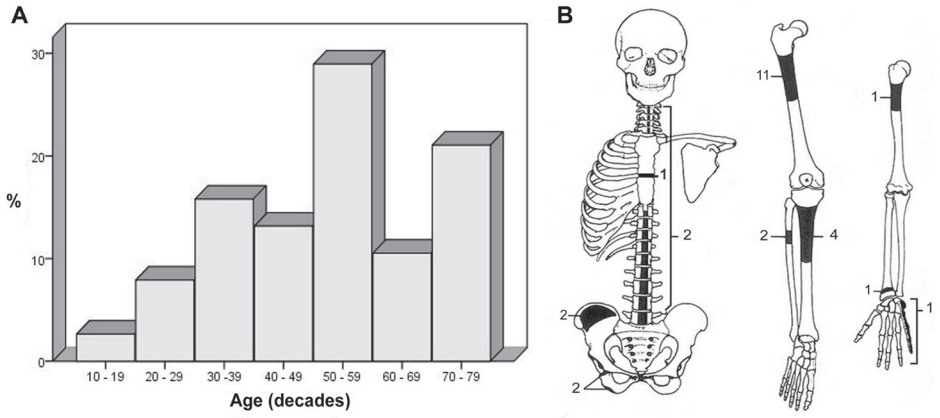

Figure 2 A, Age distribution of patients with angiosarcoma of bone (n = 38) and **B,** Skeletal distribution of solitary angiosarcoma of bone in 27 patients.

Histology

Angiosarcoma of bone showed variable histological patterns, of which the features were assessed systematically, and shown in Table 1. The majority (85.7%) of the cases showed a solid growth pattern and 57.1% demonstrated the presence of mature blood vessels, all lined by atypical endothelial cells, within the tumour (Figure 3A). Ninety-three per cent of the angiosarcomas displayed epithelioid morphology (Figure 3B). The most common histological feature was the presence of intracytoplasmatic vacuoles, either filled with erythrocytes or empty (Figure 3E). In 95.2% of the cases there was extravasation of erythrocytes resulting in iron deposition. All tumours showed cytonuclear atypia, which was scored mild, moderate or severe. Most of the angiosarcomas showed vesicular nuclei (95.2%). In 64.3% these nuclei contained one to three small nucleoli (Figure 3C), while in 35.7% macronucleoli (Figure 3D) were found. When the

tumour contained a nucleolus that was half the size of a small lymphocyte or larger (≥ 4μm) in 75% of the tumour cells or more, the tumour was labelled as having a macronuclolus. Half of the tumours contained necrotic areas. A variable inflammatory infiltrate was present in all cases, consisting generally of lymphocytes, although an eosinophilic infiltrate (five or more eosinophilic granulocytes/10 HPF) was present in 45.2% of the cases (Figure 3F). The infiltrate ranged from 5 to 60 eosinophilic granulocytes/10HPF. Mitotic activity ranged from 0 to 9 per 10 HPF (median: 2).

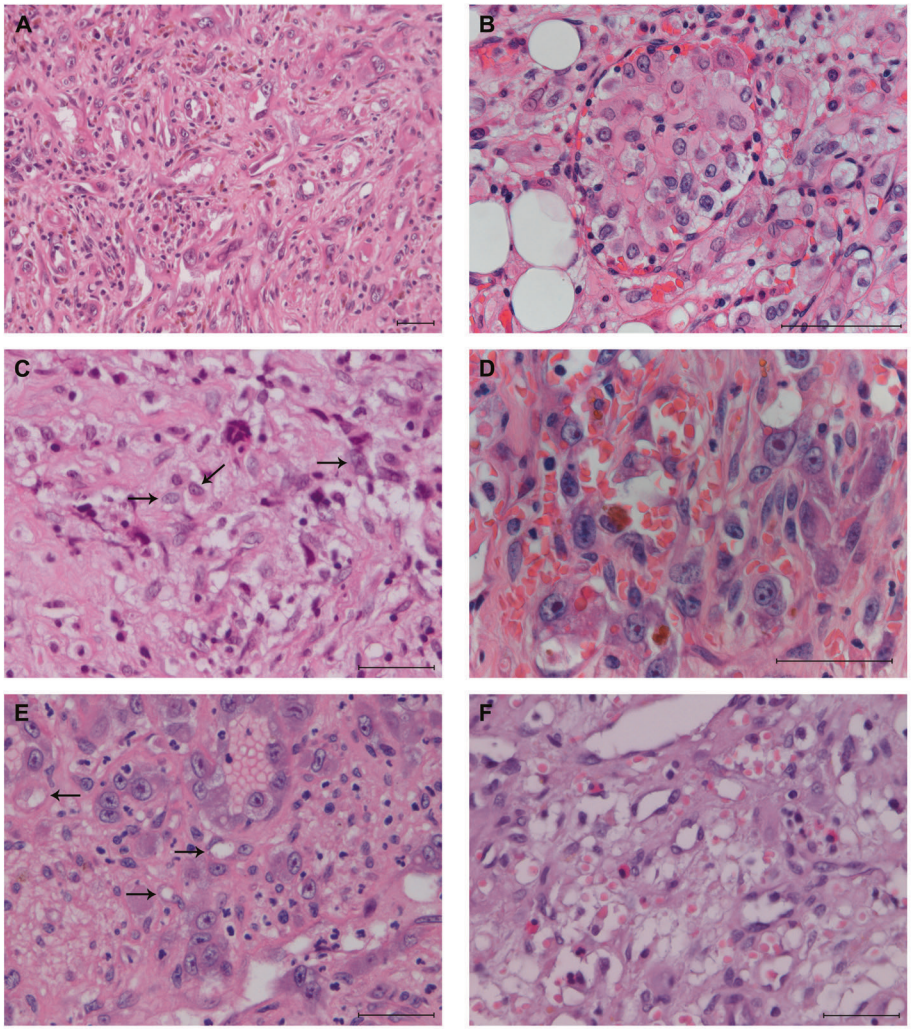

Figure 3. Different histological features of vascular tumours of bone: **A,** the presence of mature bloodvessels; **B,** epithelioid morphology; **C,** one to three small nucleoli in tumour cells (arrows); **D,** macronucleoli in tumour cells; **E,** presence of intracytoplasmatic vacuoles (arrows); **F,** presence of an eosinophilic infiltrate (A-F: haematoxylin and eosin staining; scale bar: 50 μm).

Immunohistochemistry

Angiosarcomas of bone were positive for CD31 in 38 of 40 (95%), for CD34 in 15 of 38 (39%), and for vWF in 21 of 35 (60%) cases. All tumours stained positively for at least one endothelial marker. Twenty-two of 36 tumours (61%) showed positive staining of individual tumour cells for SMA. D2-40 immunoreactivity was seen in 11 of 35 angiosarcomas (31%), while 27 of 39 cases (69%) were positive for keratin. All keratin-positive angiosarcomas showed an epithelioid morphology. However, nine angiosarcomas with epithelioid morphology did not show any positive staining for keratin. Due to tissue loss we were not able to evaluate two tumours (one patient) for CD31, tree tumours (three patients) for keratin, four tumours (three patients) for CD34, six tumours (five patients) for SMA, seven tumours (six patients) for D2-40 and seven tumours (seven patients) for vWF staining.

Statistical analysis

Median follow-up, counted from the date of diagnosis, was 12 months (range 1.4- 207.7 months). Within six months after diagnosis 32% of the patients had died of disease. The 1-, 2- and 5-year survival rates were 55%, 43% and 33%, respectively (Figure 4). Univariate analysis of the histological parameters associated with clinical outcome (alive or dead of disease) are documented in Table 1.

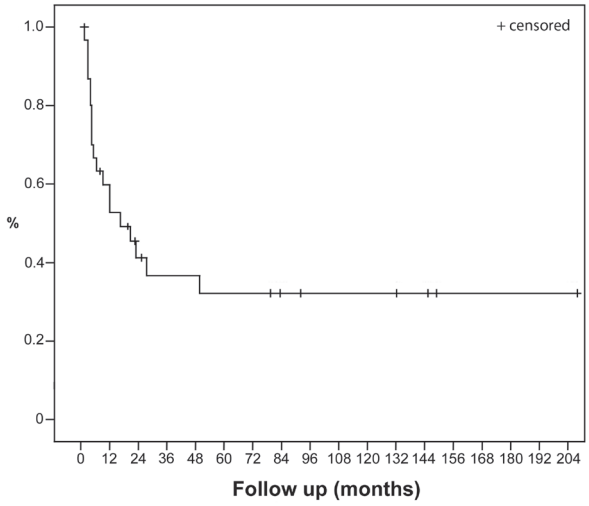

Figure 4. Kaplan-Meier overall survival curve of all patients for whom follow-up data were available (n = 31).

Kaplan-Meier survival analysis revealed a significant difference in overall survival when the tumour had three or more mitoses per 10 HPF (P = 0.0001) or a macronucleolus (P = 0.010). Moreover, the presence of fewer than five eosinophilic granulocytes/10 HPF was also associated with poor survival (P = 0.003). Kaplan-Meier survival analysis in relation to immunophenotype

did not reveal any significant differences for CD31, CD34, vWF, SMA or keratin. However, angiosarcomas of bone that were positive for D2-40 showed a worse outcome ($P = 0.046$). Cox-regression analysis showed that the most predictive combination of histological variables for poor survival was three or more mitotic figures per 10 HPF, a macronucleolus and the presence of fewer than five eosinophilic granulocytes/10 HPF (Table 3). Angiosarcomas containing these three histologic features showed an even more aggressive course compared to the overall group, with a 5-year survival rate of 0%.

Table 3. Cox-regression analysis of independent variables associated with poor survival.

Histological variables	Odds Ratio	95% CI	*P*-value
Macronucleolus	2.46	0.813 - 7.440	0.111
Mitotic figures ≥ 3/ 10 HPF	3.86	1.076 - 13.847	0.038
Eosinophilic infiltrate < 5/ 10 HPF	4.21	1.418 - 12.497	0.010

OR, Odds ratio; 95% CI, 95% confidence interval; HPF, high-power field.

Discussion

Vascular tumours of bone consist of a spectrum of clinicopathological entities ranging from benign haemangioma at one end to angiosarcoma at the other end. Intermediate between haemangioma and angiosarcoma is the clinicopathological entity epithelioid haemangioma which has been well described recently[14] and which is now generally accepted.[24] There is no consistency in the literature on the exact histological criteria with which to diagnose angiosarcoma of bone.[4-6] In addition, it is uncertain if there still remains a clinically delineated category between epithelioid haemangioma and angiosarcoma. We therefore attempted to delineate further malignant vascular tumours of bone by studying a large series of angiosarcomas. Within the EuroBoNet consortium we collected a large series of vascular tumours with previous diagnoses other than (epithelioid) haemangioma, epithelioid haemangioendothelioma and haemangiopericytoma of bone.

Twelve cases (15%) originally diagnosed as angiosarcoma were reclassified as epithelioid haemangioma. Follow-up data available of 11 patients revealed that all of these patients survived (follow-up range: 8.2 - 449 months) and none of them developed a local recurrence or metastasis. There were no tumours that were intermediate between epithelioid haemangioma and angiosarcoma. This is consistent with the recent reclassification of 23 vascular tumours of bone within the International Society for the Study of Vascular Anomalies (ISSVA),[24] although in this study three cases were reported that could not be classified, while one case was still labelled haemangioendothelioma of bone.

In our series, the presence of metastases are not very well documented, on the one side due to rapidly progressive disease leading to death from disease without further clinical investigation and on the other side due to the fact that for some of the older cases more limited clinical or radiological investigations were performed. However, in patients with rapidly progressive disease

leading to death from disease, presumably metastases were presumed to be present. Therefore, the presumed metastatic rate is 61%.

To date, there is no consensus in the literature regarding the exact histological criteria defining angiosarcoma of bone. Wenger and Wold[4] stated that tumours with high-grade cytological atypia and brisk mitotic activity should be classified as angiosarcomas. O'Connell *et al.*[6] proposed marked nuclear atypia and abundant mitotic figures, including atypical forms, as the most important histological criteria. However Evans *et al.*[5] recommended nuclear hyperchromasia, pleomorphism and high mitotic activity (>5 mitoses/10 HPF) as histological criteria to define angiosarcoma of bone. In our series of angiosarcomas of bone, cytonuclear atypia was found in all tumours, nuclear hyperchromasia in 45.2% and mitotic activity ranged from 0 to 9 mitoses per 10 HPF, thus qualifying for the diagnosis of angiosarcoma of bone. In three tumours no mitoses could be detected, although for those cases only a very small biopsy specimen was available. In these three cases cytonuclear atypia was prominent.

Within the group of angiosarcomas of bone we identified three high-risk histological parameters. Three or more mitoses per 10 HPF ($P = 0.0001$), a macronucleolus ($P = 0.010$) and the presence of fewer than five eosinophilic granulocytes per 10 HPF ($P = 0.003$) correlated with poor prognosis on multivariate analysis. Furthermore, a subset of angiosarcomas of bone containing the combination of these features showed an even more aggressive course. Therefore, our data show that within the spectrum of vascular tumours of bone three or more mitoses per 10 HPF, macronucleoli and fewer than five eosinophilic granulocytes per 10 HPF predict aggressive behaviour and poor outcome (Figure 1).

Almost one-third of the patients had multifocal disease, which is consistent with the literature.[4, 17, 25] Although the cause and biological mechanism of multifocal disease still remains unknown, in our series multifocal disease is not associated with a better prognosis as suggested in some reports for haemangioendothelioma and epithelioid haemangioendothelioma of soft issue.[4, 13, 18]

Vascular tumours variably express CD31, CD34 and vWF.[26-28] In our series 95% showed positive staining for CD31, suggesting that this is the best diagnostic marker for vascular tumours of bone. However, two out of 42 (5%) vascular tumours of bone showed positive staining for CD34 but lacked staining of CD31 or vWF. Therefore, a panel of these three markers is suggested for diagnostic use. The keratin positivity, seen in 69% of the cases which we examined, was very striking. Keratin expression has been documented in non neoplastic endothelial cells,[29] in epithelioid vascular neoplasms,[11, 30] and in up to 35% of angiosarcomas of soft tissue, independent of an epithelioid morphology,[11, 31-35] as well as in various other sarcomas, such as Ewing sarcoma.[36] Our findings suggest a higher rate of keratin positivity in angiosarcomas of bone. Because these lesions have a tendency to occur multifocally (contiguous or disseminated), based on likelihood the radiologist will suggest metastatic carcinoma or multiple myeloma. This, combined with the histology demonstrating epithelioid cells (92.9%), a solid growth pattern (85.7%) and keratin positivity (69%), may easily lead to an erroneous diagnosis of metastatic carcinoma (Figure 5).

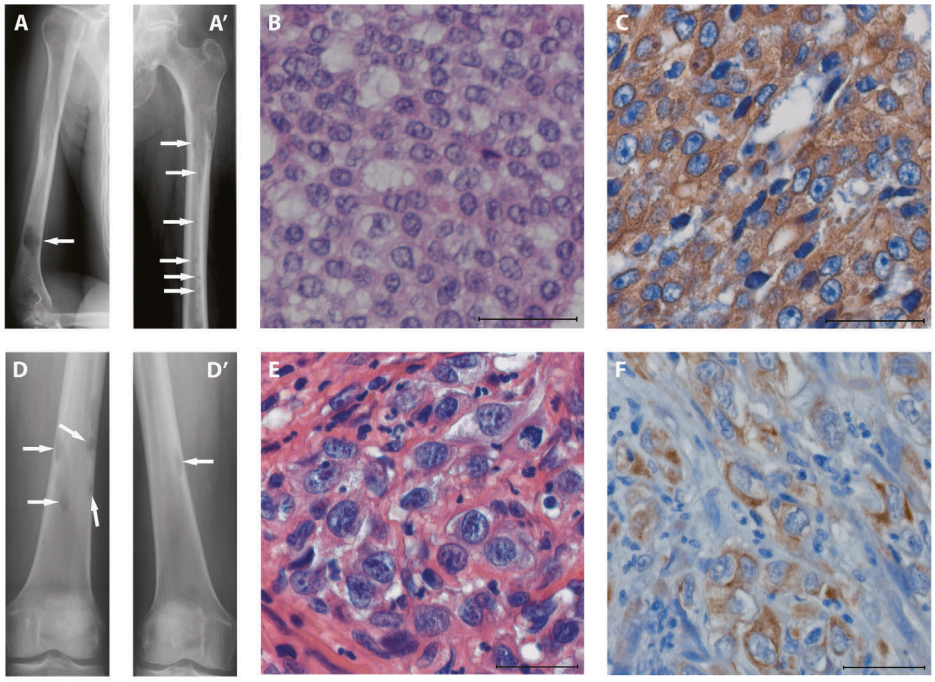

Figure 5. Illustrating the radiological and histological similarities between a metastatic carcinoma (**A**, **A'**, **B** and **C**) and a multifocal angiosarcoma of bone (**D**, **D'**, **E, F**). **A, A'** and **D, D'**: conventional X-rays exhibiting multifocal osteolytic bone lesions (arrows); **B** and **E**: haematoxylin and eosin staining of the tumour cells (scale bar: 50 μm); **C** and **F**: keratin AE1/AE3 staining of the tumour cells (scale bar: 50 μm).

D2-40 is a monoclonal mouse antibody reacting against human podoplanin, a mucine transmembrane protein.[37] It was first described as a selective marker against lymphatic endothelium; however, today it is known to be present in a variety of neoplasms.[37-40] The expression of D2-40 in a subset of soft tissue angiosarcomas has been reported in the literature, mainly in angiosarcomas exhibiting a hobnail or epithelioid morphology.[41, 42] This indicates that a subset of angiosarcomas display lymphangiogenic differentiation, and are therefore designated as lymphangiosarcoma by some authors.[41, 43] Lymphangiogenic differentiation as displayed by D2-40 expression was suggested to be an indicator of aggressive behaviour for angiosarcoma of soft tissue.[44] We demonstrate the existence of lymphangiogenic differentiation in 31% of angiosarcomas of bone, even though it has been shown previously that normal lymphatic vessels are absent in bone.[45] Similar to its soft tissue counterpart, D2-40 expression is associated with a worse prognosis of angiosarcoma of bone.

In conclusion, we describe a series of angiosarcomas of bone characterized by mitotic activity and nuclear atypia, defining the malignant end of the spectrum of vascular tumours of bone. These tumours have a 5-years survival rate of 33%. More specifically, three or more mitosis per 10 HPF, the presence of a macronucleolus and the presence of fewer than five eosinophilic granulocytes per 10 HPF, are risk factors for poor prognosis, and the finding of all three of

these histological risk factors in the same lesion decreases the 5-year survival rate to 0%. The majority of angiosarcomas of bone show epithelioid morphology and positive staining for cytokeratin is seen in a large subset of these tumours. As these lesions have the tendency to occur multifocally (29% of cases in this series), radiologists and pathologists should be aware not to misinterpret them as metastatic carcinoma (Figure 5). Although a panel of vascular markers is useful to diagnose these lesions, CD31 is the most commonly expressed vascular marker and lymphangiogenic differentiation, as shown by the presence of D2-40, seems to predict a more aggressive course.

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4

Active TGF-β signaling and decreased expression of PTEN separates angiosarcoma of bone from its soft tissue counterpart

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Abstract

Angiosarcomas constitute a heterogeneous group of highly malignant vascular tumors. Angiosarcoma of bone is rare and poorly characterized. For angiosarcoma of soft tissue, some pathways seem to be involved in tumor development. Our aim was to evaluate the role of these pathways in angiosarcoma of bone. We collected 37 primary angiosarcomas of bone and used 20 angiosarcomas of soft tissue for comparison. Immunohistochemistry was performed on constructed tissue microarrays to evaluate expression of CDKN2A, TP53, PTEN, BCL2, CDK4, MDM2, cyclin D1, β-catenin, transforming growth factor-β (TGF-β), CD105, phospho-Smad1, phospho-Smad2, hypoxia-inducible factor-1α (HIF-1α), plasminogen activator inhibitor type 1 (PAI-1), VEGF, CD117 and glucose transporter-1 (GLUT-1). *PIK3CA* was screened for hotspot mutations in 19 angiosarcomas. In nearly 55% of the angiosarcoma of bone, the retinoblastoma (Rb) pathway was affected. Loss of CDKN2A expression was associated with a significantly worse prognosis. No overexpression of TP53 or MDM2 was found, suggesting that the TP53 pathway is not important in angiosarcoma of bone. Angiosarcoma of bone showed highly active TGF-β signaling with immunoreactivity for phospho-Smad2 and PAI-1. Although the phosphatidylinositol 3-kinase (PI3K)/Akt pathway seems to be active in both tumor groups, different mechanisms were involved: 41% of angiosarcoma of bone showed a decrease in expression of PTEN, whereas in angiosarcoma of soft tissue overexpression of KIT was found (90%). *PIK3CA* hotspot mutations were absent. In conclusion, the Rb pathway is involved in tumorigenesis of angiosarcoma of bone. The PI3K/Akt pathway is activated in both angiosarcoma of bone and soft tissue, however, with a different cause; PTEN expression is decreased in angiosarcoma of bone, whereas angiosarcomas of soft tissue show overexpression of KIT. Our findings support that angiosarcomas are a heterogeneous group of vascular malignancies. Both angiosarcoma of bone and soft tissue may benefit from therapeutic strategies targeting the PI3K/Akt pathway. However, interference with TGF-β signaling may be specifically relevant in angiosarcoma of bone.

Introduction

Angiosarcoma is a rare malignant neoplasm composed of cells that demonstrate endothelial differentiation accounting for less than 1% of all sarcomas.[1,2] Although angiosarcoma can occur at any anatomical site throughout the body, it is most commonly found in the skin of the head and neck region and its underlying superficial soft tissue.[1,2] In all, 20-40% are located within the deep muscles of the extremities.[1-4] Angiosarcoma of bone is extremely rare and accounts for <1% of all malignant bone tumors.[5-7] Angiosarcomas present with widely varying signs and symptoms. Although their clinical behavior is rather unpredictable, it has been generally accepted that they can be extremely aggressive.[1,2,8-11]

Angiosarcomas constitute a highly heterogeneous group of tumors. At present, there is an increasing role for cellular and molecular features in the classification of tumors.[1] These features do not only have an impact on the identification and diagnosis of tumors, but can as well have some therapeutic implications.[12] So far, there are relatively few studies investigating cellular and molecular changes within angiosarcoma of bone. Single-case reports and small studies have shown the possible involvement of cell cycle regulators, such as cyclin D1,[13] and tumor-suppressor genes such as *CDKN2A* and *TP53*,[1,14-21] in sporadic angiosarcoma of skin, soft tissue and visceral angiosarcomas suggesting a possible role in tumorigenesis in a subset of angiosarcomas. Moreover, a subset of angiosarcomas harbor specific genetic alterations based on either their anatomical site or exposure to radiation.[22-24] In that perspective, high levels of *MYC* amplification are found in 55-100% of angiosarcomas secondary to radiation exposure or chronic lymphedema after breast surgery and radiation, which is in 25% associated with *FLT4* (FMS-like tyrosine kinase-4 encoding for VEGFR3) amplification.[22-24] In addition, *KDR* (kinase insert domain receptor, VEGFR2) mutations are present in 10% of angiosarcoma of the breast, either primary or secondary to radiation exposure.[22,23] These findings implicate that, based on tumor-specific alterations, angiosarcoma of soft tissue is highly heterogeneous. In angiosarcoma of bone, molecular studies are absent. Therefore, it is still unclear whether angiosarcoma of bone is a true separate entity or is similar to primary angiosarcoma of deep soft tissues. We therefore examined the expression of a large panel of oncogenes, tumor-suppressor genes, and signaling molecules and performed hotspot mutation analysis for *PIK3CA i*n angiosarcoma of bone. Results were compared to angiosarcoma of soft tissue. Our aim was to identify potential pathways involved in the development of angiosarcoma of bone that could serve as potential target for treatment.

Material and methods

Clinicopathological Data

Formalin-fixed paraffin-embedded tissue of 37 tumors from 33 patients, with the confirmed diagnosis of angiosarcoma of bone were collected from the archives of the Departments of Pathology of the Rizzoli Institute, Bologna, Italy (33 tumors from 30 patients), and University

Hospitals, Leuven, Belgium (4 tumors from 3 patients) as previously well characterized and described.[25] For comparison, 20 angiosarcomas of soft tissue from 20 patients were collected from the archives of the Department of Pathology of University Hospitals, Leuven, Belgium (14 tumors from 14 patients) and Leiden University Medical Center (6 tumors from 6 patients). Fresh frozen tumor tissue of six angiosarcomas of soft tissue (from six patients) was available for mutation analysis. All specimens were handled according to the ethical guidelines described in 'Code for Proper Secondary Use of Human Tissue in the Netherlands' of the Dutch Federation of Medical Scientific Societies.

Tissue Microarray

Four tissue microarrays were assembled by standard procedures[26,27] using a 2 mm-diameter punch (3DHistech, Budapest, Hungary; three tissue microarrays) or a 0.6 mm-diameter punch (Beecher Instruments, Silver Spring, MD, USA; one tissue microarray) containing 37 angiosarcomas of bone and 20 angiosarcomas of soft tissue with four cores of each lesion whenever possible. Also six tissue cores of tonsil, seven tissue cores of bowel and three cores of liver were included for orientation and control purposes. Using a tape-transfer system (Instrumedics, Hackensack, NJ, USA), 4-μm sections were transferred to glass slides.

Immunohistochemistry

Immunohistochemistry was performed on tissue microarray slides with different antibodies for oncogenes, tumor-suppressor genes, and signaling molecules which were selected based on their possible role in angiosarcoma of soft tissue, or based on their role in angiogenesis (Table 1). Immunohistochemical reactions were performed according to standard laboratory methods.[28] For each antibody a positive and negative external control was included. The antibodies, their sources, antigen retrieval methods, dilutions, positive and negative external controls used are documented in Table 1. As negative control, sections were stained without adding the primary antibody. The intensity (0 = no staining, 1 = weak, 2 = moderate, 3 = strong) and percentage of positive neoplastic cells (0 = 0%, 1 = 1-24%, 2 = 25-49%, 3 = 50-74%, 4 = 75-100%) were evaluated. Lost tissue cores were excluded from the analysis. Since decalcification could compromise the immunohistochemical result, we attempt to counteract this phenomenon by excluding the tissue samples with a negative internal control. The sum of intensity and percentage was used for analysis. Data were collected through homemade software.[29]

DNA Isolation and Mutation Detection

DNA was isolated from formalin-fixed paraffin-embedded material from 13 angiosarcomas of bone, and from fresh frozen material from six angiosarcomas of soft tissue, as described previously.[30] The Custom TaqMan® Assay Design Tool (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands) was used to detect seven different *KRAS*, three *PIK3CA* and one *BRAF* mutations as described previously.[31] Raw data from the LC480 software were imported into an inhouse–created Microsoft Excel 2003 spreadsheet to define the mutation status as described in detail previously.[31]

Table 1. List of antibodies used for immunohistochemical analysis

	Function	Clone	Dilution	AR	Blocking	Source	Positive control
CDKN2A	Cell cycle regulator	JC8	1:200	Citrate	-	Immunologic	Cervical carcinoma
TP53	Cell cycle regulator	D0-7	1:1000	Citrate	-	Dako	Adenocarcinoma
PTEN	Tumor-suppressor gene	138G6	1:200	Citrate	-	Cell Signaling	Thyroid
CDK4	Oncogene	DCS-31	1:800	Citrate	-	Biosource	Dedifferentiated Liposarcoma
Cyclin D1	Oncogene	SP4	1:400	EDTA	-	Dako	Tonsil
BCL2	Anti-apoptotic protein	124	1:200	Citrate	-	Dako	Tonsil
MDM2	P53-binding protein	IF2	1:200	Citrate	-	Zymed	Dedifferentiated Liposarcoma
β-Catenin	Wnt signaling pathway	beta-Catenin-1	1:1600	Citrate	-	Dako	Skin
CD117	Tyrosine kinase receptor	-	1:2000	-	-	Dako	Gastrointestinal stromal tumour
GLUT-1	Glucose transporter	-	1:1000	Citrate	-	Abcam	Esophagus
HIF-1alpha	Transcription factor	H1alpha67	1:2500	Dako TRS	-	Abcam	Chondrosarcoma
PAI-1	TGF-β pathway	3785	1:200	-	-	American Diagnostica	Cervical carcinoma
phospho-Smad1	BMP signalling	-	1:100	Citrate	5% Elk milk	Cell Signaling	Colon
phospho-Smad2	TGF-β pathway	Ser465/467	1:250	Citrate	NGS 10%	Cell Signaling	Kidney
TGF-β1	TGF-β pathway	9016	1:20	Citrate	-	R&D Systems	Breast carcinoma
CD105	TGF-β pathway	NCL-CD105	1:100	Citrate	-	Novocastra	Tonsil
VEGF	Growth factor	JH121	1:100	-	-	Thermoscientific Cat.	Breast carcinoma

Abbreviations: NGS, normal goat serum; TRS, target retrieval Solution.

Statistical Analysis

The results of the immunohistochemical analysis were correlated to clinicopathological data available for the angiosarcomas of bone, which were documented previously.[25] The correlation between immunohistochemical markers and the specific location of the tumors (bone *versus* soft tissue) was performed using global testing and the non-parametric Mann-Whitney *U* two-independent sample test. Global testing was used to determine whether a pre-specified group of genes were differentially expressed between angiosarcoma of bone and soft tissue.[32] Kruskal-Wallis H test (non parametric K independent sample test) was performed to evaluate the correlation between immunohistochemical markers within the different subgroups based on the origin (bone, soft tissue, head and neck region, or visceral involvement). Subsequently, immunohistochemical markers were combined into different pathways and by global testing we evaluated the correlation between these pathways and the two different groups of angiosarcoma (bone *versus* soft tissue) and corrected the results for multiple testing. Values of $P<0.005$ (corrected for multiple testing) were considered statistically significant. The data were analyzed using R (R Development Core Team (2011). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/), Global testing[32] and SPSS 17.0 software (SPSS inc., Chicago, Illinois, USA). As described previously, follow-up data were available of 31 patients with angiosarcoma of bone.[25] Survival was evaluated by Kaplan-Meier analysis and logrank test. Values of $P \leq 0.05$ were considered statistically significant.

Results

Patient Characteristics

Patient characteristics of both angiosarcoma of bone and soft tissue are summarized in Table 2. All patient characteristics for angiosarcoma of bone have been described in detail previously.[25] Based on the location of the angiosarcoma of soft tissue, the patients could be divided into three different subgroups: seven patients had an angiosarcoma located within the head and neck region, eight patients had an angiosarcoma of deep soft tissue and five patients had a visceral angiosarcoma. None of the patients had a history of radiation, that is, for other cancers, and/ or chronic lymphedema at the location of the angiosarcoma.

Table 2. Clinical and treatment characteristics of angiosarcoma of bone compared to angiosarcoma of soft tissue

Angiosarcoma	Bone *n* (%)	Soft tissue *n* (%)
Total number of patients	33	20
Male	22 (67)	11 (55)
Female	11 (33)	9 (45)
Age range	12-75 y	30-91 y
Mean	51.6 y	67.4 y
Median	54.6 y	69.5 y
Follow-up (months) range	1.7 - 207.7	NK
mean follow-up (months)	37.3	NK
median follow-up (months)	12	NK
Local recurrence	8 (24)	4 (20)
Metastases		
at time of diagnosis	1	-
during course	5	8
not known	3	12
Treatment	30 (91)	20 (100)
Surgery	12 (37)	17 (85)
Surgery + chemotherapy	8 (24)	1 (5)
Surgery + radiotherapy	4 (12)	-
Surgery + chemo- and radiotherapy	1 (3)	-
Chemotherapy	1 (3)	2 (10)
Radiotherapy	4 (12)	-
No treatment	2 (6)	-
Treatment not known	1 (3)	-
5-Year survival	33%	NK

Abbreviations: *n*, patient numbers; NK, not known; y, year.

Cell Cycle Regulators in Angiosarcoma of Bone

Nearly 55% of the angiosarcoma of bone showed a defect in the retinoblastoma (Rb) pathway, affecting either CDKN2A, cyclin D1 or both. A total loss of protein expression of CDKN2A (sumscore 0, with positive internal control) was detected in 49% (16/33) of angiosarcomas of bone. Six percent (2/33) of the angiosarcomas of bone demonstrated overexpression of cyclin D1 (sumscore ≥ 6), of which one tumor showed normal CDKN2A expression and one tumor showed a total loss of CDKN2A protein expression. Kaplan-Meier survival analysis demonstrates that patients showing loss of CDKN2A expression (sumscore 0) have a significantly worse survival ($P = 0.017$; Figure 1). Overexpression of CDK4 (sumscore ≥ 6) did not occur in angiosarcoma of bone (0%). None of the angiosarcomas of bone showed overexpression (sumscore ≥ 6) of TP53 (Figure 2) or MDM2. The anti-apoptotic protein BCL-2 was only weakly expressed in the majority of angiosarcoma of bone.

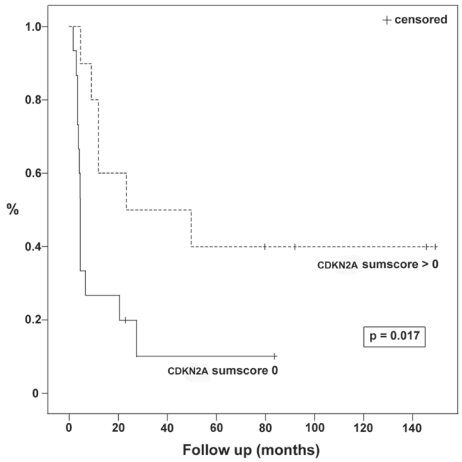

Figure 1. Kaplan-Meier survival analysis demonstrates a significantly worse survival for patients with angiosarcoma of bone showing loss of CDKN2A expression (sumscore 0).

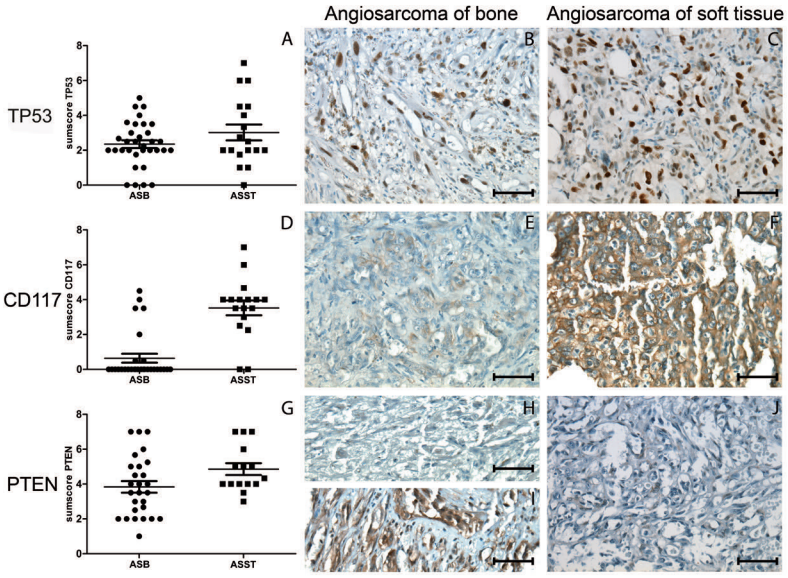

Figure 2. Dot plots and immunohistochemical results for TP53, CD117 en PTEN in angiosarcoma of bone (ASB) compared with its soft tissue (ASST) counterpart. (**a**) TP53 shows no overexpression in angiosarcoma of bone, whereas three angiosarcomas of soft tissue show clear TP53 overexpression (sumscore > 6). Although some angiosarcomas of bone show positivity for TP53, clear overexpression was not seen (**b**), in contrast to angiosarcoma of soft tissue (**c**); (**d**). Angiosarcomas of bone show a significant lower expression of CD117 than angiosarcomas of soft tissue; (**e**) weak expression of CD117 in angiosarcoma of bone compared to high expression in angiosarcoma of soft tissue (**f**); (**g**) A subset of angiosarcomas of bone show a decrease in expression (sumscore ≤ 3) of PTEN. Low expression (**h**) and normal expression (**i**) of PTEN in angiosarcoma of bone, as well as low expression of PTEN in an angiosarcoma of soft tissue (**j**) (scale bars 50 μm).

Signaling Pathways in Angiosarcoma of Bone

Transforming growth factor-β (TGF-β) signaling was highly active in angiosarcoma of bone; all tumors showed immunoreactivity for the ligand TGF-β1 and the downstream targets phospho-Smad2 and plasminogen activator inhibitor type-1 (PAI-1; Figure 3). Dot plots for all antibodies are shown in Figure 4. In addition, nuclear expression of phospho-Smad1, which is indicative of active BMP signaling, was found in 68% of the angiosarcomas of bone (sumscore > 0). Endoglin (CD105) was expressed (sumscore > 0) by the tumor cells in the majority (97%) of the tumors. Moreover, 41% of the angiosarcomas of bone showed a decrease in expression of PTEN (sumscore ≤ 3) suggesting involvement of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Figure 2). There was no evidence of active canonical Wnt signaling as we did not find nuclear localization of β-catenin in the vast majority of the tumors. As for cell survival signaling, both the hypoxia-inducible factor)-1α as well as the glucose transporter-1 were expressed in only a few tumors. On the other hand, VEGF was widely and strongly expressed (Figure 4).

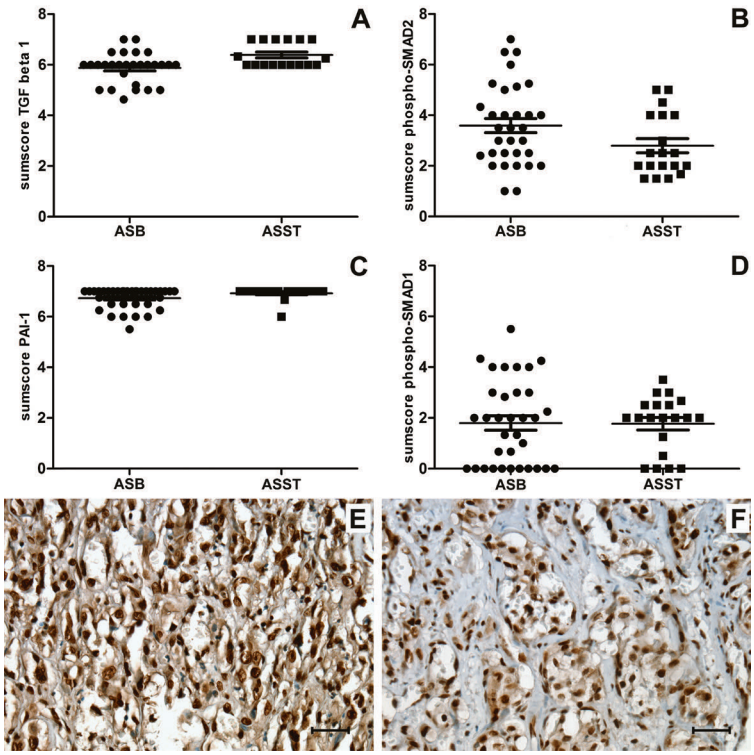

Figure 3. Difference in protein expression (sumscore) for TGF-β (**a**), phosphoSmad2 (**b**), PAI-1 (**c**) and phosphoSmad1 (**d**) between angiosarcoma of bone (ASB) and soft tissue (ASST) illustrated by dot plots; (**e**) Angiosarcoma of bone showing a strong TGF-β expression; (**f**) Angiosarcoma of bone showing a strong phosphoSmad2 expression (scale bars: 50 μm).

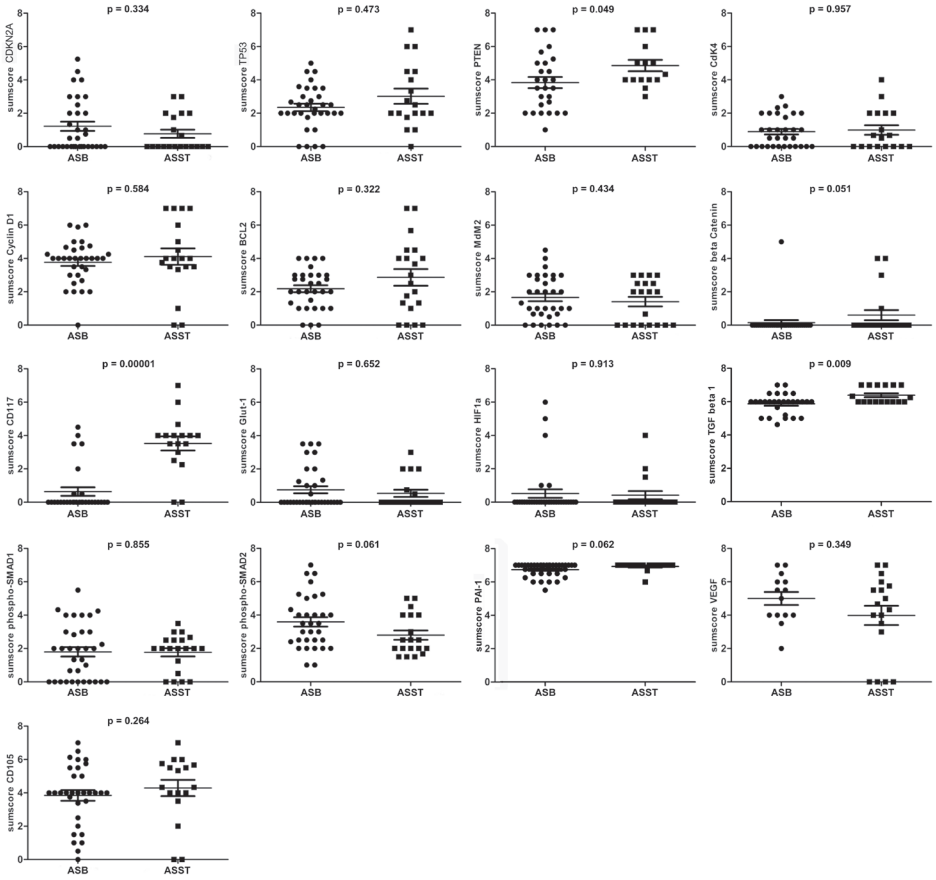

Figure 4. The difference in protein expression (sumscores) of all studied antibodies between angiosarcoma of bone (ASB) and soft tissue (ASST) illustrated by dot plots and their corresponding *P*-value.

Angiosarcoma of Bone *versus* Angiosarcoma of Soft tissue

When combining immunohistochemical markers belonging to the same pathway essential in cell signaling, proliferation and survival, global testing demonstrates a significant difference in expression for TGF-β pathway and PI3K/Akt pathway between angiosarcoma of bone and soft tissue (Table 3). The TGF-β signaling is more active in angiosarcoma of bone compared to its soft tissue counterpart as shown by global testing, which is based on difference in phospho-Smad2 expression between angiosarcoma of bone and soft tissue (Figure 5). The PI3K/Akt pathway seems to be involved in both angiosarcoma of bone and soft tissue, but through a different mechanism. The PI3K/Akt pathway in angiosarcoma of bone is affected by decreased expression of PTEN (sumscore ≤ 3), whereas in angiosarcoma of soft tissue this is mainly associated with overexpression of CD117. Decreased expression of PTEN is seen in 11 of 27 angiosarcomas of bone (41%) as compared to 1 of 15 angiosarcomas of soft tissue (7%) ($P = 0.049$; (Figure 2). Only 17% (5/29) of the angiosarcomas of bone show expression of CD117, compared to

90% (17/19) of the angiosarcomas of soft tissue (Figure 2) Global testing indeed demonstrates a significant difference in the overall immunohistochemical expression pattern (including all markers tested) between angiosarcoma of bone and soft tissue ($P = 0.0004$), which is mainly due to CD117 being predominantly expressed in soft tissue angiosarcoma ($P = 0.00001$; Figure 5). In addition, global testing shows that there is an apparent difference in expression for phospho-Smad2, PTEN and TGF-β between both tumor groups (Figure 5). The difference in protein expression between angiosarcoma of bone and soft tissue of all antibodies are illustrated by dot plots documented in Figure 4. In 75% of the angiosarcomas of soft tissue the Rb pathway is affected, either by loss of CDKN2A (sumscore 0, 12/20; 60%) and/or overexpression of cyclin D1 (sumscore ≥ 6, 5/19; 26%). Only six percent (3/18) of the angiosarcomas of soft tissue show overexpression of TP53 (sumscore ≥ 6). Tumors overexpressing TP53 were mainly located in the head and neck region (2/3).

Table 3. The different pathways based on a combination of different immunohistochemical markers that are used for global testing between angiosarcoma of bone and angiosarcoma of soft tissue.

Pathways	Markers	*P*-value adjusted
TGF-β	TGF-β+phospho-Smad2+PAI-1	**0,003**
BMP	phospho-Smad1	0.946
Rb	Cyclin D1+CDK4+CDKN2A	0.505
TP53	BCL2+MDM2+TP53	0.166
PI3K/Akt	CD117 + PTEN	**0.0000002**

The significant *P*-values are shown in bold.

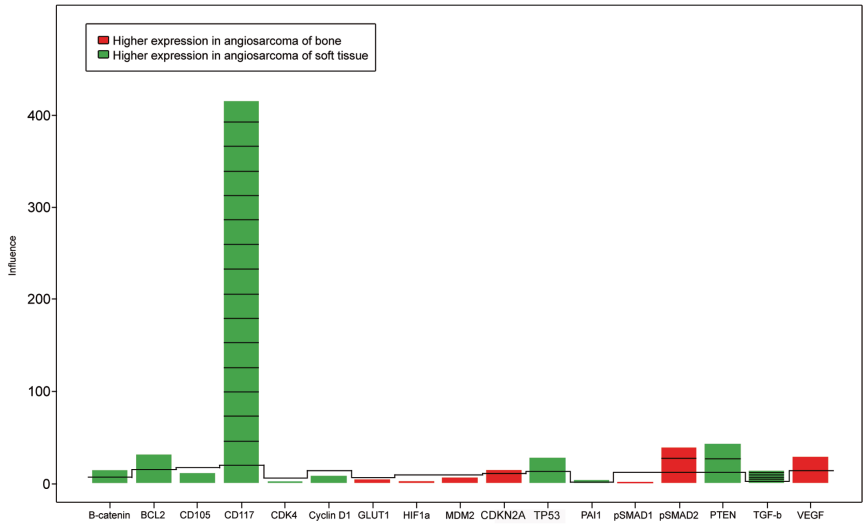

Figure 5. Global testing (R) shows a significant difference ($P = 0.00001$) in immunohistochemical expression of the different assessed antibodies between angiosarcoma of bone and angiosarcoma of soft tissue.

Mutation Analysis in Angiosarcoma

Since the PI3K/Akt signaling pathway seems to be differently activated between angiosarcoma of bone and soft tissue, we continued to analyze for mutations in *PIK3CA*. However, no hotspot mutations were detected in angiosarcoma of bone or soft tissue. Moreover, there were no mutations detected for *KRAS* and *BRAF*.

Discussion

Dysregulation of the Rb/CDKN2A and/or TP53 pathway are the most common alterations found in almost all types of human cancer.[33] Our results show that in angiosarcoma of bone, the Rb pathway is disrupted in 55% of the cases, mainly by loss of protein expression of CDKN2A (49%) or overexpression of cyclin D1 (6%), contributing to uncontrolled cell proliferation and tumorigenesis. We additionally show that, unlike in angiosarcoma of soft tissue, in angiosarcoma of bone the TP53 pathway is not important. The involvement of the Rb pathway in a small majority of angiosarcoma of bone is consistent with the hypothesis that angiosarcomas belong to the category of sarcomas with complex genomic profiles, which frequently have alterations in the Rb/CDKN2A and/or TP53 pathways.[34,35]

Moreover, inactivation of *CDKN2A* is associated with a more aggressive behavior in many cancers, including for instance Ewing sarcoma,[34,36,37] and osteosarcoma.[38,39] Here, we demonstrate that also patients with angiosarcoma of bone showing loss of expression of CDKN2A have a significantly worse prognosis. To date, few studies have reported the possible role of *CDKN2A* and *TP53* in tumor development in a subset of angiosarcomas of soft tissue.[15-20,37,40-42] Recently, it has been shown that *Ink4/Arf* deficiency in mice is associated with the development of angiosarcoma, lymphoma and fibrosarcoma.[43]

By global testing, we demonstrate that TGF-β pathway (TGF-β1/phospho-Smad2/PAI-1) is highly active in angiosarcoma of bone and therefore could play an important role in tumorigenesis. TGF-β1 is the major isoform present in bone and, among others, involved in bone formation.[44] It binds and activates ALK5, one of its specific type I receptors, and thereby inducing phosphorylation of Smad2/Smad3, resulting in the expression of PAI-1.[45] To date, it is accepted that the TGF-β signaling pathway plays an important role during embryogenesis, cell differentiation and maintenance of homeostasis during adult life, but it also prohibits uncontrolled cell proliferation.[45,46] Deregulation of this pathway causes resistance to the TGF-β mediated growth arrest and gives rise to malignancies.[47-49] However, the molecular mechanisms provoking this deregulation, that is, genetic and epigenetic aberrations, and the underlying oncogenic activities of TGF-β still remain unclear.[49] Our findings suggest possible therapeutic options for angiosarcoma of bone, because inhibitors of the TGF-β pathway (Figure 6) are available.[50] By now, multiple pre-clinical and clinical studies are investigating the inhibition of the oncogenic TGF-β signaling as a therapeutic approach in different types of cancer.[50]

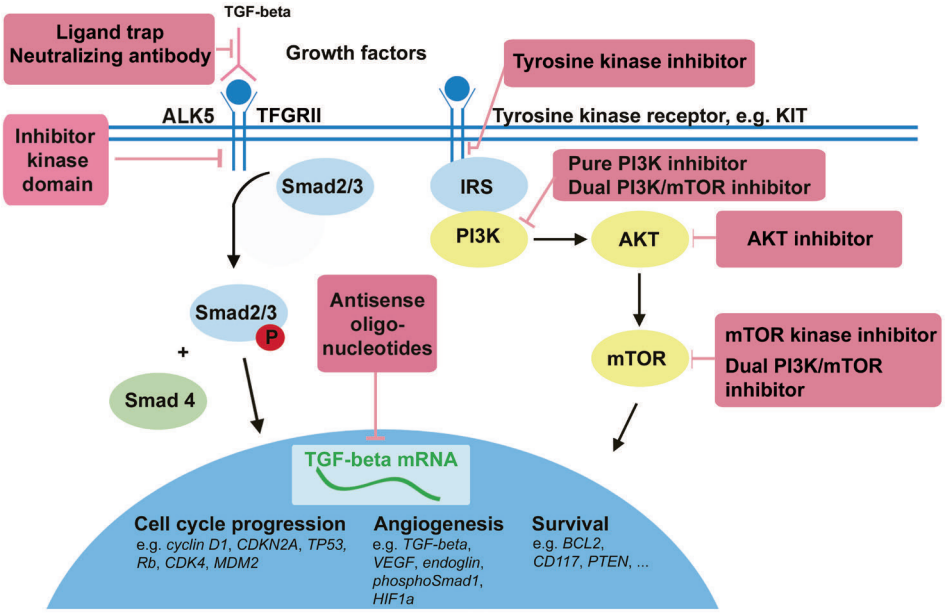

Figure 6. A simplified overview of the TGF-β pathway (left) and the PI3K/Akt (right) pathway and possible therapeutic strategies to inhibit the pathway. TGF-β can be inhibited by ligand traps (soluble receptor ectodomain constructs), neutralizing antibodies and ALK5/TβRII kinase inhibitors. In addition, ligand production can be prevented by blocking translation of TGF-β mRNA with antisense oligonucleotides. The PI3K/Akt pathway can be inhibited by pure PI3K inhibitors, dual PI3K/mTOR inhibitors, AKT inhibitors, and mTOR inhibitors e.g. rapamycin and analogs (rapalogs). More specifically, receptor tyrosine kinases such as KIT can be blocked by a tyrosine kinase inhibitor (TKI).

Pathway analysis further demonstrates that the PI3K/Akt pathway, is highly active in both angiosarcoma of bone and soft tissue (Figure 6). However, the differences in protein expression suggest that activation occurs through different mechanisms. In angiosarcoma of bone 41% show decreased expression of the tumor-suppressor PTEN, compared to only 7% in angiosarcoma of soft tissue. However, in angiosarcoma of soft tissue, the tyrosine kinase receptor KIT is overexpressed in 90%, whereas this is infrequent (17%) in angiosarcoma of bone. This is corresponding to previous studies, reporting that 20% to 58% of the soft tissue angiosarcomas show CD117 expression,[51,52] whereas normal adult endothelial cells lack CD117 overexpression.[52-54] To date, there are no *KIT* and/or *PDGFRA* mutations detected in angiosarcoma of soft tissue.[52,54,55] Of note, there is one report of a very good response to imatinib (Glivec, a specific tyrosine kinase inhibitor) in angiosarcoma of soft tissue.[55] We show limited expression of CD117 in a minority of angiosarcoma of bone, suggesting that a specific tyrosine kinase-inhibitor, such as imatinib, could be a therapeutic option for angiosarcoma of soft tissue but not for angiosarcoma of bone. In angiosarcoma of bone on the other hand we demonstrate decreased expression of PTEN. To date, there is only one report of a human *PTEN* gene mutation in one out of two angiosarcomas of the liver containing a single nucleotide substitution in exon 7.[56] A recent publication demonstrates that about 10% of the *PTEN*-haploinsufficient zebrafishes

develop hemangiosarcomas and these tumors have an activated PI3K/Akt signaling,[57] thereby illustrating the possible role of *PTEN* in the development of vascular tumors. Although it would be interesting to evaluate possible *PTEN* mutations within angiosarcoma of bone, mutation analysis could not be performed in our study, since there was not enough more DNA available. The PI3K/Akt pathway is a signal transduction cascade that plays a role and regulates a large variety of important physiological processes, such as cell proliferation and metabolism, adhesion, survival, protein synthesis, motility and angiogenesis.[58] Genetic aberrations, such as *PIK3CA* mutation, have been described, which constitutively activate this pathway and thereby potentially induce malignancies.[58,59] As the PI3K/Akt pathway is activated in both angiosarcoma of bone and soft tissue, we additionally examined *PIK3CA* for hotspot mutations. No mutations were found. Of note, recent publications have shown the presence of *PIK3CA* mutations in vascular anomalies and benign vascular lesions.[60,61]

The activated PI3K/Akt pathway in angiosarcomas provides a rationale for the use of inhibitors of the PI3K/Akt pathway in these patients (Figure 6).[62,63] The serine/ threonine kinase mammalian target of rapamycin (mTOR) is a kinase downstream in the PI3K/Akt pathway and therefore an important regulator of cell proliferation, metabolism, and protein synthesis. The mTOR activity can be inhibited by rapamycin (sirolimus) and analogs (rapalogs), for example, Temsirolimus/CCI-779 and everolimus/RAD001. Beuvinck *et al* demonstrated in human tumor xenografts that everolimus inhibits angiogenesis by preventing the proliferation of endothelial cells.[63] So far, there is only one report demonstrating the anti-proliferative effect of rapamycin in angiosarcoma cell lines.[64] Additionally, new drugs such as pure PI3K inhibitors, dual PI3K-mTOR inhibitors, Akt inhibitors and mTOR kinase inhibitors are evolving and passing through the early phases of clinical development.[58] Our findings would support further studies into the effectiveness of these inhibitors in angiosarcoma of bone. However, thus far, no cell lines derived from angiosarcoma of bone are available.

In conclusion, we demonstrate that angiosarcoma of bone is different from angiosarcoma of soft tissue at the molecular level, suggesting that the underlying mechanism of tumorigenesis between both groups may be different. This supports the previous finding that angiosarcomas are a heterogeneous group of malignant vascular tumors with possible different subgroups and corresponding expression profiles.[22,23] We demonstrate that in 55% of the angiosarcoma of bone the Rb pathway is involved in tumorigenesis, mainly by the loss of protein expression of CDKN2A which seems to be related with a significantly worse prognosis. The TP53 pathway seems of no importance in angiosarcoma of bone. Moreover, by global testing we demonstrate that the TGF-β pathway is more active in angiosarcoma compared to its soft tissue counterpart. The PI3K/Akt pathway is active in both angiosarcoma of bone and soft tissue, and we here demonstrate that while in angiosarcoma of soft tissue overexpression of KIT is involved, in angiosarcoma of bone PTEN expression is decreased. Our results provide rationale for therapeutic strategies including TGF-β and/or PI3K/mTOR inhibitors to improve the generally poor prognosis of patients with angiosarcoma of bone.

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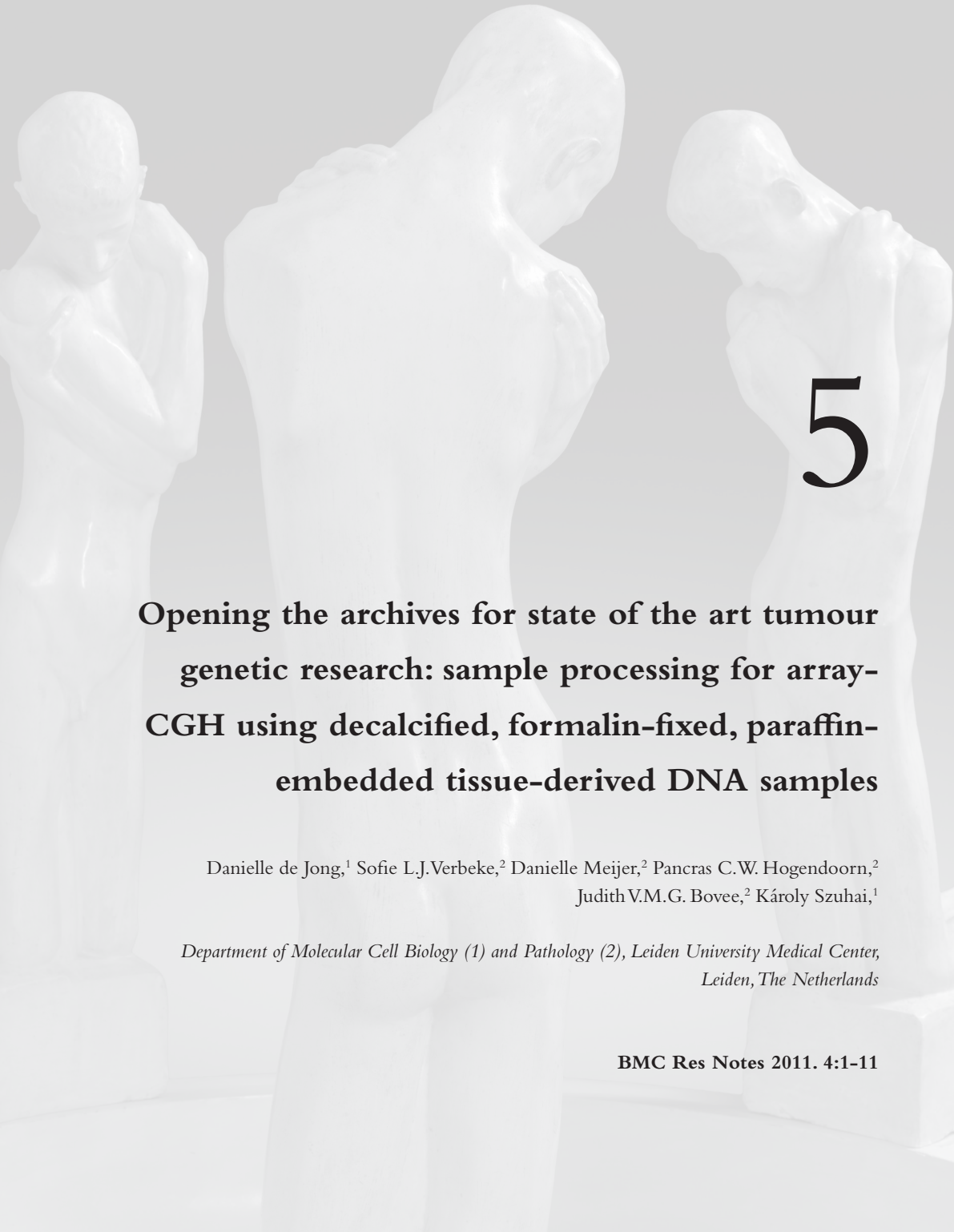

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Opening the archives for state of the art tumour genetic research: sample processing for array-CGH using decalcified, formalin-fixed, paraffin-embedded tissue-derived DNA samples

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Abstract

Background: Molecular genetic studies on rare tumour entities, such as bone tumours, often require the use of decalcified, formalin-fixed, paraffin-embedded tissue (dFFPE) samples. Regardless of which decalcification procedure is used, this introduces a vast breakdown of DNA that precludes the possibility of further molecular genetic testing. We set out to establish a robust protocol that would overcome these intrinsic hurdles for bone tumour research.

Findings: The goal of our study was to establish a protocol, using a modified DNA isolation procedure and quality controls, to select decalcified samples suitable for array-CGH testing. Archival paraffin blocks were obtained from 9 different pathology departments throughout Europe, using different fixation, embedding and decalcification procedures, in order to preclude a bias for certain lab protocols. Isolated DNA samples were subjected to direct chemical labelling and enzymatic labelling systems and were hybridised on a high resolution oligonucleotide chip containing 44,000 reporter elements.
Genomic alterations (gains and losses) were readily detected in most of the samples analysed. For example, both homozygous deletions of 0.6 Mb and high level of amplifications of 0.7 Mb were identified.

Conclusions: We established a robust protocol for molecular genetic testing of dFFPE derived DNA, irrespective of fixation, decalcification or sample type used. This approach may greatly facilitate further genetic testing on rare tumour entities where archival decalcified, formalin fixed samples are the only source.

Background

The introduction of high-throughput, high-resolution molecular screening tools had tremendous impact on molecular genetic studies both for constitutional and tumour genetic investigations [1,2]. Whilst the accessibility of good quality samples for constitutional genetic studies is often achievable, for cancer genetic investigations it has remained a hurdle especially for those dealing with rare tumour entities. A comprehensive study of rare cancers, such as bone tumours, requires the use of archived tissue materials such as formalin fixed paraffin embedded tissue (FFPE) [3-5]. It is well known that the quality of FPPE-derived DNA is both fixation time- and fixative-dependent and is highly variable between different institutions. 10% buffered formalin is a commonly used fixative in routine diagnostic labs. Long term storage of this fixative leads to the formation of formic acid and methanol by the Cannizzaro-reaction. Formic acid promotes the breakdown of the DNA and thus inferior quality is extracted from these tissue samples. To process bone derived tumour samples, an extra decalcification step is necessary to remove the Ca^{2+} containing matrix part of the tissue. This can be achieved either by EDTA treatment or by an extensive formic acid treatment. EDTA treatment is a labour-intensive procedure and takes up to several weeks of incubation. The treatment introduces limited breakdown of DNA but because of its lengthy procedure it is impractical for routine diagnostics. The formic acid-based decalcification procedure introduces a tremendous breakdown of DNA within these samples. As a result, most of these samples are usually regarded as unsuited for molecular biological testing. The formic acid-based decalcification has been the golden standard procedure at many institutions, meaning that most of the archival material collected from multiple sources has been treated in this way. The goal of our study was was to establish a modified DNA isolation protocol with quality controls enabling array-CGH testing on decalcified samples irrespective of fixation and decalcification steps used. Isolated DNA samples were labelled using two FFPE labelling kit systems and were hybridised on a high resolution oligonucleotide chip containing 44k reporter elements.

Material and Methods

Sample selection

Samples were selected for molecular cytogenetic testing from various partner institutions within the EuroBoNet consortium http://www.eurobonet.eu for different projects (rare chondrosarcoma subtypes of bone and primary angiosarcoma of bone) dealing with decalcified FFPE (dFFPE) samples. Samples used in this study represent both tumours with high cellularity and a low extracellular matrix proportion as well as samples with low cellularity and an excessive extracellular matrix composition. Sample collection dates varied from 1990 until 2008. Samples were all fixed in 10% buffered formalin but the exact fixation times and conditions are not known (Table 1). For one case (Nr 10) array comparison using DNA isolated from dFFPE tissue and the corresponding frozen tissue part was possible. All samples were handled in a

coded fashion, and all procedures were performed according to the ethical guidelines, ''Code for Proper Secondary Use of Human Tissue in the Netherlands'' (Dutch Federation of Medical Scientific Societies).

Table 1. Overview of samples included in this study

Sample ID	Diagnosis	Collection date	Material	Cellularity	Extracellular matrix	Decalcification
1	Rare chondrosarcoma	2004	dFFPE	High	Low	Formic acid
2	Rare chondrosarcoma	2007	dFFPE	High	Low	Formic acid
3	Rare chondrosarcoma	2007	dFFPE	Low	High	Formic acid
4	Rare chondrosarcoma	1996	dFFPE	Low	High	Formic acid
5	Rare chondrosarcoma	1997	dFFPE	High	Low	Formic acid
6	Rare chondrosarcoma	2005	dFFPE	Low	High	Formic acid
7	Rare chondrosarcoma	2005	dFFPE	High	Low	Formic acid
8	Rare chondrosarcoma	2004	dFFPE	Moderate	Moderate	Formic acid
9	Rare chondrosarcoma	2006	dFFPE	Moderate	Moderate	Formic acid
10	Rare chondrosarcoma	2000	dFFPE	Moderate	Moderate	Formic acid
11	Rare chondrosarcoma	1996	dFFPE	Moderate	Moderate	Formic acid
12	Rare chondrosarcoma	NA	dFFPE	High	Moderate	Formic acid
13	Rare chondrosarcoma	NA	dFFPE	High	Moderate	Formic acid
14	Rare chondrosarcoma	1994	dFFPE	High	Moderate	Formic acid
15	Rare chondrosarcoma	NA	dFFPE	High	Moderate	Formic acid
16	Rare chondrosarcoma	2007	dFFPE	High	Moderate	Formic acid
17	Rare chondrosarcoma	NA	dFFPE	High	Moderate	Formic acid
18	Rare chondrosarcoma	2001	dFFPE	High	Moderate	Formic acid
19	Rare chondrosarcoma	1994	dFFPE	High	Moderate	Formic acid
20	Rare chondrosarcoma	1996	dFFPE	High	Moderate	Formic acid
21*	Rare chondrosarcoma	2000	Frozen	Moderate	Moderate	None
22**	Rare chondrosarcoma	1996	Frozen	Moderate	Moderate	None
23	Chondrosarcoma	2001	Frozen	High	High	None
24	Chondrosarcoma	2003	Frozen	low	High	None
25	Primary angiosarcoma	2007	Frozen	High	Low	None
26	Primary angiosarcoma	NA	dFFPE	High	Low	Formic acid
27	Primary angiosarcoma	2007	dFFPE	High	Low	Formic acid
28	Primary angiosarcoma	2007	FFPE	High	Low	None
29	Primary angiosarcoma	NA	FFPE	High	Low	None
30	Primary angiosarcoma	2007	FFPE	High	Low	None
31	Primary angiosarcoma	NA	dFFPE	High	Low	Formic acid
32	Chondrosarcoma	1990	dFFPE	Low	High	Formic acid

* Corresponding frozen sample Nr 10
** Corresponding frozen sample Nr 11
***NA: not available

DNA isolation

Five to ten 0.2 mm FFPE punches or two to five 20 μm thick dFFPE sections were collected depending on tissue type and tumour content. From each block a 4 μm consecutive section was cut and stained using standard haematoxylin and eosin (HE) staining to visualise target cells and served as control. An optimized DNA isolation protocol was developed based on the use of Macherey-Nagel Nucleospin Tissue kit. Briefly, sections/punches were collected into an Eppendorf tube and were deparaffinised using two cycles of xylene incubation, 15 min each at room temperature, followed by two steps of 100% ethanol incubation, 15 min each. Samples were then dried and 200 μl PK1 buffer supplemented with Proteinase K (0.4 mg/ml) was added to each tube and incubated for 18 hours at 56 °C. On day two, 200 μl buffer B3 was added to each vial. Samples were vortexed vigorously, incubated at 70°C for 10 min and vortexed again. By these means, most tissue pieces were dissolved. When visible particles were left (typically bone remnants), samples were centrifuged for 5 min at 11.000 × g and supernatant was transferred to a new tube. Before loading samples to a DNA binding column, 210 μl 100% ethanol was added. At this step, a partial precipitation within the solution was observed in some of the samples. For DNA binding, samples were centrifuged for 1 min at 11.000 × g. In some cases, repeated centrifugation steps were necessary. Flow-through was discarded and columns were washed by adding 500 μl BW solution followed by 1 min 11.000 × g centrifugation step, followed by a second wash step using 600 μl B5 buffer and centrifugation. To elute the DNA 50 μl preheated (70°C) MQ solution was added to the column and incubated at room temperature for 5 min followed by a centrifugation step at 11000 × g for 1 min. DNA isolation from frozen tissue was performed as described earlier [6].

Sample assessment

DNA concentrations were measured using a Nanodrop ND-1000 spectrophotometer and 500 ng was electrophoresed in a 1% agarose gel stained with ethidium bromide.

Sample labelling

Agilent Oligo aCGH Labeling Kit for FFPE Samples (Agilent) utilising ULS labelling system

Labelling was done according to the manufacturer's recommendations with some modifications. In brief, for 44k Agilent arrays (Agilent Technologies, Santa Clara, CA), 500 ng DNA was chemically labelled with Universal Linkage System (ULS) Cy3 (test) or Cy5 (reference)-dyes. Before labelling, reference samples were heat fragmented in order to achieve equal fragment sizes in both test and reference sample. The labelled samples were then purified using the Agilent KREApure columns. Labelling efficiency was calculated using a Nanodrop Spectrophotometer measuring A260 (DNA), A550 (Cy3) and A649 (Cy5).

BioPrime Total FFPE Genomic Labelling System (Invitrogen)

Labelling was done according to the manufacturer's recommendations with some modifications. In brief, 500 ng DNA was used for labelling, instead of the recommended 1 μg DNA. Labelling with both 150 ng and 500 ng DNA was done for one sample (Nr 13). Random

prime (RP) labelling was done by using the BioPrime Total FFPE Genomic Labelling System (Invitrogen Corporation, Carlsbad, CA) Labelling efficiency was calculated using a Nanodrop Spectrophotometer measuring A260 (DNA), A550 (Cy3) and A649 (Cy5). Heat-fragmented DNA from a commercial source (Promega Corporation, Madison, WI) was used as a reference. Samples were labelled with Alexa Fluor 3 mix (test sample) and Alexa Fluor 5 mix (reference sample). For both ULS- and RP-system-labelled test and reference samples were mixed and hybridized as a gender mismatch to show dynamic range of hybridisation on the X and Y chromosomes. Two samples were labelled both with the random prime kit and with ULS (Nr 10 and Nr 18).

Hybridisation, scanning and, data extraction

Hybridisation was performed on a 4 × 44k Agilent oligo array Chip at 65°C for 40 hours. Slides were washed with Oligo aCGH Wash Buffer 1 at room temperature for 5 min followed by a 1 min wash with Oligo aCGH Wash Buffer 2 at 37°C. Finally, slides were dried without using the stabilisation and drying solution. Slides were scanned using an Agilent Scanner with 5 μm scan resolution. Scan images were processed with Feature Extraction Software and Genomic Workbench (Agilent Technologies, Santa Clara, CA). All samples, irrespective of quality, were processed for further comparisons.

Interphase FISH confirmation

To confirm one of the array-CGH results of case Nr 26, a two-colour interphase FISH experiment was done. A BAC-clone (RP1-80K22) located at 8q24.21 covering the MYC gene locus (detected in red) in combination with an alpha satellite probe specific to the centromeric region of chromosome 8 (detected in green) were used as described earlier [7].

Statistical analysis

Log2 transformed ratio values were extracted from the scan images and processed using the Feature Extraction Software package and Genomic Workbench (Agilent). The exported log2 transformed ratio values were used for further comparison. Correlations were calculated using Pearson coefficients and systematic bias calculations were done by using Bland-Altman plots using the SPSS 16.0 for Windows software package. For the Bland-Altman plots the differences between the two individual reporters measured by two experiments on the y axis were plotted against the mean log2 ratio of the two on the x axis. This test allows the investigation of systematic bias. Relatively small differences and little bias are represented by a "flat profile". For the comparison of the resulting array-CGH profiles we used the CGHCall R script developed by van de Wiel et al. [8].

Results

DNA quality and quantity assessment

DNA concentration was estimated using the Nanodrop system and equal amounts of DNA were electrophoresed in a 1% agarose gel. The absorption based measurement using the Nanodrop system showed inconsistent results when values were compared to agarose gel images. Figure 1 shows a diverse range of DNA fragment sizes for all samples. Samples with moderate (for example nr 3 and 27) to severe (sample nr 17, and 31) DNA degradation showed acceptable CGH profiles.

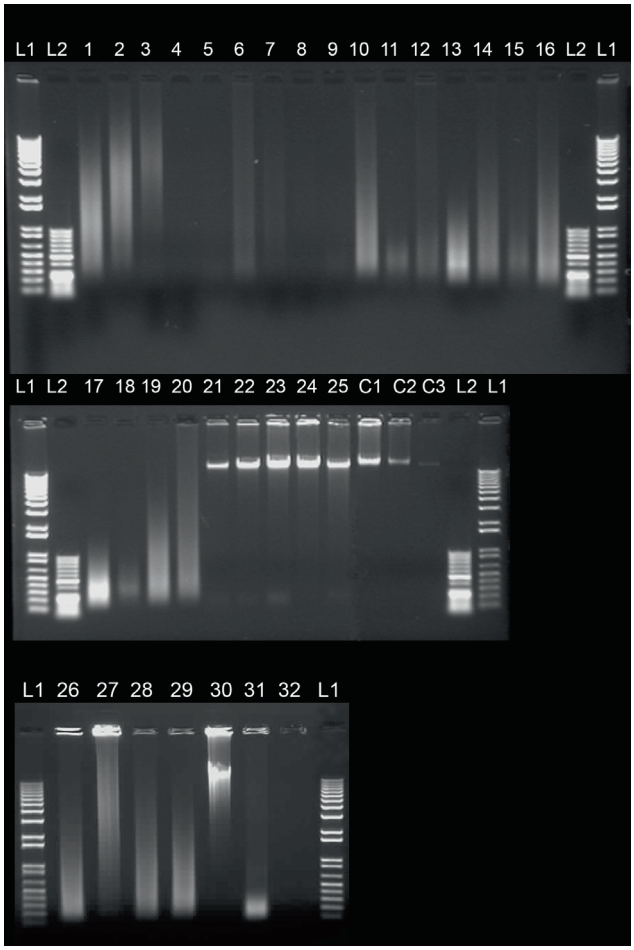


Figure 1. Quality and quantity assessment of DNA samples: Image of a 1% agarose gel separation after ethidium bromide staining depicting several representative tumour samples for testing. L1 and L2 represent 1kb+ and 50bp ladders, respectively. C1, C2, C3 are high molecular weight genomic DNA samples with known concentrations of 500, 250 and 50ng, respectively. Detailed sample characteristics are provided in Table 2.

Table 2. Overview of DNA concentrations using Nanodrop and Gel based estimation

Sample ID*	Nanodrop conc (ng/µl)	260/280 ratio	260/230 ratio	Gel-based conc (ng/µl)	Correction factor**	Array QC***
1	131.0	1.84	2.47	134.5	0.97	OK
2	153.0	1.84	2.42	130.7	1.17	OK
3	31.9	1.88	2.67	24.9	1.28	OK
4	10.9	1.66	0.41	4.5	2.41	NP
5	13.6	2.08	1.57	3.9	3.51	NP
6	44.7	1.8	1.38	20.5	2.18	NP
7	9.8	2.06	1.92	9.0	1.08	NP
8	44.0	1.1	0.3	3.1	14.36	NP
9	57.5	1.57	0.82	3.8	14.94	Poor
10	540.0	1.78	1.9	346.9	1.56	OK
11	164.0	1.63	1.24	19.5	8.42	Poor
12	102.0	1.76	1.98	19.3	5.27	OK
13	485.0	1.84	2.34	270.5	1.79	OK
14	291.0	1.8	2.3	127.8	2.28	OK
15	430.0	1.69	2.17	76.3	5.64	Poor
16	208.0	1.81	2.21	135.9	1.53	OK
17	269.0	1.84	2.35	192.3	1.40	OK
18	63.0	1.73	2.11	9.7	6.48	Poor
19	215.0	1.75	2.12	146.6	1.47	OK
20	285.0	1.7	2.37	170.4	1.67	OK
21	1376.0	1.78	1.28	565.6	2.43	OK
22	88.6	1.67	0.91	68.1	1.30	OK
23	259.8	1.8	1.75	286.8	0.91	OK
24	496.4	1.7	1.8	523.9	0.95	OK
25	30.6	1.7	1.6	25.2	1.22	NP
26	172.0	1.8	2.11	NP	NP	OK
27	306.0	1.75	1.98	NP	NP	OK
28	203.0	1.89	2.31	NP	NP	OK
29	132.9	1.78	2.05	NP	NP	OK
30	300.0	1.8	1.96	NP	NP	OK
31	71.0	1.67	0.89	NP	NP	OK
32	47.5	1.71	0.68	NP	NP	Poor
C1	500	1.8	1.95	462.9	1.08	NP
C2	250	1.8	1.95	238	1.05	NP
C3	50	1.8	1.95	45	1.11	NP

* Sample ID corresponds to the sample label in Figure 1
** Nanodrop concentration/Gel-based concentration
*** NP: Not performed

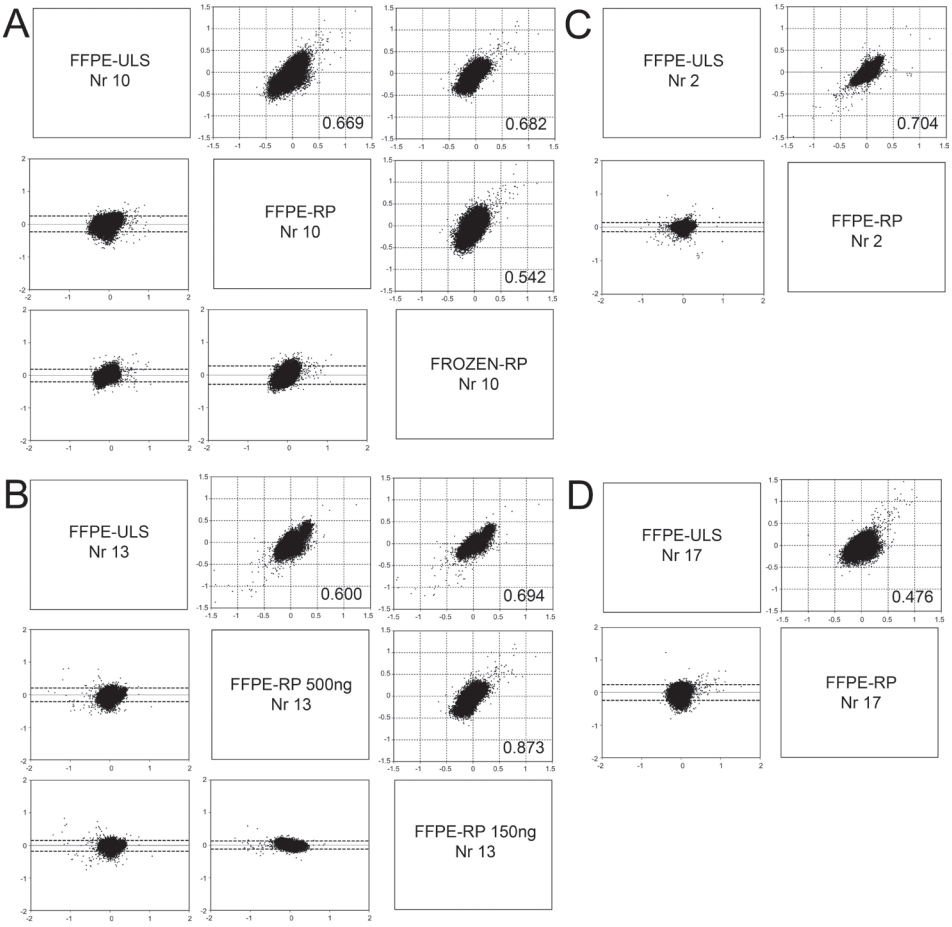
In general, DNA concentration was overestimated particularly for cartilaginous tumour samples with high extracellular matrix composition. In these cases relatively low concentrations were measured (typically in the range of 2-15 ng/µl) (Table 2) but determining the concentration based on the corresponding gel image suggested that these measurements were an over estimate

(Figure 1) (for example: samples nr 9, 11, 15 and 18). As for all labelling reactions, the initial amount of starting material is a crucial factor. We corrected the DNA concentration measured by Nanodrop using the integral of the UV-excited ethidium bromide fluorescence obtained from the agarose gel images. For these measurements, known amounts of reference DNA samples were loaded. The correction factor between the two types of measurements, especially at the lower concentration range, was as high as 10 fold resulting in significant over estimation of sample concentration for labelling and consecutive testing.

Comparison of different labelling approaches

Different comparisons were made based on the type of samples available. A three-way comparison was made for Nr 10 with DNA collected from both frozen and dFFPE material. DNA from frozen tissue was labelled using a random primer labelling kit and DNA from dFFPE tissue was labelled with both the random primer labelling kit designed for FFPE samples and ULS labelling kit for FFPE samples (Figure 2A, Figure 3). The different labelling schemes showed an overall good correlation, the Pearson correlation coefficient varied between 0.542 and 0.682 and showed a better correlation between the ULS-FFPE vs RP frozen (0.682) than the RP frozen vs RP-FFPE reaction (0.542). Very good agreement was observed between the two different labelling reactions using dFFPE samples (0.669). Side-by-side comparative whole genome overview of the array-CGH results showed the variation of the reporter signals was highest (black dots represent individual reporter elements) in the case of FFPE-RP labelling, followed by FFPE-ULS and Fr-RP. In all three profiles almost identical aberrations were present (see Table 3 for an overview of the genome-wide genomic aberrations). Since for routine applications the amount of DNA for testing is often limited, we compared the influence of lower amounts of starting material for labelling using 500 ng and 150 ng dFFPE-isolated DNA for the FFPERP kit (Nr 13). These results were compared to ULS labelling reaction using 500 ng of DNA (Figure 2B). Based on the comparison of the overall profiles, the best correlation was observed between the two FFPE-RP reactions (0.873) using different input for labelling (150 vs 500 ng dFFPE DNA for FFPE-RP kit) followed by a 0.694 between the 500 ng FFPE-ULS and 150 ng FFPERP. This correlation shows that the type of labelling bias, introduced by the labelling kit of choice, makes the overall profile more alike suggesting that ULS labelling of samples will result in a comparable profile of other ULS samples while the FFPE-RP kit will have its own bias and similar profiles for comparison between different samples. In contrast to this, the influence of sample storage (FPPE vs frozen) was stronger than the influence of labelling kit used (FFPE-RP or frozen-RP vs ULS) as we observed better correlation between the independent labelling reaction (ULS, FFPE-RP) than between the frozen RP and FFPE-RP labelling reaction (Figure 2A). Poor correlations and corresponding array profiles were seen for samples with very low amounts of DNA irrespective of labelling reactions (Figure 2E and samples 9, 11, 15 and 18 in Table 2). For these reactions a minimum of 50 to 100 ng DNA was used. These results indicate the possible presence of substances influencing the efficiency of both the chemical and enzymatic labelling reactions. Because of the poor array performance using very low DNA concentrations (samples 9, 11, 15 and 18), five other samples with similarly

low DNA concentrations (samples 4, 5, 6, 7, and 8) were not tested as indicated in Table 2. For sample nr 2 and nr 17 the Pearson correlation coefficient varied between 0.704 and 0.476 (Figure 2C and 2D), respectively. Despite the weaker correlation for case 17, both arrays showed similar profiles and similar gains and losses were detected.



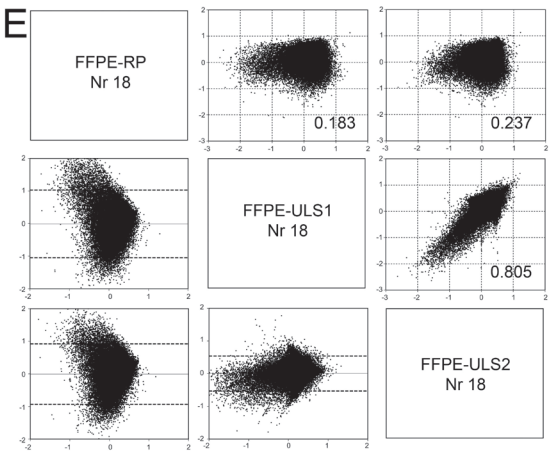


Figure 2. Array-CGH plots of decalcified FFPE samples after ULS or RP labelling. For all plots: Upper right: Correlation plots of log2 ratios for each reporter between experiments, with linear regression and Pearson's correlation coefficients given. Lower left: Bland-Altman plots of the differences between two reporters measured by two experiments on the y axis against the mean log2 ratio of the two on the x axis. Standard deviation of mean differences is plotted in purple, the mean in black.
A: Correlation plots of sample Nr 10 comparing hybridisation of random prime and ULS based labelling of FFPE and RP labelling of frozen tissue derived DNA samples. B: Correlation plots of sample Nr 13 using FFPE isolated DNA samples with ULS, 150ng RP and 500ng RP labelling. C, D Correlation plots of samples Nr 2 and Nr 17 using FFPE isolated DNA samples with ULS or RP labelling reactions. E: Correlation plot of sample Nr 18. This sample showed a great degree of discrepancy for the estimated DNA concentration between the absorption based and the gel based measurements.

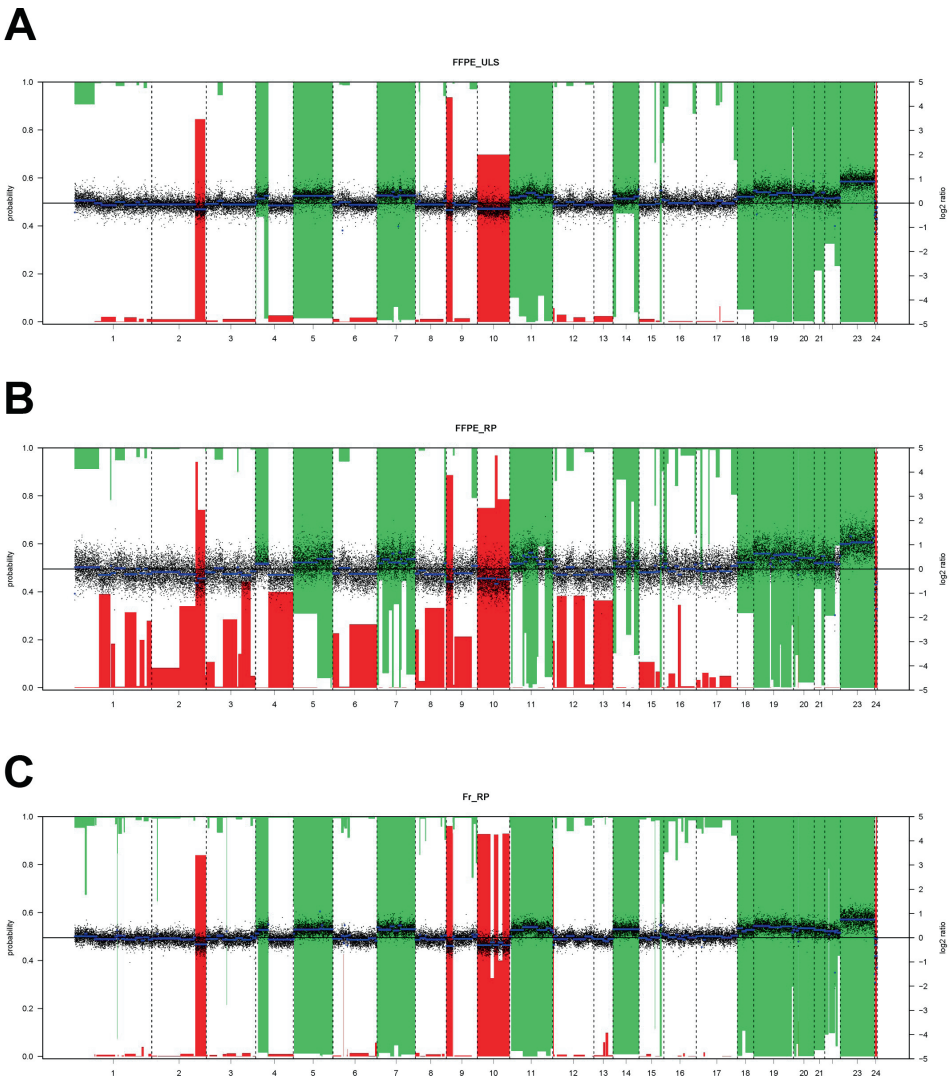


Figure 3. Side-by-side comparative whole genome overview of the array-CGH results from case Nr 10. A: Array CGH profile of FFPE tissue isolated DNA sample after ULS labelling (FFPE-ULS). B: Array CGH profile of FFPE tissue isolated DNA sample after using a Random Prime labelling especially designed to label FFPE samples (FFPE-RP). C: Array-CGH profile of frozen tissue isolated DNA after standard Random Prime Labelling reaction (Fr-RP).
Normalized log2-ratios are plotted with the scale on the right axis. Vertical bars indicate loss and gain probabilities. Probability scale is on the left axis; reversed ('1–') for the gains. Segments are plotted as horizontal blue lines. Segments with a bar extending beyond the middle axis (probability >0.5) are called as gain or losses. All plots were generated using the CGHCall R software package.
The variation of the reporter signals was the highest (black dots represent individual reporter elements) in case of FFPE-RP labelling (see also Figures 2a) followed by FFPE-ULS and Fr-RP. In all three profiles almost identical calls were present (see Table 3 for details on the called regions).

Table 3. Overview of genome-wide genomic aberrations of dFFPE sample Nr 10 and the corresponding frozen sample

Chr	Cyto band	Start (bp)*	Stop (bp)*	Nr of Probes	Gain (+)/ Deletion (-)	FrozenRP	dFFPERP	dFFPEULS
1	p36.33 - p33	749422	46786807	1053	+	+	+	+
1	p12 - q23.3	119416284	160645328	539	+	NC**	+	+
2	q33.2 - q37.3	204593489	242169652	575	-	+	+	NC**
4	p16.3 - q13.3	146653	70631034	683	+	+	+	+
5	p15.33 - q35.3	1163403	180617248	2104	+	+	+	+
6	p22.1 - p21.1	26128906	44328148	558	+	NC**	+	+
7	p22.3 - q36.3	289341	158602640	2056	+	+	+	+
9	p24.3 - p13.3	322256	33155616	370	-	+	+	+
9	q33.3 - q34.3	129159725	140128884	293	+	NC**	+	+
10	p15.3 - q26.3	138006	135222624	1739	-	+	+	+
11	p15.4 - q25	2906039	133951511	2213	+	+	+	+
12	q13.11 - q14.1	47340134	56637091	379	+	+	+	+
14	q11.2 - q32.33	19508645	106330010	1394	+	+	+	+
15	q25.3 - q26.1	86577905	91761128	104	+	+	+	+
16	p13.3 - q24.3	36566	88572953	1741	+	+	+	+
17	p13.3 - q25.3	295150	78154619	2163	+	+	+	+
18	p11.32 - q23	170029	76083258	875	+	+	+	+
19	p13.3 - q13.43	231880	63389940	2096	+	+	+	+
20	p13 - q13.33	73854	62363774	1115	+	+	+	+
21	p11.1 - q22.3	10013063	46646924	549	+	+	+	+
22	q11.1 - q13.33	14433273	49525271	833	+	+	+	+
22	q13.1	37688858	37715585	3	-	+	NC**	+

* according to NCBI hg18
** NC: not called

Interphase FISH

In two different samples, we readily detected a high level of amplification of the MYC locus and a homozygous deletion of the CDKN2A/CDKN2B loci with estimated sizes of 0.7 Mb and 0.6 Mb, respectively (Figure 4A, C). The two-colour Interphase FISH performed on dFFPE tissue sections of case 26 showed a significant increase of signal involving BAC-clone RP1-80K22 on one chromosome arm only (Figure 4B). This pattern is compatible with the amplification of the MYC locus as was detected by the array-CGH test using the corresponding dFFPE tissue isolated DNA.

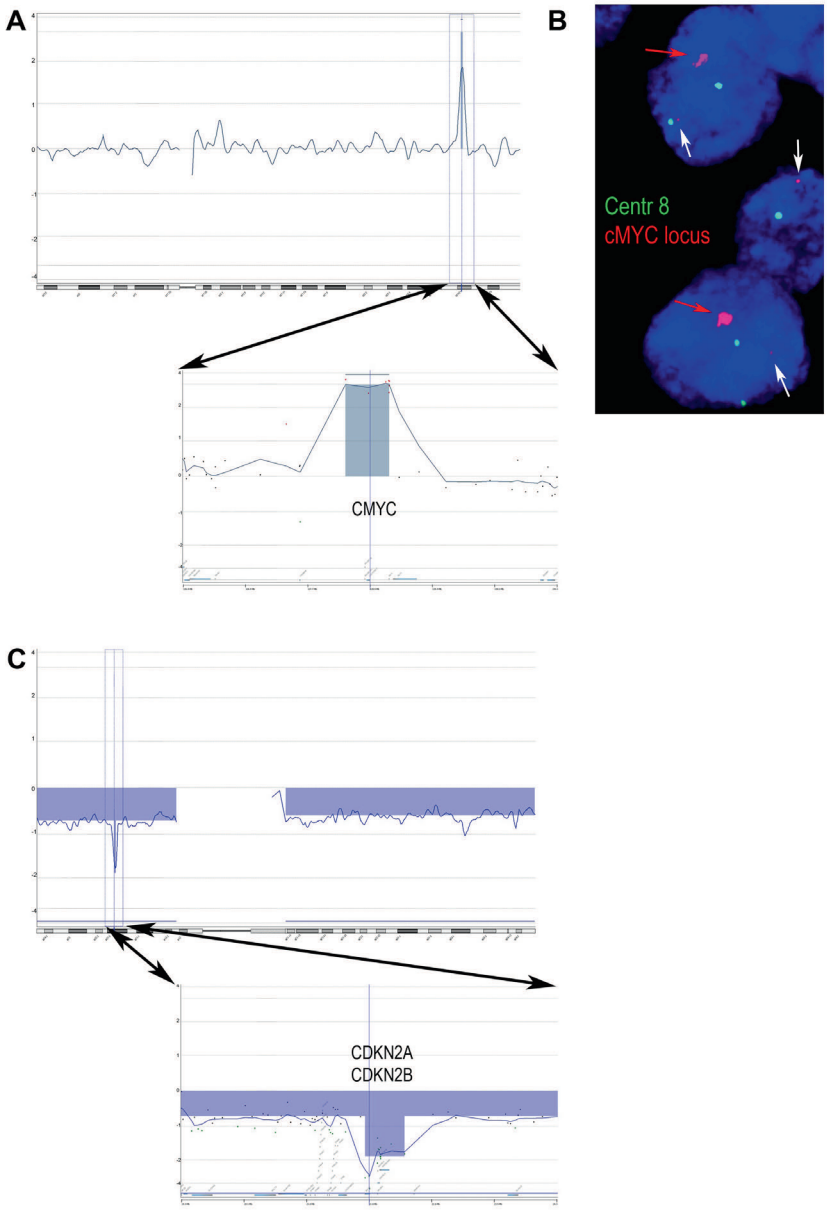


Figure 4. Detection of small aberrations in *MYC* and *CDKN2A*/CDKN2B loci. A: Array-CGH showed a high level of amplification. The amplified region of was about 700kb in size involving the *MYC* locus on the long arm of chromosome 8. Arrows point out an enlargement of the CMYC locus. B: Interphase FISH verification using centromere 8 specific probe (green) and *MYC* locus specific BAC probe (red) on the corresponding dFFPE section. The red arrow indicates signals of the amplified *MYC* locus and white arrow points to the normal locus. C: Array-CGH result of a case with homozygous deletion. The estimated size of the homozygous deleted area was about 600kb involving the *CDKN2A/CDKN2B* loci on the short arm of chromosome 9. Arrows point out the region of the homozygous deletion, containing the *CDKN2A/CDKN2B* loci.

Discussion

We have established and successfully applied a robust protocol to study heavily degraded DNA, obtained from decalcified FFPE samples, collected from various institutions using an oligonucleotide-based chip platform. Both formic acid based decalcification and fixation with non-buffered formalin solution similarly degrade tissue DNA. As the average fragment length of the DNA obtained from these samples is often less than 200 bps, these are regarded as unsuited for further molecular DNA testing [4,5,9]. In this study we used oligonucleotide based array chips containing reporter elements of ~60 bps. For optimal hybridisation the fragment length of the labeled DNA sample should be similar in size as the reporter elements (60-150 bps) [3]. Because enzymatic labelling is introducing further fragmentation during labelling, we applied the Universal Linkage System (ULS) labelling technology, which is a direct chemical labelling, without introducing further fragmentation [9,10]. In addition, we compared the ULS labelling system to a commercially available random primer (RP) labelling kit especially developed for FFPE tissue derived DNA. The overall reproducibility of the two FFPE labelling systems tested was excellent (Figure 2). With both kits we were able to obtain good results using 500 ng of starting material in contrast to the 1 μg DNA recommended by the vendors. The RP labelling has the benefit of amplifying the samples during the labelling reaction. By using as little as 150 ng degraded dFFPE DNA template for the reaction, we obtained similar results to using 500 ng (Figure 2B). However, further reduction of the starting material, especially in cases with discrepancies between estimated DNA concentrations in different measuring methods, resulted in poor results. The use of less than 500 ng DNA for ULS labelling resulted in too weak signals and is therefore not recommended. Samples labelled with the RP kit showed higher fluorescence intensities after scanning as compared to the ULS labelled samples. However, the overall variance of the log2 ratio distribution of the signal was higher as compared to the ULS system (Figure 3A, B). For one case (Nr 10), we had access to both frozen and dFFPE samples. By comparing three kinds of labelling systems a good correlation was observed between all labelling systems and samples (Figure 3, Table 3). We showed that, irrespective of the fragment size of the DNA, all samples with sufficient quantity were eligible for testing. Since correctly estimated DNA oncentration is more critical for successful testing than the quality of the DNA (i.e. fragment size), DNA concentrations were established by using two independent approaches. For some samples we observed discrepancies between the absorption-based DNA concentration measurement and the estimation based on ethidium bromide stained gel imaging. In general, the absorption based system tends to overestimate the final DNA yield resulting in a suboptimal amount for testing (Figure 1). This observed difference might, in part, be explained by the presence of negatively charged matrix glycoproteins such as chondroitin 4-sulphate, chondroitin 6-sulphate and keratan sulphate in some of the tumour samples. Some of these matrix glycoproteins may have similar charges as DNA and consequently could bind to the purification columns when the total DNA content of the sample was low. None of the used labelling systems gave reliable array profiles in cases with high over estimates of concentration. In these cases, in addition to the low DNA concentration, other factors might interfere with

the labelling reaction and could be responsible for the failure. The low amount of DNA might be compensated for by a whole genome amplification step using DOP-PCR, GenomePlex or Phi29 polymerase based reactions. However, it has been shown by others that when using good quality FFPE samples, DOP-PCR results in amplification biases and GenomePlex was suitable in only 58% of the analysed cases [9,11]. The use of multiplex PCR based pre-screening of FFPE samples may be used to select samples, however, it is noteworthy that most of our samples were degraded beyond the exclusion limits of those QC reactions and would not provide a good prediction [4,5,9]. There are several reports using FFPE samples for genomic profiling either on BAC array [4], oligonucleotide based array or the Illumina Golden Gate SNP array systems [6,12,13]. The Golden Gate system has a relatively low resolution consisting of approximately 6000 SNP reporter elements with an average physical distance of about 500 kb. Due to the increased variation of signal ratio values, extensive smoothing steps (i.e. averaging of multiple probes for a given segment) are routinely applied to even out these variations. In turn, the overall resolution of these platforms decreases and most of the changes reported will concern whole chromosome arms or chromosome regions over at least 15-20 Mb in size. In contrast to these limitations, the procedure we established readily detected both homozygous deletions and high level of amplifications of 0.6 and 0.7 Mb in size, respectively (Figure 4).

Conclusions

We developed a reliable DNA isolation and labelling procedure using decalcified, formalin-fixed, paraffinembedded tissue from various clinical specimens. Using two independent techniques (gel-based and absorptionbased), we showed that the estimation of DNA concentration is a more critical step in sample quality assessment than DNA quality (assessed by the degree of fragmentation). In our assessment, both the directchemical-labelling-based ULS kit and the modified random-prime labelling kit worked equally well.

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6

Array-CGH analysis identifies two distinct subgroups of primary angiosarcoma of bone

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Abstract

Molecular genetic studies on vascular tumors are rare. Recently, possible involvement of MYC and KDR has been documented in a subset of angiosarcomas of soft tissue. We performed a cytogenetic analysis of primary angiosarcomas of bone (n=13) and soft tissue (n=5) using high density array-Comparative Genomic Hybridization (array-CGH). Regions of interest were validated by Fluorescence *In Situ* Hybridization (FISH). Antibodies for candidate genes (SKI, MYC, KDR, and MAPK9) were selected and immunohistochemistry was performed. Six angiosarcomas of bone and four angiosarcomas of soft tissue showed chromosomal losses, gains and high level of amplifications. Cluster analysis identified two groups: a group with a complex genetic profile and a group with only few genetic aberrations. Five regions of interest were selected, which were located at chromosomes 1p36.23, 2q32-34, 5q35, 8q24 and 17q21.32-24.2. Interphase FISH confirmed the high-level of amplifications. Immunohistochemical analysis showed high expression of *MYC*, MAPK9 and SKI in 26%, 95% and 83%, of the tumors, respectively. There were no differences between the two groups with regards to location, immunohistochemical expression nor survival. In summary, we identified two subgroups of angiosarcomas: those with few or no gross aberrations and those which show numerous genetic aberrations consisting of chromosomal losses, gains and high level of amplifications or complex aberrations. The most common finding was amplification of 2q and 17q in both angiosarcoma of bone and soft tissue, suggesting overlap in tumorigenesis irrespective of their location. We show MYC amplification in primary angiosarcoma indicating this is not entirely specific for radiation induced angiosarcoma.

Introduction

Angiosarcoma is a rare malignant neoplasm composed of cells that demonstrate endothelial differentiation, accounting for less than 1% of all sarcomas (Fletcher et al., 2013; Weiss and Goldblum, 2008). The exact mechanism of tumorigenesis still remains unclear. However, one presumes that these tumors may arise from normal endothelium or at least cells with features of normal endothelium (Fletcher et al., 2013; Manner et al., 2010). Angiosarcoma occurs most frequently at the skin in the head and neck region, however it can arise at any anatomical site including the viscera and deep soft tissue (Fletcher et al., 2013; Weiss and Goldblum, 2008). Angiosarcoma primary of bone is extremely rare (Dorfman et al., 1971; Huvos, 1991; Mulder et al., 1993), and has a tendency to occur multifocal and has a more aggressive course(Verbeke et al., 2011; Vermaat et al., 2011). Thus, angiosarcomas are heterogeneous at the clinical and morphological level. Recent studies have suggested that this heterogeneity might be based on a different underlying molecular mechanism associated with the anatomical site of the tumor. *KDR* mutations are seen in angiosarcoma of the breast whereas high-levels of *MYC* amplification, with or without co-amplification of FLT4, are seen in secondary angiosarcoma after irradiation or chronic lymphedema (Antonescu et al., 2009; Guo et al., 2011; Manner et al., 2010).

Sarcomas are divided in two main categories based on their genetic abnormalities: i) tumors with a near-diploid karyotype and simple genetic alterations, such as specific translocations (e.g. synovial sarcoma) and mutations (e.g. gastro-intestinal stromal tumor sarcoma), which cause transcriptional deregulation or altered signaling and ii) tumors with complex and unbalanced karyotypes (e.g. osteosarcoma) (Borden et al., 2003; Helman and Meltzer, 2003; Taylor et al., 2011). To date, only a few number of angiosarcomas, mostly of soft tissue, have been genetically studied. To this point, only complex karyotypes and no recurrent chromosomal alterations have been described in angiosarcoma of soft tissue (Baumhoer et al., 2005; Cerilli et al., 1998; Fletcher et al., 1991; Gil-Benso et al., 1994; Kindblom et al., 1991; Schuborg et al., 1998;Van den Berg et al., 1994;Wong et al., 2001; Zu et al., 2001). It is therefore suggested that angiosarcomas belong to the group of sarcomas with a complex genetic profile (Helman and Meltzer, 2003). However, it may be that tumors lacking chromosomal genetic alterations were not reported, resulting in a biased representation of the literature. Dunlap and colleagues reported the first cytogenetic aberration in angiosarcoma of bone. They identified a unique clonal chromosomal rearrangement t(1;14)(p21;q24) in a primary angiosarcoma of the tibia, although a germline translocation was not excluded here (Dunlap et al., 2009). Here we report the first series of primary bone angiosarcomas with full molecular characterization. Our aim was to elucidate whether these tumors harbor recurrent genetic aberrations, or that these are tumors with complex karyotypes.

Material and Methods

Tumor tissue

Twenty five tumor samples (from 22 patients), either frozen or formalin-fixed paraffin-embedded (FFPE) material, with the diagnosis of angiosarcoma of bone were collected from the archives of the departments of Pathology of the Rizzoli Institute, Bologna, Italy (20 tumors, all FFPE material), University Hospitals Leuven, Leuven, Belgium (one tumor, frozen material), and Leiden University Medical Center, Leiden, the Netherlands (four tumor samples from one patient with multifocal disease, frozen material). Six tumor samples of angiosarcoma of soft tissue (from six patients) were collected from the archives of the department of Pathology of University Hospitals Leuven, Leuven, Belgium (three tumors, both frozen and FFPE material) and Leiden University Medical Center (three tumors, frozen or FFPE material) for comparison. The cases were originally diagnosed between 1964 and 2008. All clinical, radiodiagnostical and pathological data were reviewed, as described previously (Verbeke et al., 2011). All tumor samples were originally revised by 3 pathologists (Verbeke et al., 2011) and included in this study when the tumor had a clear-cut histology and tumor cells stained at least for one endothelial marker. Based on the histology (epithelioid hemangioma (n=1), epithelioid hemangioendothelioma (n=1), or other diagnosis (n=2) such as epithelioid sarcoma) and/or the presence of a disease specific genetic aberration (n=1; Ewing-like sarcoma with EWSR1-NFATc2 amplification) five tumor samples (from five patients) were excluded, leaving 20 tumor samples of angiosarcoma of bone (from 17 patients) and six tumor samples from angiosarcoma of soft tissue (from six patients). All specimens were handled according to the ethical guidelines described in "Code for Proper Secondary Use of Human Tissue in the Netherlands" of the Dutch Federation of Medical Scientific Societies.

Tissue microarray (TMA) construction

Four TMAs were assembled from formalin-fixed, paraffin embedded tissue using standard procedures (Verbeke et al., 2011) using a 2 mm-diameter punch (3DHistech Ltd., Budapest, Hungary; 3 TMAs) or a 0.6 mm-diameter punch (Beecher Instruments, Silver Spring, MD, USA; 1TMA) as previously extensively described (Verbeke et al., 2011; Verbeke et al., 2013) containing 42 angiosarcomas of bone and 19 angiosarcomas of soft tissue. Using a tape-transfer system (Instrumedics, Hackensack, NJ, USA), 4-μm sections were transferred to glass slides.

DNA isolation

DNA was isolated from 20 angiosarcomas of bone and six angiosarcomas of soft tissue, as described previously (de Jong D. et al., 2011). Four μm consecutive sections were cut, either from frozen tissue and/ or FFPE, and stained using standard hematoxylin and eosin (HE) to confirm a tumor content of at least 70%. DNA concentrations were measured using a Nanodrop ND-1000 spectrophotometer and quality was checked after electrophoresis separation using a 1% agarose gel stained with ethidium bromide.

Multicolor (COBRA)-FISH karyotyping

From case L2258, a multifocal angiosarcoma, life cells were available after surgical intervention (amputation). Tumor samples were collected from five different sites and samples subjected to short term culture followed by chromosome harvesting according to protocols described (Szuhai et al., 2009). Metaphase cells were obtained from three samples (annotated as L2258 A, D and E) and subjected to multicolor COBRA-FISH karyotyping analysis as described earlier (Szuhai and Tanke, 2006).

Array-CGH analysis

ULS labeling of DNA, derived from decalcified FFPE tissue sections, was performed as previously described (de Jong D. et al., 2011) using the Agilent Oligo array-CGH Labeling Kit for FFPE Samples (Agilent Technologies, Santa Clara, CA). Labeling of DNA, derived from frozen tissue sections, was done using the BioPrime Total Genomic Labeling System (Invitrogen Corporation**,** Carlsbad**,** CA). Labeling efficiency was calculated using a Nanodrop ND-1000 spectrophotometer measuring A_{260} (DNA), A_{550} (Cy3) and A_{649} (Cy5). As a reference, DNA from a commercial source (Promega Corporation, Madison, WI) was used. Labeled test and reference samples were mixed and hybridized as a gender mismatch to show dynamic range of hybridization on the X and Y chromosomes. Hybridization was performed on an Agilent 4x44k oligo array at 65°C for 40 hours. Slides were washed with Oligo array-CGH Wash Buffer 1 at room temperature for 5 min followed by a 1 min wash with Oligo array-CGH Wash Buffer 2 at 37°C. Finally, slides were dried without using the stabilization and drying solution. Slides were scanned using Agilent Scanner with 5μm scan resolution. Scan images were processed with the Feature Extraction Software and analyzed by using Genomic Workbench (Agilent Technologies, Santa Clara, CA) and data was deposited via CanGEM (Scheinin et al. (2008) Nucleic Acids Research 36:D830-D835). Altered regions were calculated using the array-CGH analysis tool in Chipster v1.4.6 (http://chipster.csc.fi/). The minimum number of probes per called segment was 5. To identify common regions the maximum amount of information loss allowed was 0.01, as described by van de Wiel et al (van de Wiel and Wieringen, 2007). Samples were divided into 2 groups, angiosarcoma of bone (black) and angiosarcoma of soft tissue (red) to see if these would cluster together in the wecca (weighted clustering of called array-CGH data) plot (Figure 1A).

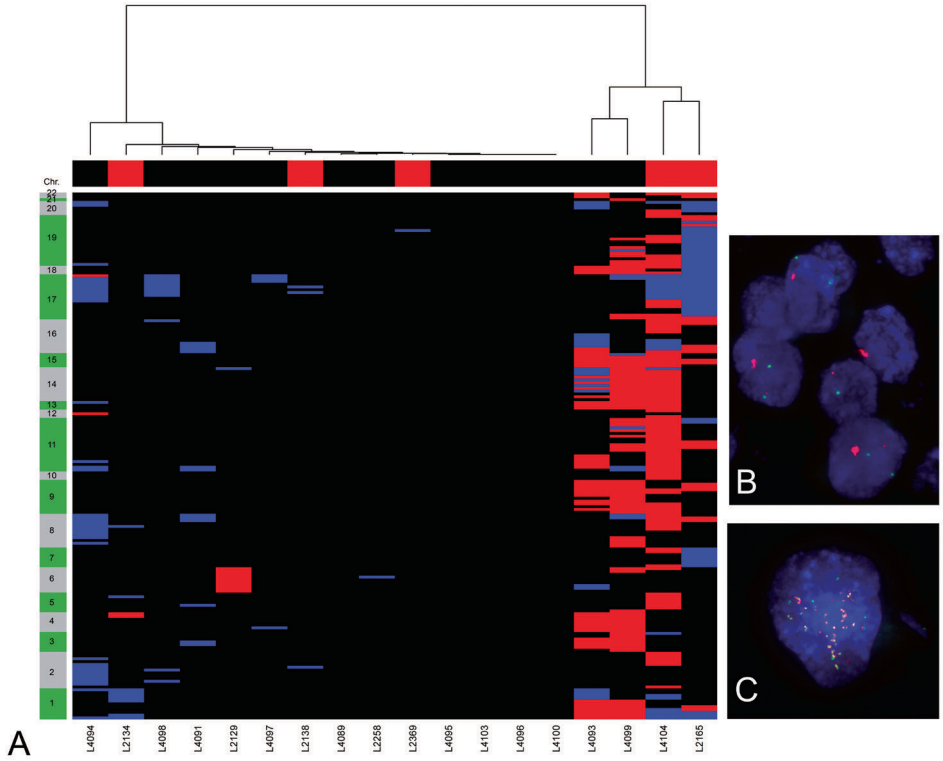

Figure 1A. Weighted clustering of called array-CGH data (WECCA) for the individual samples. The samples are divided into two groups: angiosarcoma of bone in black and angiosarcoma of soft tissue in red. On top the clustering is depicted of these groups. The green and grey bars on the left represent different chromosome segments with their called aberrations. Called losses are shown in red, gains in blue, and unaltered regions in black. Note that the size of a displayed chromosome is proportional to the size of the called region and not to the actual size; **1B.** Amplification of the *MYC* gene locus (detected in red) was confirmed by FISH on L2134. In green the centromeric region of chromosome 8 is detected; **1C.** High level amplification of chromosomes 2q31.1and 17q23.2 and a co-localization of the two probes was observed in L2138.

Confirmatory Fluorescence *In Situ* Hybridization (FISH)

Based on the array-CGH results, interphase FISH using region specific BAC clones on the tissue samples and the TMAs was performed to confirm the results. In short, slides were deparaffinized for 2 x 10 min in xylene, following dehydration and 30 min incubation in NaSCN at 90°C. After washing with PBS slides were treated for 30 min with 0.4% pepsin. Slides were washed with PBS, dehydrated and air-dried. To confirm some of the array-CGH results, two-color interphase FISH experiments were done. A BAC-clone (RP1-80K22) located at 8q24.21 covering the *MYC* gene locus (detected in red) in combination with an alpha satellite probe specific to the centromeric region of chromosome 8 (detected in green) was used as described earlier (Rossi et al., 2007). In order to validate the amplification of 2q31.1 and 17q23.2 in L2138, BAC-clones were used covering these regions. RP11-472A3 (detected in green) was located on 2q31.1 and

RP11-651H20 (detected in red) was located on 17q23.2. After validation of the amplification on whole sections from the two respective cases, these probes were also hybridized on the TMAs. All evaluable nuclei of the tissue cores were examined.

Immunohistochemistry

Based on the array-CGH results, antibodies for some candidate genes (SKI, MYC and MAPK9) were selected and immunohistochemistry was performed on the TMAs. Because *KDR* (*kinase insert domain receptor*, a.k.a. *VEGFR2*) mutations were described in primary angiosarcoma of the breast (Antonescu et al., 2009), the KDR immunoreactivity was also analyzed on the TMAs. Immunohistochemical reactions were performed according to standard laboratory methods (Pansuriya et al., 2011). For each antibody a positive and negative external control was included. The antibodies, their sources, antigen retrieval methods, dilutions, positive and negative external controls used are documented in Table 1. As negative control, sections were stained without adding the primary antibody. The intensity (0 = no staining, 1 = weak, 2 = moderate, 3 = strong) and percentage of positive neoplastic cells (0 = 0%, 1 = 1-24%, 2 = 25-49%, 3 = 50-74%, 4 = 75-100%) were evaluated. Lost tissue cores were excluded from the analysis. As decalcification could compromise the immunohistochemical result, we attempt to counteract this phenomenon by excluding the tissue samples in which an expected positive internal control was negative and tissue samples without a positive internal control that were negative for multiple antibodies. The sum of intensity and percentage was used for analysis.

Table 1. List of antibodies used for immunohistochemical analysis

Antibody	Clone	Dilution	AR	Blocking	Source	Positive control
c-MYC	Y69	1:8000	EDTA	-	Epitomics	Burkitt lymphoma
MAPK9		1:2000	Citrate	-	Abcam	Breast carcinoma
SKI		1:6000	Citraat	NGS 10%	Bioconnect	Kidney
KDR	55B11	1:125	EDTA	-	Cell Signaling	Tonsil

Results

Patient Characteristics

Patient characteristics of both angiosarcoma of bone and soft tissue are summarized in Table 2.

Table 2. Summary of clinicopathological characteristics of all angiosarcoma of bone and soft tissue

Sample ID	F/FFPE*	Age/sex	Diagnosis	Primary localisation
L2129	F	46/F	Multifocal epitheloid angiosarcoma	Bone - foot
L2258	F	39/F	Multifocal angiosarcoma	Bone - foot
L4089	FFPE	41/M	Angiosarcoma	Bone - metacarpal bone
L4091	FFPE	59/M	Angiosarcoma	Bone - femur
L4093	FFPE	72/M	Angiosarcoma	Bone - pubic bone
L4094	FFPE	56/M	Angiosarcoma	Bone - femur
L4095	FFPE	60/M	Angiosarcoma	Bone - femur
L4096	FFPE	35/M	Angiosarcoma	Bone - tibia
L4097	FFPE	74/F	Angiosarcoma	Bone - femur
L4098	FFPE	32/M	Angiosarcoma	Bone - sacrum
L4099	FFPE	54/F	Angiosarcoma	Bone - tibia
L4100	FFPE	54/M	Angiosarcoma	Bone - femur
L4103	FFPE	52/M	Angiosarcoma	Bone - femur
L2134	F/FFPE	79/F	Angiosarcoma	Soft tissue - leg
L2138	F/FFPE	67/M	Angiosarcoma	Soft tissue - retroperitoneum
L2165	F	37/M	Angiosarcoma	Soft tissue - heart
L2369	F	31/M	Angiosarcoma	Soft tissue - muscle arm
L4104	FFPE	42/F	Angiosarcoma	Soft tissue - spleen

F: frozen; FFPE: formalin-fixed paraffin-embedded

Array-CGH analysis

Twenty tumor samples of angiosarcoma of bone and six tumor samples of angiosarcoma of soft tissue were analyzed on a 44K oligonucleotide array chip (Agilent). However due to bad DNA quality the array-CGH failed in seven tumor samples of angiosarcoma of bone (from 5 patients) and in one tumor sample of angiosarcoma of soft tissue (from 1 patient). Alterations were observed in 6 out of 13 angiosarcomas of bone and 4 out of 5 angiosarcomas of soft tissue, varying from full complex genetic profiles with random gains and losses of whole chromosomes (2 angiosarcomas of bone: L4093 and L4099; and 2 angiosarcomas of soft tissue: L2165 and L4104) to tumors showing only smaller altered regions (2 angiosarcomas of bone: L4094 and L4098; and 2 angiosarcomas of soft tissue: L2134 and L2138). Two tumors (L2129 and L4097, both angiosarcoma of bone) only showed a single chromosome gain as aberration. Seven angiosarcomas of bone (L2258, L4089, L4091, L4095, L4096, L4100 and L4103) and one angiosarcoma of soft tissue (L2369) did not show any alterations, despite a percentage of tumor cells >70%. Cluster analysis demonstrated two distinct subgroups of angiosarcoma: one group of tumors with a complex genetic profile and a second group with only few genetic aberrations or a normal genetic profile (Figure 1A).

Alterations found in more than 25% of the cases were considered as recurrent genomic changes. However, none of the genomic alterations reached the 25% cut off. An overview of all genomic changes and wecca plots of all analyzed samples are shown in Figure 1A. Cluster analysis did not show a genetic difference between angiosarcoma of bone and soft tissue, indicating the absence of a genomic pattern specific for either bone or soft tissue angiosarcoma.

High level of amplifications/ homozygous deletions

High level of amplification was observed in two angiosarcomas of bone (L4094 and L4098) at 2q31.1 and at 17q23.2 (Table 3). Also in one angiosarcoma of soft tissue (L2138), amplicons located in these regions were observed (Table 3). The minimal common region on chromosome 2 includes several genes (listed in Table 3). The amplicon on chromosome 17 harbors even more genes (amongst others *USP32*, listed in Table 3). One angiosarcoma of soft tissue (L2134) showed high level amplification at region 5q35 (Table 3), which includes the *MAPK9* gene, and an amplicon at region 8q24.2, containing the *MYC* gene (amongst others). One angiosarcoma (L2165) of soft tissue showed an amplicon at region 1p36, containing the *SKI* gene (Figure 1A and Table 3). In two cases a homozygous deletion of the *CDKN2A/CDKN2B* locus was found (L4093 and L4094). Start and end position of the critical minimal region of amplification and homozygous deletion are provided in Table 3.

Multicolor (COBRA)-FISH karyotyping

In total 22 metaphase cells were analyzed, from L2258 A (12 metaphase cells), D (5 metaphase cells) and E (5 metaphase cells), respectively. All analyzed cells showed a seemingly normal karyotype, no balanced rearrangements were detected by this technique (data not shown). These results were in line with the array-CGH findings.

Confirmatory FISH

To confirm some of the array-CGH results, two-color interphase FISH experiments were done. The amplification of the *MYC* gene locus could be verified in case L2134 (Figure 1B), however, no other cases on the TMAs showed a *MYC* amplification. The amplification of 2q31.1 and 17q23.2 was verified in L2138. Also a clear co-localization was observed between the two probes used, suggesting a more complex rearrangement in this tumor (Figure 1C) that leads to co-amplification of the joined genomic regions. Interphase FISH with these probes on the TMAs did not reveal a second case with similar amplification and co-localization pattern of the probes. Amplification of both probes was confirmed in two tumor samples that were also analyzed with array-CGH (L4094, L4098) (Data not shown).

Immunohistochemistry

Irrespective of the interphase FISH results, 18.5% (5/27) of the angiosarcomas of bone showed expression of c-MYC (sumscore ≥ 3) as compared to one third (11/33) of the angiosarcomas of soft tissue. An overview of the number of positive tumors and percentages for the different antibodies used on the TMAs is given in in Table 4. The majority of angiosarcomas of bone showed expression for MAPK9 (sumscore ≥ 3; 87.5%; 28/32) and SKI (sumscore ≥ 4; 85.1%; 23/27). Also angiosarcoma of soft tissue showed a similar expression percentage for MAPK9 (94.6%; 35/37) and SKI (82.9%, 29/35).

Table 3. Overview of the tumor samples with an aberrant array-CGH result, either exhibiting homozygous deletions and/ or high level of amplifications, and the related genes of interest in angiosarcoma of bone and soft tissue

Sample ID	Overall aCGH result	Homozygous deletion	High level of amplification*	Genes of interest
L4093	Complex genomic pattern, Homozygous deletion	chr9		***CDKN2A/2B***
L4094	Homozygous deletion,	chr9		***CDKN2A/2B***
	High level of amplification on chr2q + chr17q		chr2:171,979,233-173,263,789	HAT1, MAP1D, DLX1, DLX2, U61089,
				ITGA6, PDK1, RAPGEF4, ZAK
			chr17:60,222,436-63,379,832	USP32, C17orf64, APPBP2, PPM1D,
				BCAS3, TBX2, TBX4, BRIP1, INTS2,
				THRAP1, EFCAB3, METTL2A, TLK2,
				MRC2, RNF190, BC040294, AB046856
L4098	High level of amplification on chr2q + chr17q		chr2:171,979,233-173,263,789	HAT1, MAP1D, DLX1, DLX2, U61089,
				ITGA6, PDK1, RAPGEF4, ZAK
			chr17:60,222,436-63,379,832	USP32, C17orf64, APPBP2, PPM1D,
				BCAS3, TBX2, TBX4, BRIP1, INTS2,
				THRAP1, EFCAB3, METTL2A, TLK2,
				MRC2, RNF190, BC040294, AB046856
L2134	High level of amplification on chr5 + chr8		chr5:178,611,827-180,846,638	CLK4, ZNF354A, BC024222, ZNF354B, ZFP2,
				ZNF454, GRM6, BX648737, ZNF354C,
				ADAMTS2, RUFY1, HNRPH1, CANX,
				MAML1, AB209135, LTC4S, MGAT4B,
				BC001874, SQSTM1, LOC51149, TBC1D9B,
				RNF130, RASGEF1C, **MAPK9**, GFPT2,
				CNOT6, FLT4, MGAT1, AK057194
			chr8:127,161,861-127,741,027	DQ515897, BC042052, M13930, ***MYC***
L2138	High level of amplification on chr2q + chr17q		chr2:171,979,233-173,263,789	HAT1, MAP1D, DLX1, DLX2, U61089,
				ITGA6, PDK1, RAPGEF4, ZAK
			chr17:60,222,436-63,379,832	USP32, C17orf64, APPBP2, PPM1D,
				BCAS3, TBX2, TBX4, BRIP1, INTS2,
				THRAP1, EFCAB3, METTL2A, TLK2,
				MRC2, RNF190, BC040294, AB046856

L2165	Complex genomic pattern, High level of amplification	chr1:1,569,923-3,759,635	SSU72, MIB2, CDC2L1, SLC35E2, NADK, AK096614, GNB1, PRKCZ, AK127994, AK126870, **SKI**, MORN1, RER1, PEX10, PLCH2, PANK4, TNFRSF14, C1orf93, MMEL1, AL713743, PRDM16, ARHGEF16, FAM79A, WDR8, TP73 , BC044603, CCDC27
L4104	Complex genomic pattern		
L4099	Complex genomic pattern		

*UCSC Genome Browser on Human Dec. 2013 (GRCh/hg38) Assembly

Table 4. Immunohistochemistry results of all analyzed angiosarcomas of bone and soft tissue present on the different TMAs.

	Angiosarcoma of bone		Angiosarcoma of soft tissue		*P*-value
	n	%	n	%	
c-MYC	5/27	18,5	11/33	33,3	ns
MAPK9	28/32	87,5	35/37	94,6	ns
SKI	23/27	85,1	29/35	82,9	ns
KDR	8/27	29,6	23/33	69,7	0,002

ns = not significant

As mutations in KDR were previously described in angiosarcomas of soft tissue, especially of the breast (Antonescu et al., 2009), we investigated whether KDR overexpression was also present in angiosarcoma of bone, and especially in the subgroup with relatively few genetic aberrations. The expression of KDR was significantly different ($p = 0.002$) between angiosarcoma of bone and soft tissue: Only 29.6% (8/27) of the angiosarcomas of bone showed overexpression of KDR (sumscore ≥ 6) as compared to more than two third (69.7%; 23/33) of the angiosarcomas of soft tissue. There was however no statistically significant difference in expression of KDR, nor of MYC, MAPK9, SKI, or any of the previously identified markers p53, p16, D2-40 and no difference in survival (performed previously (Verbeke et al., 2011;Verbeke et al., 2013)) between tumors with few genomic aberrations and tumors with complex genetic changes although numbers of cases in the different subgroups are very small (Table 5).

Table 5. Correlation of the cluster analysis, based on the array-CGH results, with present (SKI, KDR, MAPK9 and MYC) and previously (TP53, CDKN2A and D2-40) described immunohistochemical analysis and the relation with the corresponding survival data as published previously (Verbeke et al., 2011; Verbeke et al., 2013)

	Simple karyotype	Complex karyotype	*P*-value
Bone versus Soft Tissue	11 versus 3	2 versus 2	
Range median overall survival (months)	2 - 207	4 - 49	
TP53 overexpression	1/10 (10%)	0/3 (0%)	0,640
Lack of CDKN2A expression	4/10 (40%)	1/3 (33%)	0,793
D2-40 expression	6/8 (75%)	2/2 (100%)	0,429
MYC amplification	3/10 (30%)	1/3 (33%)	0,913
Overexpression of KDR	3/10 (30%)	1/3 (33%)	0,913
Expression of SKI	8/10 (80%)	2/3 (66%)	0,631
Expression of MAPK9	10/10 (100%)	3/3 (100%)	-

Discussion

We report the first large series of primary bone angiosarcomas with full molecular characterization, as cytogenetic studies of these tumors so far are limited to case reports (Dunlap et al., 2009). We previously demonstrated that the TGF-beta pathway is more active in angiosarcoma of bone compared to its soft tissue counterpart and that the PI3K/Akt pathway is involved in both angiosarcoma of bone and soft tissue, but through a different mechanism: decreased expression of PTEN in angiosarcoma of bone and overexpression of KIT in angiosarcoma of soft tissue (Verbeke et al., 2013). In the current study we show that there is no evident molecular genetic difference between angiosarcoma of bone and angiosarcoma occurring primarily in soft tissue (Figure 1A). This suggests that either alterations not detectable by array-CGH, or the microenvironment which differs between bone and soft tissue, may cause the previously detected differences in signaling pathways in angiosarcomas. Instead, we identified two different subgroups of primary angiosarcomas: one group of angiosarcomas with a complex genetic profile and a second group of angiosarcomas with only few genetic aberrations (Figure 1A). Since sarcomas with complex karyotypes usually have a defective Retinoblastoma (Rb) and/or TP53 pathway (Helman and Meltzer, 2003; Perot et al., 2010), we questioned whether the angiosarcomas with complex changes also more often demonstrated alterations in the Rb and/or TP53 pathway, results which were available from our previous immunohistochemical study (Verbeke et al., 2013). We described that the Rb pathway was disrupted in 55% of angiosarcomas of bone which was mainly caused by lack of protein expression of CDKN2A. Interestingly, only two of the 18 cases showed a detectable involvement of the CDKN2A/2B region (homozygous deletion in L4093 and L4094, one with a complex and one with a simple genetic profile, respectively) indicating the involvement of other mechanisms leading to the loss of CDKN2A protein expression. Angiosarcomas of bone showing lack of expression of CDKN2A had also a significantly worse prognosis (Verbeke et al., 2013). In contrast to angiosarcoma of soft tissue, the TP53 pathway seemed not important in angiosarcoma of bone (Verbeke et al., 2013). Here we show that there is no statistically significant difference in lack of CDKN2A protein expression nor TP53 overexpression between angiosarcomas with complex genetic alterations and those that are genetically more simple (Table 5). Interestingly, neither was correlation found with survival, location in bone or soft tissue, nor with expression of D2-40, a marker of lymphangiogenic differentiation which previously showed to be an indicator of aggressive behavior and worse prognosis (Table 5) (Verbeke et al., 2011).

It is interesting that a subset of angiosarcomas lacks genetic aberrations detectable at array-CGH. Pre-analytic inaccuracies might be anticipated as we included tumor samples with a tumor percentage of 70% or more. As all cases were reviewed by three expert pathologists (Verbeke et al., 2011) and since both groups of tumors, with or without a complex genetic profile, showed a similar overall survival, misdiagnosis seems unlikely. Balanced genomic rearrangements, such as balanced translocations and inversions, and point mutations in the DNA are not detected by array-CGH. However, multicolor (COBRA)-FISH karyotyping did not detect any cytogenetically visible balanced rearrangements in case L2258 (A, D and E)

displaying multifocal lesions. Although this supports our array-CGH findings, the presence of cryptic structural rearrangements or point mutations cannot be excluded. We investigated the expression of KDR, which was shown to be mutated in angiosarcoma of soft tissue, especially the breast (Antonescu et al., 2009), and did not detect a higher number of cases with KDR overexpression in the angiosarcomas with simple karyotypes.

Previously, a clear genomic difference between primary angiosarcomas and angiosarcomas secondary to radiation or lymphedema was well documented (Guo et al., 2011). It was initially suggested that *MYC* amplification is a specific feature of secondary angiosarcomas (Guo et al., 2011). *MYC* amplification was shown to upregulate the miR-17–92 cluster, which subsequently downregulates thrombospondin-1 (THBS1), a potent endogenous inhibitor of angiogenesis, and thereby mediating the angiogenic phenotype of angiosarcoma (Italiano et al., 2012).

We here report the presence of *MYC* amplification in one angiosarcoma of soft tissue (L2134) without any history of radiation therapy or chronic lymphedema. This is in line with recent studies also demonstrating the presence of *MYC* amplification in a small subset of non-radiation induced angiosarcomas, primary cutaneous angiosarcomas and a subset of primary hepatic angiosarcomas(Ginter et al., 2014; Italiano et al., 2012; Shon et al., 2014). In addition to MYC, coamplification of *FLT4* (encoding *VEGFR3*) at 5q35 was identified in 25% of secondary angiosarcomas, and not in other types (Guo et al., 2011). Our case (L2134) with *MYC* amplification also revealed a high level of amplification at region 5q, containing the *FLT4* and the *MAPK9* (*JNK2*) gene. The protein JNK2 belongs to the JNK kinase family and in response to different stimuli, such as cytokines and growth factors, these proteins are activated by a series of phosphorylation events and subsequently activate several nuclear and non-nuclear proteins, including MYC, p53, and cell death regulators of the Bcl-2 family in the mitochondria (Bubici and Papa, 2014). Therefore, JNK signaling pathways do not only play a role in normal physiological processes, but are also involved in cancer pathogenesis (Bubici and Papa, 2014). Several studies have shown a potential role for JNK2 in tumor cell survival (Barbarulo et al., 2013; Raciti et al., 2012). We demonstrated high protein expression of MAPK9 (JNK2) in 87-94% of the angiosarcomas, irrespective of genomic amplification. This may be explained by the high expression of TGF-beta in angiosarcoma of bone that we previously identified (Verbeke et al., 2013), as TGF-beta can activate JNK2 (Galliher et al., 2006) thereby contributing to increased tumor cell survival. A potential role of JNK2 in angiogenesis has not been described yet.

Very recently, a next generation sequencing (NGS) approach revealed recurrent mutations in *PTPRB* in 26% of the analyzed cases with co-occurring *PLCG1* mutations in 3 of those cases (Behjati et al., 2014). Interestingly, these mutations only occurred in either known secondary angiosarcomas and/ or angiosarcomas with a *MYC* amplification. These data again confirm that secondary and/or *MYC* amplified angiosarcomas should be regarded as a separate subgroup. Our study included only one such case, as we selected primary angiosarcomas. It would be interesting to subject these cases to NGS, however, the archival formic acid decalcified FFPE material is not suited for NGS yet.

One angiosarcoma of soft tissue (L2165) showed an amplicon at region 1p36. Amongst many other genes, this region contains *SKI* (Sloan Kettering Institute proto-oncoprotein). The Ski proto-oncogene (alternative name Proto-oncogene c-Ski or c-Ski) is involved in many signaling pathways (Bonnon and Atanasoski, 2012) and its most well-known function is to negatively regulate TGF-beta signaling. In this study we demonstrate that both angiosarcoma of bone and soft tissue show a high expression of Ski. This finding is consistent with a previous report of Ski expression in hemangiomas (TM et al., 2009). Interestingly, our previous findings show that the TGF-beta pathway is highly active in angiosarcoma especially in angiosarcoma of bone (Verbeke et al., 2013). Thus, despite high expression of Ski, TGF-beta signaling is not suppressed. Although the precise mechanism is unclear one could speculate that other mechanisms are operable overruling the suppression of TGF-beta signaling by Ski in angiosarcoma of bone, and that Ski may contribute to tumorigenesis through other mechanisms. It has been demonstrated that Ski can promote cancer progression and is highly expressed in different human solid tumors, e.g. leukemia, gastro-intestinal cancers and melanoma (Bonnon and Atanasoski, 2012; Wang et al., 2013). It has been suggested that overexpression of Ski plays a role in tumor growth and angiogenesis in diffuse type gastric cancer (Kiyono et al., 2009), however its exact role in angiogenesis remains unclear.

Although the threshold for recurrent aberrations was not reached, three cases showed a high level of amplification of chromosome 2q and chromosome 17q (Table 3). By gene amplification cancer cells are allowed to promote expression of genes that are involved in tumor development and progression. High level amplification of 17q23 is described in many tumor types (Andersen et al., 2002; Parssinen et al., 2007) and especially in breast carcinomas it is associated with tumor progression and poor prognosis (Andersen et al., 2002; Barlund et al., 1997; Isola et al., 1995; Parssinen et al., 2007). So far, the high level amplification of chromosome 2q32-34 has not been described. Both amplified regions contain a large number of possible candidate genes (Table 3). To date, multiple genes have been proposed that could play a role in tumor development and progression (Parssinen et al., 2007). It has been hypothesized that not a single target gene is responsible for, or contributes to, tumor pathogenesis, but the simultaneous overexpression of multiple genes could lead to growth advantages of cancer cells (Parssinen et al., 2007).

In conclusion, we report the molecular genetic characterization of the first series of primary angiosarcoma of bone, in comparison to soft tissue. We identified two different subgroups: one group of angiosarcomas with a complex genetic profile and a second group of angiosarcomas with only few genetic aberrations: mainly high level amplifications. No evident molecular difference was found between both angiosarcoma of bone and soft tissue. We confirm that although *MYC* amplification first was described in radiation therapy and chronic lymphedema associated angiosarcomas, it can occur in a subset of primary angiosarcomas and is therefore not entirely specific for radiation induced angiosarcoma. Moreover, our data indicate that in addition to secondary/ *MYC* amplified angiosarcomas, also primary angiosarcomas are genetically heterogeneous.

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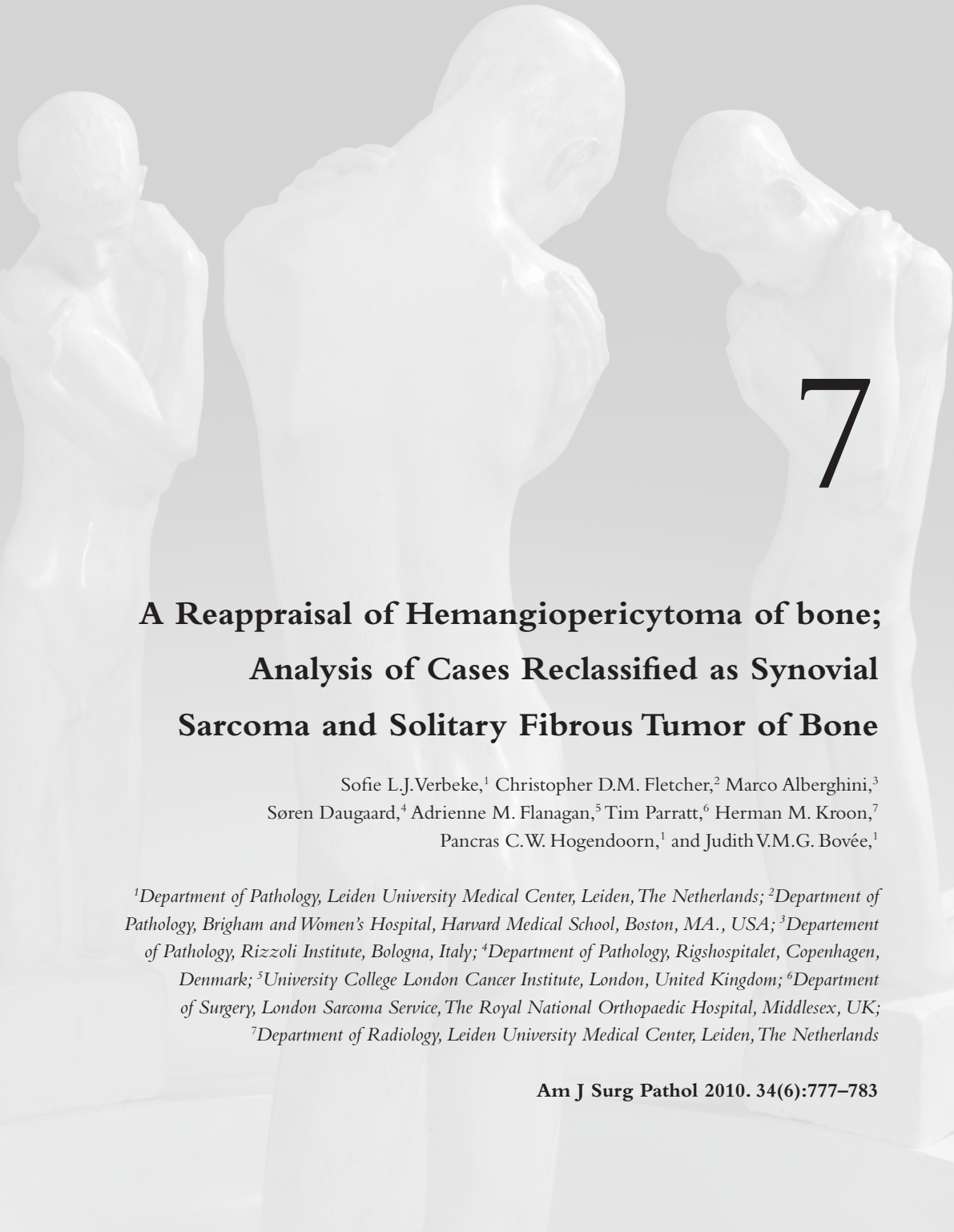

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A Reappraisal of Hemangiopericytoma of bone; Analysis of Cases Reclassified as Synovial Sarcoma and Solitary Fibrous Tumor of Bone

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Abstract

Hemangiopericytoma (HPC) was first described as a neoplasm with distinct morphologic features, presumably composed of pericytes. In soft tissue, it is accepted that most such lesions are solitary fibrous tumors (SFTs), monophasic synovial sarcomas (SSs) or myofibromatoses. It is unclear whether HPC of bone exists. We reviewed nine primary "HPC" of bone from 4 institutions diagnosed between 1952 and 2002. Immunohistochemistry was performed for CD31, CD34, von Willebrand factor, smooth muscle actin, keratin AE1/AE3 and epithelial membrane antigen. There were 4 male and 5 female patients between 21 and 73 years. All tumors were located within bone, either sited within spine or extremities. All tumors showed thin-walled branching vessels surrounded by undifferentiated spindle or round cells. These cells showed variation in their morphologic pattern: 6 tumors showed a pattern-less architecture and varying cellularity, consistent with SFT; 3 of 5 cases examined were CD34-positive. Three tumors showed more densely packed sheets and fascicles of poorly differentiated cells, resembling SS, of which 2 showed focal staining for keratin AE1/AE3 or epithelial membrane antigen. Fluorescent in-situ hybridization confirmed the presence of SS18 rearrangement in 1 of 2 tumors examined. In conclusion, similar to their soft-tissue counterpart, HPC-like features in bone are a non-specific growth pattern rather than a true diagnosis. We confirm the existence of 2 entities: SFT and SS of bone. Both are characterized by distinct morphology and immunohistochemical profile. SFT of bone is located within spine and has a better prognosis, whereas SS of bone is located within long bones having a poor prognosis.

Introduction

Hemangiopericytoma (HPC) was first described as a type of vascular tumor by Stout and Murray in 1942.[29] It was suggested that these lesions were derived from Zimmerman's pericytes, so-called modified smooth muscle cells.[29] Histologically, this tumor was characterized by endothelial-lined tubes or endothelial sprouts surrounded by rounded or spindle-shaped cells typically supported by a meshwork of reticulin fibers.[29] Originally, this tumor was primarily described within soft tissue, but over the years investigators reported occasional cases occurring as solitary lesions within the bone as well.

For many years, HPC was a generally accepted histologic entity, although the diagnosis was merely based on its architectural pattern. However, Fletcher[8] and others investigators[13,23] showed that many soft tissue tumors could mimic a HPC-like growth pattern, including, among others, lesions such as synovial sarcoma (SS) and mesenchymal chondrosarcoma, and therefore, stated that HPC should be regarded as a nonspecific growth pattern instead of a true histologic diagnosis. This is, however, a controversial issue.[7] Electron microscopy showed pericytic differentiation in only a minority of cases diagnosed as HPC.[5] Basal lamina-like materials, cytoplasmic processes, cytoplasmic filaments, discrete basal lamina, and poorly formed intercellular junctions were the most frequently found features in these lesions and some soft tissue pathologists have used the presence of basement membrane at electron microscopy to distinguish HPCs from solitary fibrous tumor (SFT).[5]

Since the era of immunohistochemistry, true pericytic differentiation could not be confirmed in the majority of the cases[4,20,28] and on the basis of the immunohistochemical profile, a subgroup of HPCs could be reclassified as infantile myofibromatosis.[8,19] Furthermore, the majority of so-called HPCs seemed to be indistinguishable from SFTs, a spindle-cell tumor most commonly arising in the pleura although extrapleural locations are now extensively described,[19] leading to a substantial overlap between both entities.

Today, HPC is no longer recognized in the 2002 World Health Organization Classification of Soft Tissue and Bone Tumors, and as stated, so-called HPCs of soft tissue should be better classified as typically rather cellular examples of SFT.[9,10] However, it is not clear whether HPC of bone represents a true, separate entity or is comparable with its soft-tissue counterpart, and should be regarded as a nonspecific growth pattern and therefore reclassified as, for example, SFT of bone. We collected a series of cases diagnosed as HPC of the bone between 1952 and 2005 in 4 different bone tumor referral centers with the goal of reviewing the histology and radiology. Immunohistochemistry and Fluorescent in-situ hybridization (FISH) were performed to investigate whether HPC of the bone is a distinct entity, or, similar to its soft-tissue counterpart, represents a growth pattern that can be observed in SFT, SS or myofibromatosis arising in the bone.

Material and Methods

Clinico-pathologic Data and Histologic Review

Sixteen tumors (from 16 patients) diagnosed as HPC of bone were collected from the archives of the departments of pathology of the Rizzoli Institute, Bologna, Italy (8 tumors), The Netherlands Committee on Bone Tumours (4 tumors), Rigshospitalet, Copenhagen, Denmark (2 tumors) and The Royal National Orthopaedic Hospital, London, UK (2 tumors). The cases were originally diagnosed between 1952 and 2005. All clinical, radiologic and pathologic data were reviewed. All tumor samples were reviewed by three pathologists (J.V.M.G.B., P.C.W.H. and C.D.M.F.) who were blinded towards any clinical data except for age, sex, and affected bone. Only cases with delicate, thin-walled branching blood vessels with a staghorn-like architecture surrounded by non-atypical, round to spindle-shaped cells with uniform, bland nuclei were included in the study. As 2 tumors did not show this true HPC-like growth pattern, they were excluded from this study. Furthermore, tumors were excluded when no tissue blocks were available (2 cases), the histologic appearance was too badly preserved due to heavily decalcification (2 cases) or a primary meningeal HPC had been documented prior to the bone lesion (1 case). Nine remaining cases were included in the study (Table 1). All specimens were handled according to the ethical guidelines described in "Code for Proper Secondary Use of Human Tissue in the Netherlands" of the Dutch federation of Medical Scientific Societies.

Table 1. Clinical Data in 9 Cases of So-called Hemangiopericytoma of Bone.

Case No.	Sex, Age (y)	Location	Therapy	LR	Metastases	LFU (y)	Status
1	F, 21	sacrum	curettage	-	-	5	NED
2	M, 40	sacrum	resection	-	lung, bone, after 3y	5	NED
3	M, 50	vertebra Th12	resection	yes, after 15, 17, 19 and 20y	lung, liver, ST, after 21y	22	DOD
4	M, 44	humerus	resection	yes, after 4, 5 and 20y	lung, after 20y	13	NED
5	F, 73	fibula	amputation	-	lung, after 0.25y	1.5	DOD
6	F, 31	vertebra L4	resection	-	-	4	NED
7	M, 44	sacrum	chemo+radiation	-	-	7	DOD
8	F, 21	humerus	resection	-	lung, after 6y	7	DOD
9	F, 55	sacrum	radiation	-	-	11	NED

Case No.: case number; F: female; M: male; LR: local recurrence; y: years; ST: soft tissue; LFU (y): length follow-up in years; DOD: death of disease; NED: no evidence of disease.

Radiology

All available radiologic data were reviewed. In one case, radiologic data was not accessible any more. In one patient only a magnetic resonance imaging scan was available and therefore the imaging was suboptimal for radiologic evaluation.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue (FFPE) was available for all tumors and immunohistochemistry was performed for CD31, CD34, von Willebrand factor, smooth muscle actin (SMA), keratin AE1/AE3, and epithelial membrane antigen (EMA). For each antibody a positive and negative control was included. Immunohistochemical reactions were performed according to standard laboratory methods.[3] The antibodies, their sources, antigen retrieval methods, and dilutions used are documented in Table 2. Immunoreactivity was evaluated as focal positive, diffuse positive or negative.

Table 2. Antibodies Used for Immunohistochemical Analysis

Antibody	Clone	Dilution	AR	Source	+ & - control
CD31	JC70A	1:10000	Citrate	Dakocytomation	Tonsil
CD34	QBEnd/10	1:30000	-	Neomarkers	Tonsil
Cytokeratin AE1/AE3	-	1:500	Citrate	Neomarkers	Colon
Epithelial membrane antigen	E-29	1:10000	Citrate	Dako	Tonsil
Smooth muscle actin	ASM-1	1:8000	-	Progen	Colon
Von Willebrand factor	-	1:8000	Citrate	Dako	Tonsil

AR: antigen retrieval; + & - control: positive and negative control.

FISH

FISH was performed on paraffin-embedded 4 μm sections of tumor tissue of all patients according to previously described standard laboratory methods.[27] A 10μL Locus Specific Identifier *SS18 (SYT)* probe (Vysis, Inc, Downers Grove, IL) was used. Tissue from 2 tumors was repeatedly lost during this process; therefore, nuclei were isolated from the FFPE material according to standard laboratory methods.[17] Fluorescence signals were analyzed using a Leica DM RXA fluorescence microscope (Leica Mikroskopie & Systeme GmbH, Wetzlar, Germany) equipped with an appropriate filter set. One hundred non-overlapping nuclei were counted.

Results

Patient Data

Histology

Based on histology, 2 principal morphologic patterns were recognized (Fig. 1). Six tumors demonstrated a pattern-less architecture and varying cellularity (hypo- and hypercellular areas) (Fig.1A). These tumors consisted of monomorphic, round to spindle-shaped tumor cells (Fig. 1B). All tumor cells had uniform, bland nuclei and limited amounts of palely eosinophilic cytoplasm with indistinct cell borders. None of the 6 tumors showed nuclear overlapping. Five tumors had less than 4 mitoses per 10 high-power fields (HPF) and only 1 tumor had 5 mitoses/10 HPF. This pattern closely resembled SFT of soft tissue.[10] In contrast, 3 tumors were characterized by a more fascicular arrangement of quite uniform nonpleomorphic spindle-

shaped tumor cells (Figs. 1G, H). The tumor cells had a sparse amount of cytoplasm, indistinct cell borders, and showed sometimes overlapping nuclei. The mitotic activity ranged from 3 to 5 mitoses/10 HPF. Necrosis was not seen. This pattern resembled monophasic SS of soft tissue.[10] The cases and their suggested diagnosis based on histomorphology are listed in Table 3. No distinctive "grungy" calcified matrix, fat, or osteoclasts were seen which would suggest phosphaturic mesenchymal tumor.[11]

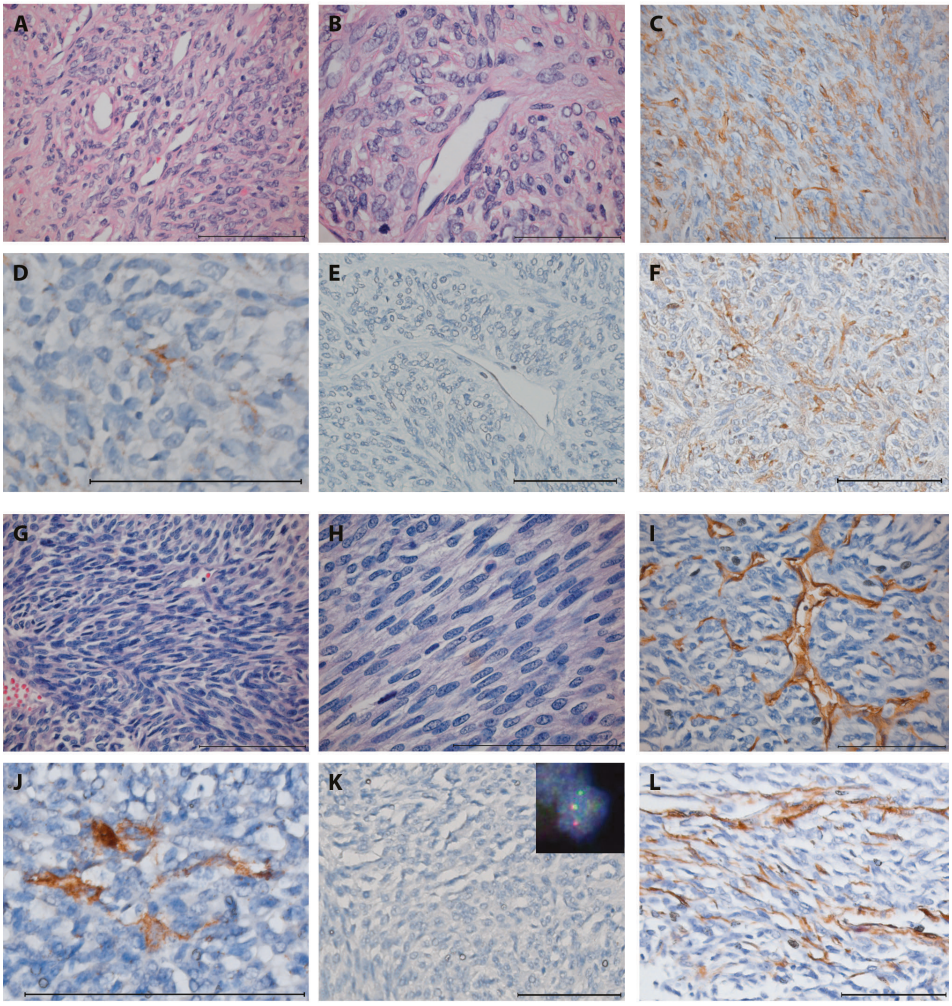

Figure 1. The 2 principal morphologic patterns that are recognized: solitary fibrous tumor-like tumor showing a varying cellularity and a monomorphic tumor cell population [(A-B) haematoxylin and eosin staining; scale bar: 50μm; case number 2] and SS-like tumor showing a fascicular arrangement of non-atypical, spindle-shaped tumor cells [(G-H) haematoxylin and eosin staining; scale bar: 50μm; case number 5], and their corresponding immunohistochemical profile as listed in table 3: (C, I): CD34; (D, J): EMA; (E, K): keratin AE1/AE3; (F, L): SMA (scale bars: 50μm). Inset in (K): SS18-fluorescent in-situ hybridization showing a break-apart of the probes and confirming the diagnosis of SS. SS indicates synovial sarcoma.

Table 3. Histologic and Immunohistochemical Data in 9 Cases of So-called Hemangiopericytoma of Bone.

Case No.	Histology				Immunohistochemistry							
	Cellularity	Growth Pattern	Nuclear Overlap	Mitoses (per 10 HPF)	CD31	CD34	vWF	KerAE1/3	EMA	SMA	SS18-FISH	New Diagnosis
1	variable	patternless	no	3	-*	-*	-	+	-	-	-	SFT
2	variable	patternless	no	5	-	++	-	-	+	+	-	SFT
3	variable	patternless	no	1	-	++	-	+	+	-	-	SFT
4	constant	fascicular	yes	3	-	+	-	+	-	+	-	SS
5	constant	fascicular	yes	5	-	-	-	-	+	+	+	SS
6	variable	patternless	no	2	-	-	-	-	-	-	-	SFT
7	variable	patternless	no	1	-	+	-	-	+	-	-	SFT
8	constant	fascicular	yes	4	-	-	-	-	-	+	failed	SS
9	variable	patternless	no	0^{+}	-	-	-	-	-	-	-	SFT

Cs. No.: case number; $0/10HPF^{+}$: only biopsy available; *: internal control negative; ++: diffuse positive; +: focal positive.

Patient Characteristics

All clinical data are summarized in Table 1. Tumors morphologically resembling SFT of bone had a wide age distribution, ranging from the third till the sixth decade (range: 21 to 55y; median: 42y) and an equal sex distribution (3 males and 3 females). Tumors morphologically resembling SS of bone had an age distribution ranging from the second till the eighth decade (range: 21 to 73y; median: 44y). There were 2 females and 1 male.

Radiologic Appearance and Localization

All patients presented with a solitary, osteolytic bone lesion with a geographic or moth-eaten pattern of bone destruction. Furthermore, in all 7 tumors, for which conventional radiology was available, cortical disruption and extension into the adjacent soft tissue was present. In all tumors, the bulk of the tumor mass was located within the bone and the majority of tumors showed only limited extension into the soft tissue, in keeping with a primary bone tumor with soft tissue extension rather than a soft tissue tumor with bone invasion (Fig. 2). In only 2 of 6 SFT-like tumors, small areas of mineralization were seen, suggestive of residual pre-existent bone fragments rather than mineralization by the tumor itself, whereas 1 of 2 SS-like tumors showed focal mineralization. However, none of these radiologic criteria were characteristic for either SFT or SS-like tumors.

All SFT-like tumors were located within the spine: either the sacrum (4 tumors), the lumbar spine (1 tumor), or the dorsal spine (1 tumor). Both vertebral tumors were located within the corpus of the vertebra and showed expansion into the arch and an adjacent vertebra. All SS-like tumors were located in the extremities, especially the long tubular bones, without any predilection for diaphysis, metaphysis, or epiphysis. Clinically and radiologically, there were no signs of osteomalacia or any other metabolic bone disorder.

Patients Follow-up

In all patients, follow-up data was available, ranging from 1.5 to 22 years (median: 7 y; Table 1). All patients were treated, either with surgery: curettage (case 1) or margin free resection/amputation (cases 2 to 6 and 8), or when inoperable with chemotherapy combined with radiotherapy (case 7) or radiotherapy only (case 9) (Table 1). Only one patient with an SFT-like tumor (case 3) and one with an SS-like tumor (case 4) developed a local recurrence after 15 and 4 years, respectively. Five patients developed metastases ranging from 0.25 to 21 years after the initial diagnosis (median: 6 y). All patients with SS-like tumors developed lung metastases, whereas only 2 patients with a tumor resembling SFT developed metastases. In one of these SFT patients (case 2), the metastases were located within the lungs and bone, whereas the other (case 3) developed metastases within the lungs, liver, and soft tissue. Only the primary tumor in case 3 had more than 4 mitoses/10 HPF.

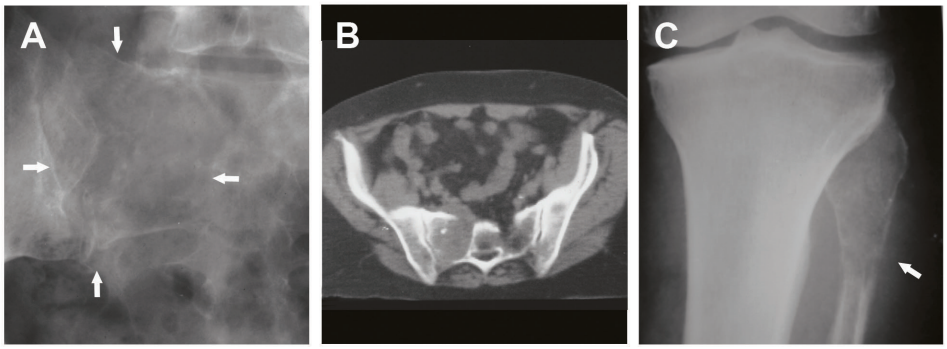

Figure 2. Radiologic appearance of the two different entities: solitary fibrous tumors of bone [(A, B) case number 9] and synovial sarcoma of bone [(C) case number 5] showing that the bulk of the tumor mass is located within the bone, corresponding with a primary bone tumor. A, Anteroposterior conventional radiograph of the sacrum showing an ill-defined osteolytic lesion (arrows) in the area of the first and second sacral vertebra with loss of delineation of the sacral foramina on the right. B, The computed tomography slice demonstrates the intra-osseous origin of the tumor, the anterior cortical interruption and the subsequent soft-tissue extension. C, Anteroposterior conventional radiograph of the knee showing an ill-defined osteolytic lesion arising in the medullar cavity of the proximal fibula. There is a permeative cortical destruction and a periosteal reaction with possible extension into the soft-tissue extension (arrow).

Immunohistochemistry

Three of 5 tumors resembling SFT showed diffuse or more focal staining for CD34 (Fig. 1C). One tumor showed focal staining for SMA (Fig. 1F). Three and 2 tumors showed focal staining for EMA and keratin AE1/AE3 (Figs. 1D, E), respectively. Two tumors resembling SS showed either focal-positive staining for EMA or keratin AE1/AE3 (Figs. 1J, K). The keratin-positive tumor showed some focal staining for CD34. The other 2 tumors were negative for CD34 (Fig. 1I). All tumors showed focal-positive staining for SMA (Fig. 1J). None of the 9 tumors showed expression of CD31 or von Willebrand factor. Results of immunohistochemical analysis for all cases are listed in Table 2.

FISH

Only 1 tumor showed in 82 nuclei a break-apart of probes flanking the *SS18 (SYT)* gene, confirming the diagnosis of SS (Fig. 1K inset). One tumor having morphology consistent with SS failed repeatedly, probably because of decalcification effects. In 6 tumors, 1 resembling SS and 5 resembling SFT (including those showing focal positivity for keratin and EMA), an SS18 rearrangement was not detected. Results of the FISH analysis for all cases are listed in Table 2.

Discussion

Although the existence of HPC of bone has been reported, mainly in single case reports, they are extremely rare.[31,33] The largest series consisted of 11 cases and was described by Wold and colleagues in 1982.[33] However, to date there is no consensus whether this entity truly exists.

Today, it is accepted that the "so-called" HPC of soft tissue merely represents a nonspecific growth pattern, instead of a true, specific entity. Over the years, it has become clear that many tumors exhibit this growth pattern.[8,10,13] As true pericytic differentiation could not be confirmed in the majority of the tumors, it has been stated that most of these lesions should be classified as SFT, monophasic SS, and (infantile) myofibromatosis or myofibroblastic lesions.[8,10,13,18] In addition, a hemangiopericytomatous vascular pattern is seen in phosphaturic mesenchymal tumor.[11] We therefore collected a series of so-called HPC of bone to see whether these were distinctive lesions, or that, similar to its soft-tissue counterpart, also in bone this is a nonspecific growth pattern. In addition, in neuropathology, there is a current shift from meningeal HPC toward meningeal SFT. It is well known that these tumors tend to metastasize to bone.[16] Therefore, we excluded all patients with brain surgery or confirmed meningeal HPC/SFT in their medical history.

The histologic review of the 9 bone tumors with a strict hemangiopericytomatous vascular pattern suggested the existence of 2 categories: 6 tumors consistent with the morphology of SFT and 3 tumors consistent with the morphology of SS. To support or confirm these diagnoses, immunohistochemistry and SS18-FISH were performed.

Within the group of tumors reminiscent of SS, 2 of 3 tumors either showed focal keratin or EMA expression, which is more or less consistent with the literature.[10] One tumor also showed focal staining for CD34. Although most SS are negative for CD34, positivity has been reported in a minority of cases, up to 7.7%, mainly of monophasic or poorly differentiated types. [2,6,22,26] Positivity for SMA has also been described in up to 21% of the SSs.[26] As SMA can be present in either SS or SFT, it is not a useful marker for distinguishing between these diagnoses.

SS is characterized by a tumor-specific translocation t(X;18)(p11.2;q11.2) leading to the *SS18-SSX* fusion gene which is present in 90 to 95% of the SSs. SS18-FISH was performed and the presence of a break in the *SS18* gene confirmed the diagnosis of SS and therewith the existence of primary SS of bone. However, we could demonstrate the tumor-specific translocation in only 1 of 2 cases evaluated. Both FISH and reverse transcription-polymerase chain reaction can be used to detect the SS-specific translocation in FFPE material.[1,30] It has been shown that FISH is slightly less sensitive compared with reverse transcription-polymerase chain reaction, which may explain the negative result in one tumor.[1,30] Alternatively, as 5% to 10% of the SSs lack this tumor-specific translocation, we can not rule out another complex genetic aberration not detectable by the FISH-probe used. Nevertheless, the morphology and the immunohistochemical profile in this SS18-negative tumor (case 4), suggest SS. In the third case the FISH failed repeatedly, most probably due to heavy decalcification methods or poor fixation of the specimen. This tumor (case 8) also did not show any immunoreactivity for keratin AE1/AE3 or EMA. Furthermore, this tumor was negative for CD34, although the numerous blood vessels present within the tumor were positive. Thus, although the morphology, mitotic rate and clinical behavior were consistent with SS, immunohistochemistry and molecular diagnostics could not definitively support reclassification of this tumor.

Immunohistochemistry in the group of tumors reminiscent of SFT revealed 3 tumors with either diffuse (2 tumors) or focal (1 tumor) staining for CD34, whereas 2 tumors were CD34 negative.

One tumor did not show any immunoreactivity for CD34, repeatedly, and also the internal control (blood vessels) was negative, suggesting loss of antigenicity for this marker possibly due to heavy decalcification. Although CD34 is the most useful marker to diagnose SFT,[10,24] it is expressed by many other spindle-cell neoplasms, such as neurofibromas, gastrointestinal stromal tumors, spindle cell lipoma and dermatofibrosarcomas protuberans.[14,25] In addition, expression of CD34 in SFT is rather variable ranging from 80% to 95% of cases in the literature,[10,13] which may explain the 2 CD34-negative tumors. So far, it has not been elucidated why a small percentage of SFT are CD34 negative. Although expression of keratin and SMA is rare, these markers have been reported to be positive in about 2% to 3% of SFT, whereas EMA can be positive in up to 20% to 35% of the tumors.[10] We found EMA positivity in 3 of 5 and keratin positivity in 2 of 5 bone tumors with SFT like morphology. It may therefore be that SFT of bone is more often negative for CD34 and more often positive for EMA as compared to morphologically similar tumors arising in soft tissue. Alternatively, these cases represent other entities. However, none of the EMA-positive cases demonstrated a break in the *SS18* gene and none of the CD34-negative cases had the characteristic features of phosphaturic mesenchymal tumor.

Unfortunately, there is no specific immunohistochemical marker or a specific genetic aberration that can be used to further support the diagnosis of primary SFT of bone. Cytogenetic aberrations are uncommon in small SFTs but are more common (and also heterogeneous) in the larger ones.[21]

Despite the fact that this is a multicenter study, numbers are small and statistical analysis is not meaningful. However, our studies suggest that SS of bone tends to have a predilection for the long tubular bones. In contrast, SFT of bone is located within the spine, in particular the sacrum or lower lumbar region. It has been reported that SS metastasizes in about 40%, most often to lungs and bone.[10] All 3 SSs in our series metastasized to the lungs, whereas only one SFT metastasized to lungs and bone and one SFT metastasized to lungs, liver, and soft tissue. According to the literature SS has a 5-year survival of 36% to 76% and a 10-year survival of 20% to 63%.[10] Although the number of SS cases in this study is very limited, the survival curve of our 3 patients shows a similar pattern. Two of 3 SS died 1.5 and 7 years after diagnosis (cases 5 and 8, respectively). For SFT there is not always a consistent correlation between morphology and biologic behavior. It has been stated that 10% to 15% of the tumors behave aggressively for which increased mitotic rate (4 or more mitoses/10 HPF) was reported to be the most useful predictive factor.[12,15,32]. In our series, 2 patients with SFT died due to progressive disease.

In conclusion, comparable to their presumed soft-tissue counterpart, we believe that HPC of bone should be regarded as a nonspecific growth pattern. Metastatic disease, in particular metastasis of meningeal HPC/SFT, should be excluded. Two entities formerly classified as HPC of bone can be distinguished: primary SFT and primary SS of bone, respectively. SFT of bone is characterized by a pattern-less architecture and variable cellularity, and often shows diffuse CD34 positivity. These tumors, mostly located within the spine, tend to have a better prognosis. SS of bone is characterized by a more spindled and fascicular morphology, often with focal keratin or EMA positivity. These tumors are located within the long bones and have a poor

prognosis. The latter can be confirmed by SS18-FISH, if nonaggressive decalcification methods are used.

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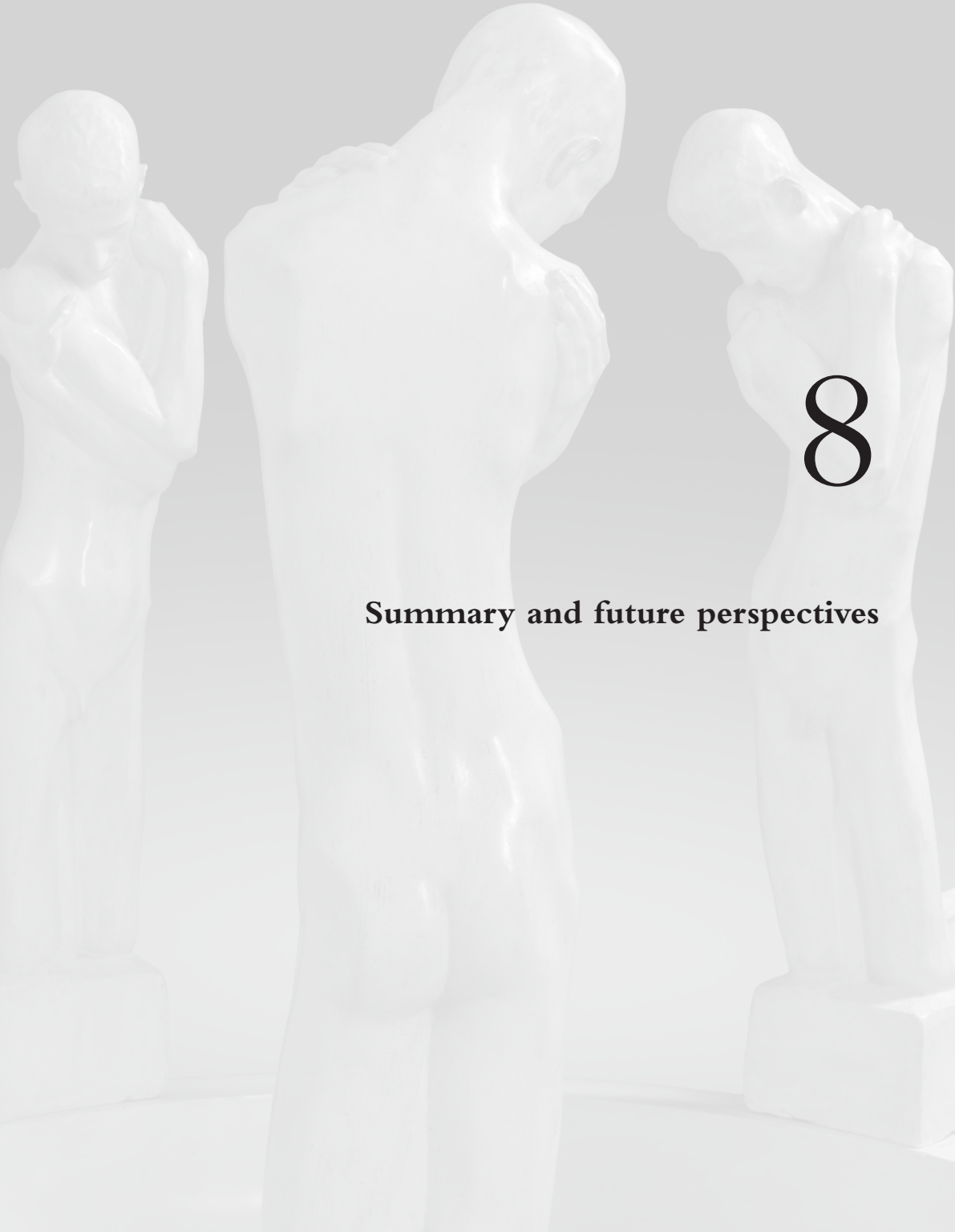

8

Summary and future perspectives

8

Introduction

Chapter 1 emphasizes the need for more specific morphological, immunohistochemical and/or molecular tools to support the classification of the different vascular tumours of bone. Because vascular tumours of soft tissue are more common and more easily accessible for research, these have been better delineated at the morphological as well as molecular level over the past years. Vascular tumours of bone need decalcification for adequate routine histological analysis, which has a severe negative impact on DNA quality. It is still unclear whether vascular tumours of bone are comparable to their soft tissue counterparts simply differing in location, or whether they should be regarded as different entities with their own molecular profile and clinical behaviour. **Chapter 2** summarizes all different histological types of vascular tumours of bone based on radiological imaging, histology and their genetic profile, as far as available, before the WHO classification 2013. Based on the available literature a classification scheme was proposed, which was later on adopted in the 2013 WHO classification[1].

Characterization of angiosarcoma of bone based on morphology

In **Chapter 3** we attempt to delineate the high-grade malignant vascular tumours of bone by systematic histological analysis of a large multi-institute series of angiosarcomas of bone. Previously, high-grade cytological or marked nuclear atypia, brisk mitotic activity with or without atypical forms were stated as important histological criteria for malignancy in vascular tumours of bone. However, exact quantifiable histological criteria were still lacking. In our series of angiosarcomas of bone, cytonuclear atypia was found in all tumours, nearly half of the tumours showed nuclear hyperchromasia and the mitotic activity ranged from 0 to 9 mitoses per 10 HPF. Within this group we identified three high-risk histological parameters that correlated with poor prognosis on multivariate analysis: three or more mitoses per 10 HPF, a macronucleolus and the presence of fewer than five eosinophilic granulocytes per 10 HPF. Furthermore, a subset of angiosarcomas of bone containing the combination of these features showed an even more aggressive course. Nearly one third of the patients demonstrated multifocal disease. Angiosarcomas of bone showed a variable expression of the vascular markers CD31, CD34 and FactorVIII and more than two third showed cytokeratin positivity. In addition, the majority of tumours showed epithelioid morphology. The multifocal appearance combined with keratin positivity can easily mislead radiologists and pathologists to misdiagnose these tumours as metastatic carcinoma. Radiologically, well demarcated, multifocal osteolytic lesions with cortical destruction and marked soft tissue changes should trigger the radiologist to add a vascular tumour in the differential diagnosis and thereby also the pathologist to use additional immunohistochemistry to confirm the vascular nature of the tumour[2].

Characterization of angiosarcoma of bone based on protein expression in comparison with angiosarcoma of soft tissue

In **Chapter 4** we characterize angiosarcomas of bone based on protein expression of a large panel of oncogenes, tumour-suppressor genes, and signalling molecules. In addition, hotspot mutation analysis of PIK3CA, KRAS and BRAF was performed. In this thesis we demonstrate

that in more than half of the angiosarcomas of bone the Rb pathway is disrupted, either by loss of expression of CDKN2A or by overexpression of Cyclin D1. Unlike angiosarcoma of soft tissue, the TP53 pathway seems of no importance in angiosarcoma of bone. Disruption of the Rb pathway would suggest that angiosarcomas belong to the category of sarcomas with complex genetic profiles. In addition, we demonstrate that the TGF-beta pathway is highly active in angiosarcoma of bone and may play a role in tumourigenesis. This finding also suggests therapeutic options for angiosarcoma of bone, since inhibitors of the TGF-beta pathway are available. We also demonstrate that the PI3K/Akt pathway is active in angiosarcoma of bone and soft tissue, but differences in protein expression suggest different mechanisms of activation. In angiosarcoma of bone this could be explained by the decreased expression of PTEN in nearly half of the tumour samples, compared to 7% in angiosarcoma of soft tissue. Although a single case concerning an angiosarcoma of the liver has been reported to contain a *PTEN* gene mutation, it is so far unclear whether PTEN mutations are present in angiosarcoma of bone. It is known that genetic aberrations in the PI3K/Akt pathway, such as PIK3CA mutations, have the potential to cause malignancies. The possible role of PI3K/Akt pathway in angiosarcoma of bone provides a rationale for the use of PI3K/Akt pathway inhibitors although future studies are needed to reveal its value. In angiosarcoma of soft tissue the tyrosine kinase receptor KIT is overexpressed in 90%, compared to only 17% in angiosarcoma of bone. Although there is one report of a very good respons to imatinib (Glivec) in angiosarcoma of soft tissue, no KIT or PDGFRA mutations have been detected in angiosarcoma of soft tissue[3].

Characterization of angiosarcoma of bone at the molecular level in comparison with angiosarcoma of soft tissue

Formic acid based decalcification which is routinely used in daily diagnostics in most pathology labs for tissues containing bone decreases the quality of the DNA by degrading it. The length of DNA fragments obtained from these samples are often less than 150-200 base pairs, hampering molecular testing. In order to characterize our retrospective series of angiosarcomas of bone, mostly consisting of decalcified FFPE blocks, we had to circumvent this technical hurdle. We therefore first optimized and validated the array CGH technique on various heavily decalcified formalin-fixed, paraffin embedded bone tumours of multiple institutions in **Chapter 5**. In this thesis we use oligonucleotide based array chips containing reporter elements of ~60 base pairs. Because enzymatic labelling can cause further fragmentation of the DNA, two different labelling techniques are tested. The first is a direct chemical labelling (Universal Linkage System) without further fragmentation of the DNA, while the second method is a commercially available random primer labelling kit especially developed for formalin-fixed, paraffin embedded tissue derived DNA. Both labelling methods show a good reproducibility and show similar results by using 500ng starting material. Furthermore, the DNA concentrations are measured by two independent methods: absorption-based DNA concentration measurements and ethidium bromide stained gel-imaging. We demonstrate that the estimation of the DNA concentration is more important for successful testing than the quality (fragment size) of DNA obtained from formalin-fixed, paraffin embedded tissue.

After optimization, in **Chapter 6** we applied this technique to create genomic profiles of the first large series of angiosarcomas of bone in comparison to a small group of angiosarcomas of soft tissue. In this study we demonstrate no evident molecular genetic difference between angiosarcoma of bone and soft tissue. In contrast, we identify two groups of angiosarcomas: angiosarcomas with a complex genetic profile and a second group of angiosarcomas with only few genetic aberrations. Although previous results in this thesis indicate a possible role of the Retinoblastoma (Rb) pathway and/or TP53 pathway, here we show no correlation between deregulation of the TP53/Rb pathway and a complex karyotype. One angiosarcoma of soft tissue demonstrates the presence of *MYC* amplification, without any history of radiation therapy or chronic lymphedema, and a high level of amplification at region 5q, containing the *FLT4* and *MAPK9* (*JNK2*) gene. We show high protein expression of MAPK9 (JNK2) in the vast majority of angiosarcomas (bone and soft tissue), irrespective of whether genomic amplification of this region was present or not. A possible role of JNK2 has not been described yet. However, it is known that TGF-beta can activate JNK2. Thus we hypothesize that the presence of JNK2 expression can be explained by the high expression of TGF-beta, which we demonstrate previously in **Chapter 4**. Another angiosarcoma of bone shows an amplicon at region 1p36, amongst others containing *SKI* (Sloan Kettering Institute proto-oncoprotein). SKI is not only involved in many signalling pathways (its most well known function is to negatively regulate TGF-beta signalling), but can also promote cancer progression and is highly expressed in different human solid tumours. Here we demonstrate high expression of SKI in both angiosarcoma of bone and soft tissue. Although previous reports suggest that overexpression of SKI plays a role in tumour growth and angiogenesis, its exact role in angiogenesis still remains unclear. Although the threshold for recurrent aberrations (25%) is not reached within our series, three angiosarcomas show a high level of amplification of chromosome 2q and 17q. High level amplification of 17q23 has been described in many tumour types, and especially in breast carcinomas it is suggested to be associated with tumour progression and poor prognosis. To date, high level amplification of 2q32-34 has not been described yet.

Haemangiopericytoma of bone: a true entity?

Since the 2002 World Health Organisation Classification of Tumours of Soft Tissue and Bone, haemangiopericytoma of soft tissue is no longer recognized as a separate entity and it has been accepted that these lesions should be classified as solitary fibrous tumour, monophasic synovial sarcoma, and (infantile) myofibromatosis or myofibroblastic lesions[4]. In **Chapter 7** we again exploit parallels between vascular tumours of bone by comparison with their soft tissue counterpart, by studying a relatively large series of cases previously diagnosed as "haemangiopericytoma of bone". Based on histological review, two principal morphological patterns are recognized: one group of six tumours demonstrate a pattern-less architecture and varying cellularity with monomorphic tumour cells with bland nuclei that do not overlap and have a limited amount of pale eosinophilic cytoplasm with indistinct cell borders, consistent with the morphology of solitary fibrous tumours. A second group of three tumours are characterized by a fascicular arrangement of uniform non-pleomorphic spindle cells with a sparse amount

of cytoplasm, nuclear overlap and indistinct cell borders, consistent with the morphology of synovial sarcoma. These histological findings could in nearly all cases be confirmed by either immunohistochemistry and/ or molecular testing by SS18-FISH. At the time of evaluation of these tumours, no specific genetic aberration and/ or specific immunohistochemical marker was known to support the diagnosis of solitary fibrous tumour. Recently, a specific *NAB2-STAT6* gene fusion product has been described in solitary fibrous tumour of soft tissue and brain[5]. Moreover, an antibody directed against *STAT6* is commercially available and proven useful to support the diagnosis of solitary fibrous tumour, as due to the fusion with NAB2, STAT6 which is normally localized in the cytoplasm, translocates to the nucleus[6]. In this thesis, we confirm that similar to its soft tissue counterpart, haemangiopericytoma in bone also merely represents a growth pattern rather than a true entity. Therefore, STAT6 immunohistochemistry and/or SS18 FISH should be used in the diagnostic work-up of spindle cell tumours of bone displaying a haemangiopericytomatous vascular pattern.

Future perspective

In this thesis we delineated the most malignant part of vascular tumours of bone, more specific angiosarcoma of bone. These are extremely rare tumours for which the collaboration with multiple different institutions is needed. Due to the partnership within the EuroBoNeT consortium, a European Commission granted Netwerk of Excellence for studying pathology and genetics of bone tumours that stimulates and promotes the multicentric collaboration within Europe, this research was possible.

Although we could identify in **Chapter 3** three high-risk histological parameters that correlated with poor prognosis, not all angiosarcomas exhibit all three histological features and a small portion of these tumours have a different, even better, clinical course. To date, however, there is no evidence or supporting data that low-grade angiosarcoma or haemangioendothelioma of bone is a distinct or truly existing entity. Array-CGH analysis, performed in **Chapter 6,** could only identify two subgroups of angiosarcoma (with or without a complex genetic profile) which did not correlate with prognosis. Therefore, the results of the array-CGH do not provide diagnostic or prognostic markers that can assist in the classification of angiosarcoma of bone or that can distinguish angiosarcoma of bone with a poor prognosis (2-years survival 0%) from angiosarcoma of bone with a slightly better prognosis (5-years survival 33%). However, balanced genomic rearrangements, such as balanced translocations and inversions, and point mutations in the DNA are not detected by array-CGH. More recent molecular studies of distinct vascular entities, such as epithelioid haemangioendothelioma, pseudomyogenic haemangioendothelioma and even epithelioid haemangioma, have shown the presence of a specific balanced translocation in a vast majority of these tumours. In this perspective, Next Generation Sequencing (NGS) of angiosarcoma of bone would be very interesting: mainly to investigate whether recurrent genetic alterations are present in angiosarcoma of bone and if so, whether these alterations correlate with morphology and clinical outcome. Moreover, these genetic alterations could

elucidate the process of tumourigenesis and subsequently lead to new and better therapeutic options. However to date, this molecular procedure is not optimized for (decalcified) formalin-fixed, paraffin embedded material.

In this thesis, we could not detect by multicolour (COBRA-)FISH karyotyping any cytogenetically visible balanced rearrangements in one case displaying multifocal lesions of angiosarcoma of bone (**Chapter 5**). However, recent studies have shown identical alterations in multifocal vascular lesions within the same patient: all multifocal lesions of an epithelioid haemangioendothelioma[7] contained a translocation with an identical breakpoint, and multiple enchondromas and spindle cell haemangiomas in patients with Maffucci syndrome[8] all contain the R132C hotspot mutation. These findings support the hypothesis of clonal disease and suggest that the tumour nodules are metastatic implants, rather than synchronous multiple neoplastic clones[7]. Testing of multiple tumour samples within a single patient using NGS could be interesting and may reveal the clonal evolution of these lesions.

Although it is still not fully elucidated whether angiosarcoma of bone is truly different from angiosarcoma of soft tissue, or should be regarded as the same entity with a different localization, we have shown in **Chapter 6** that from the perspective of the array-CGH study, we could not demonstrate any evident molecular genetic difference between these two lesions. However, based on the immunohistochemical analysis performed in **Chapter 4** there is a clear difference in protein expression between both tumour entities. Pathway analysis revealed that TGFbeta is more active in angiosarcoma of bone, whereas the PI3K/Akt pathway is active in both angiosarcoma of bone and soft tissue. Since these tumours have a similar morphology and genetic profile the difference in protein expression and pathway activation may be caused by epigenetic changes or the different tumour microenvironment, which may have possible therapeutic implications. In order to further evaluate these therapeutic options, there is an urgent need for in vitro models and it would be desirable to establish an angiosarcoma of bone cell line.

In this perspective we could conclude that the 2013 WHO classification of vascular tumours is merely a good start, but should not be regarded as an endpoint. In contrast to the 2002 WHO classification, epithelioid haemangioma is nowadays a well recognised entity with characteristic histological features and a favourable prognosis and therefore no longer merged into the group of angiosarcoma. However, angiosarcomas are still a heterogeneous group and further research (by next generation sequencing for example) is needed to elucidate underlying mechanisms that could explain these differences.

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9

Nederlandse samenvatting

9

Introductie

Hoofdstuk 1 benadrukt de noodzaak voor meer specifieke morfologische, immuunhistochemische en/of moleculaire testen die de classificatie van de verschillende vaattumoren van het bot kunnen ondersteunen. Aangezien vaattumoren gelokaliseerd in de weke delen frequenter voorkomen en dus ook gemakkelijker beschikbaar zijn voor aanvullend onderzoek, zijn deze tumoren in de loop der jaren zowel morfologisch als op moleculair niveau beter omschreven. Vaattumoren gelokaliseerd in het bot moeten eerst ontkalkt worden voordat deze in de dagelijke praktijk histologisch onderzocht kunnen worden. Deze ontkalkingstechniek heeft echter een ernstige negatieve invloed op de DNA kwaliteit. Tot op heden is het nog steeds niet duidelijk of vaattumoren van het bot vergelijkbaar zijn met hun tegenhanger in de weke delen, en dus alleen verschillen in lokalisatie, of dat deze tumoren toch beschouwd dienen te worden als twee verschillende entiteiten met een afzonderlijk moleculair profiel en een verschillend klinisch gedrag.
Hoofdstuk 2 vat alle verschillende histologische subtypes van vaattumoren van het bot samen, gebaseerd op radiologische beeldvorming, histologie en indien bekend het genetisch profiel, zoals dit voor het verschijnen van de Wereld Gezondheid Organisatie (WGO) classificatie van 2013 gebruikelijk was. Op basis van de destijds beschikbare literatuur is een classificatie schema voorgesteld welke later is overgenomen in de WGO classificatie van 2013[1].

Typering van angiosarcomen van het bot gebaseerd op morfologie

In **Hoofdstuk 3** proberen we de hooggradige kwaadaardige vaattumoren (ook wel angiosarcomen genoemd) van het bot nader te omschrijven door een systematische histologische analyse van een grote groep van angiosarcomen van het bot, afkomstig uit verschillende centra voor bottumoren. Voorheen werden uitgesproken kernatypie en veelvuldig voorkomen van celdelingen, met of zonder atypische vormen, gebruikt als belangrijke histologische criteria om kwaadaardigheid in vaattumoren van het bot vast te stellen. Exact meetbare (kwantificeerbare) histologische parameters ontbreken echter nog steeds. In onze dataset van angiosarcomen van het bot is cytonucleaire atypie aanwezig in alle tumoren. Bijna de helft van de tumoren toont kern-hyperchromasie en de celdelingen varieren van 0 tot 9 celdelingen per 10 hoge vergrotingsvelden. Binnen deze groep stelden we, via multivariaat statistisch onderzoek, drie hooggradige histologische parameters vast die samenhangen met een slechte overleving, namelijk: 3 of meer celdelingen per 10 hoge vergrotingsvelden, een macrocronucleolus en de aanwezigheid van minder dan 5 eosinofiele granulocyten per 10 hoge vergrotingsvelden. Het deel van angiosarcomen van het bot die de combinatie van deze drie eigenschappen bevatten, toonden een ernstiger klinisch beloop. Bij nagenoeg een derde van de patiënten bleken meerdere botten aangedaan door de ziekte. Angiosarcomen van het bot tonen een wisselende expressie voor de vaatmarkers CD31, CD34 en Factor VIII en meer dan twee derde toont ook aankleuring voor cytokeratine. Daarnaast toont een grote meerderheid van de tumoren ook een epitheliaal-achtige, zogenaamd epitheloide, morfologie. Het multifocaal voorkomen (meerdere botten aangedaan), de epitheloide morfologie samen met de aankleuring voor cytokeratine kan

makkelijk tot de verkeerde diagnose van een uitgezaaide epitheliale tumor leiden. Beeldvorming van goed omschreven, osteolytische (waarbij het bot verdwijnt) afwijkingen die in meerdere botten voorkomen met ook onderbreking van de cortex en opvallende reactie van de omgevende weke delen moeten de radioloog ertoe aanzetten om vaattumoren in de differentiaal diagnose op te nemen, en de patholoog ertoe aansporen om bijkomstige immuunhistochemische markers toe te voegen om de vaat-differentiatie van de tumor te bevestigen[2].

Typering van angiosarcomen van het bot gebaseerd op eiwitexpressie in vergelijking met angiosarcomen van de weke delen

In **Hoofdstuk 4** typeren we de angiosarcomen van het bot door middel van eiwitexpressie gebaseerd op een grote groep van oncogenen, tumorsupressor genen en signaalmoleculen. Daarnaast werd een hotspot mutatie analyse verricht voor PIK3CA, KRAS en BRAF. In dit proefschrift tonen we aan dat meer dan de helft van de angiosarcomen van het bot een verstoring van de retinoblastoom (Rb) route laat zien, veroorzaakt door ofwel verlies van CDKN2A expressie ofwel door overexpressie van Cycline D1. In tegenstelling tot angiosarcomen van de weke delen, lijkt de TP53 route niet belangrijk bij angiosarcomen van het bot. De verstoring van de Rb route suggereert dat angiosarcomen behoren tot de groep van sarcomen met een complex genetisch profiel. Daarnaast tonen we ook aan dat de TGF-beta route erg actief is in angiosarcomen van het bot en dus een mogelijke rol speelt in de tumorontwikkeling. Aangezien er reeds producten voorhanden zijn die deze TGF-beta route kunnen blokkeren, impliceren deze bevindingen ook mogelijke therapeutische opties voor angiosarcomen van het bot. We tonen ook aan dat de PI3K/Akt route zowel in angiosarcomen van het bot als in de weke delen actief is, maar gezien het verschil in eiwitexpressie is er vermoedelijk een ander activatiemechanisme. In bijna de helft van de angiosarcomen van het bot kan dit verklaard worden door de afname van expressie van PTEN, terwijl dit slechts in 7% van de angiosarcomen van de weke delen wordt gezien. Hoewel er in de literatuur slechts bij één angiosarcoom van de lever een *PTEN* genmutatie is gerapporteerd, is het tot op heden onduidelijk of PTEN mutaties aanwezig zijn in angiosarcomen van het bot. Het is bekend dat genetische afwijkingen in de PI3K/Akt route, zoals PIK3CA mutaties, aanleiding kunnen geven tot kwaadaardige tumoren. De mogelijke rol van de PI3K/Akt route in angiosarcomen van het bot leidt tot de achterliggende gedachte dat stoffen die de PI3K/Akt-route kunnen blokkeren behulpzaam zouden kunnen zijn in de behandeling van deze tumoren. Nader onderzoek is hiervoor echter noodzakelijk. In 90% van de angiosarcomen van de weke delen wordt de tyrosine kinase receptor KIT tot overexpressie gebracht, dit is slechts in 17% van de angiosarcomen van het bot aanwezig. Er is één publicatie van een goede respons op imatinib (Glivec) in een angiosarcoom van de weke delen, ondanks dat er geen KIT of PDGFRA mutaties in deze tumoren werden aangetoond[3].

Typering van angiosarcomen van het bot op moleculair niveau in vergelijking met angiosarcomen van de weke delen

In de meeste pathologie laboratoria gebeurt de ontkalking van botbevattend materiaal in de dagelijke praktijk op basis van mierenzuur. Deze methode zorgt voor DNA afbraak waardoor

de DNA kwaliteit sterk vermindert. De lengte van de DNA fragmenten die van deze stalen verkregen worden, zijn vaak kleiner dan 150-200 baseparen, waardoor moleculaire testen enigszins bemoeilijkt worden. Ten einde onze groep van angiosarcomen van het bot, een groep die voornamelijk bestaat uit ontkalkt en in formaline gefixeerd en in paraffine ingebedde weefselblokjes, te kunnen typeren middels een moleculaire test, moesten we eerst deze technische hindernis omzeilen. Derhalve hebben we eerst de array-CGH techniek geoptimaliseerd en gevalideerd op verschillende bottumoren die in verschillende instituten uitgebreid ontkalkt zijn en vervolgens in formaline gefixeerd en in parafine ingebed, zoals beschreven in **Hoofdstuk 5**. In dit proefschrift gebruiken we oligonucleotide gebaseerde array chips die reporter elementen van ~60 baseparen bevatten. Omdat enzymatische labeling verdere fragmentatie van het DNA kan veroorzaken, worden twee verschillende labelingstechnieken uitgetest. De eerste methode betreft een directe chemische labeling (Universal Linkage System) zonder verdere fragmentatie van het DNA, terwijl de tweede methode een commercieel verkrijgbare 'random primer labeling' set is speciaal ontworpen voor DNA verkregen uit formaline gefixeerd en in paraffine ingebed weefsel. Beide labelingsmethodes bleken goed reproduceerbaar en tonen gelijkwaardige resultaten bij gebruik van 500 ng DNA als startmateriaal. Daarnaast werden de DNA concentraties op twee verschillende manieren gemeten: een DNA concentratie meting gebaseerd op absorptie, en ethidium-bromide gekleurde gel-electroforese. We tonen aan dat een schatting van de DNA concentratie belangrijker is voor een positief testresultaat dan de DNA kwaliteit (grootte van de fragmenten) van het DNA verkregen uit formaline gefixeerd en in paraffine ingebed weefsel.

Na optimalisatie, hebben we deze techniek toegepast in **Hoofdstuk 6** om op deze manier het genomisch profiel te verkrijgen van een eerste grote groep van angiosarcomen van het bot, in vergelijking met een kleine groep van angiosarcomen van de weke delen. In deze studie tonen we aan dat er geen evident moleculair verschil is tussen beide groepen. Daarentegen stellen we vast dat er twee groepen angiosarcomen zijn, namelijk angiosarcomen met een complex genetisch profiel en angiosarcomen met weinig of geen genetische afwijkingen. Hoewel eerdere resultaten in dit proefschrift een mogelijk rol van de Rb en/of TP53 route suggereren, wordt er in deze studie geen verband aangetroffen tussen de Rb/TP53 route en een complex genetisch profiel. Eén angiosarcoom van de weke delen toont de aanwezigheid van *MYC* amplificatie, dit zonder een voorgeschiedenis van radiotherapie of chronisch lymphoedeem, en 'high-level' amplificatie van regio 5q, de regio die het *FLT4* en *MAPK9* (*JNK2*) gen bevat. In het overgrote deel van angiosarcomen (bot en weke delen) tonen wij hoge eiwit expressie aan van MAPK9 (JNK2), onafhankelijk van de aan- of afwezigheid van genomische amplificatie van deze regio. Een mogelijke rol van JNK2 bij vaattumoren werd nog niet eerder beschreven, hoewel TGF-beta JNK2 kan activeren. Daarom lijkt het aannemelijk dat de aanwezigheid van JNK2 expressie verklaard kan worden door de hoge expressie van TGF-beta, welke we reeds eerder in **Hoofdstuk 4** aangetoond hebben. Eén angiosarcoom van het bot toonde een amplificatie ter hoogte van regio 1p36, welke onder andere het SKI (Sloan Kettering Institute proto-oncoprotein)-gen bevat. SKI is niet alleen betrokken bij talrijke signaalroutes (de meest bekende functie is de negatieve regulatie van het TGF-beta signaal), maar kan ook kanker progressie bevorderen en

wordt in verschillende tumorsoorten hoog tot expressie gebracht. In dit onderzoek tonen we aan dat SKI in zowel angiosarcomen van het bot als de weke delen hoog tot expressie wordt gebracht. Hoewel er al eerdere publicaties zijn die suggereren dat de overexpressie van SKI een rol zou kunnen spelen in tumorgroei en bloedvatvorming, blijft de specifieke rol van SKI in de bloedvatvorming onduidelijk. Hoewel binnen onze groep de drempel (25%) voor terugkerende genetische afwijkingen niet bereikt werd, tonen 3 angiosarcomen een 'high-level' amplificatie van chromosoom 2q en 17q. High-level amplificatie van 17q23 is eerder al beschreven in verschillende tumorsoorten. Met name in borsttumoren wordt er verondersteld dat er een relatie is met tumor progressie en een slechte prognose. Tot op vandaag is high-level amplificatie van 2q32-34 niet eerder beschreven.

Haemangiopericytoom van het bot: een bestaande entiteit?

Sinds de 2002 WGO Classificatie voor Weke Delen en Bottumoren, is het haemangiopericytoom van de weke delen niet langer erkend als een unieke entiteit. Het is algemeen aanvaard dat deze afwijkingen beter ingedeeld worden als solitaire fibreuze tumoren, monofasisch synoviosarcoom en (infantiele) myofibromatosis of myofibroblastaire laesies[4]. In Hoofdstuk 7 vergelijken we de bottumoren met hun tegenhangers in de weke delen door een relatief grote groep van tumoren die eerder als "haemangiopericytoom van het bot" werden gediagnosticeerd te bestuderen. Op basis van een histologische herbeoordeling, worden er 2 histologische hoofdpatronen herkend: een groep van zes tumoren toont een patroonloze architectuur, variabele celrijkdom met monomorfe tumorcellen zonder opvallende kernen die ook geen overlap tonen en omgeven worden door een beperkte hoeveelheid eosinofiel cytoplasma en onscherpe celgrenzen, inpasbaar met de morfologie van een solitaire fibreuze tumor. Een tweede groep van drie tumoren wordt gekenmerkt door een bundelige opbouw van uniforme, niet pleiomorfe spoelvormige cellen met een geringe hoeveelheid cytoplasma, kernoverlap en onscherpe celgrenzen, overeenkomstig met de morfologie van een synoviosarcoom. Deze histologische bevindingen worden in de meerderheid van de gevallen ondersteund door immuunhistochemie en/of een moleculaire test, namelijk SS18-FISH. Op het moment van de evaluatie van deze tumoren was er nog geen specifieke translocatie en/of specifieke immuunhistochemische marker bekend die de diagnose van solitaire fibreuze tumor kon ondersteunen. Recent is er in solitaire fibreuze tumoren van de weke delen en de hersenen een specifieke *NAB2-STAT6* fusieproduct beschreven[5]. Daarnaast is er ook een commercieel verkrijgbaar antilichaam gericht tegen *STAT6*, waarvan bewezen is dat het behulpzaam is bij het stellen van de diagnose van solitair fibreuze tumoren. Door de fusie met NAB2 transloceert STAT6, wat normaal gesproken in het cytoplasma aanwezig is naar de kern[6]. In dit proefschrift bevestigen we dat, net zoals in de weke delen, haemangiopericytoma van het bot louter staat voor een groeipatroon in plaats van een zuivere aparte entiteit. In dit opzicht is het gebruik van STAT6 immuunhistochemie en/ of SS18-FISH wenselijk in de diagnostische opwerking van spoelcellige tumoren met een hemangiopericytoom-achtig vaatpatroon.

Toekomstmogelijkheden

In dit proefschrift hebben we het meest kwaadaardige deel van de vaattumoren van het bot omschreven. Dit zijn uiterst zeldzame tumoren waardoor samenwerking met meerdere, verschillende instituten noodzakelijk is. Dankzij een samenwerking binnen het EuroBoNeT consortium, een door de Europese Commissie gesubsidieerd Netwerk van Excellentie voor het bestuderen van pathologie en genetica van bottumoren dat de multicentrische samenwerking binnen Europa stimuleert, was dit onderzoek mogelijk.

Alhoewel we in **Hoofdstuk 3** drie hoog-risico histologische parameters konden aantonen die een verband tonen met een slechte prognose, tonen niet alle angiosarcomen deze drie kenmerken en een klein deel van deze tumoren toont een ander, zelfs iets beter, klinisch beloop. Tot op heden is er geen overtuigend bewijs voor het bestaan van laaggradige angiosarcomen van het bot, ook wel haemangioendotheliomen genoemd, als een separate entiteit. Array-CGH onderzoek, verricht in **Hoofdstuk 6**, toont enkel 2 subgroepen van angiosarcomen (met of zonder complex genomisch profiel) aan, waarbij er ook geen verband aantoonbaar was met de prognose. Hieruit leiden we af dat de resultaten van de array-CGH geen diagnostische of prognostische markers leveren die behulpzaam kunnen zijn bij de indeling van angiosarcomen van het bot of een onderscheid kunnen maken tussen angiosarcomen met een slechte prognose (2 jaarsoverleving 0%) en angiosarcomen met een enigzins betere prognose (5 jaarsoverleving 33%). Hierbij dient opgemerkt te worden dat gebalanceerde genomische veranderingen, zoals gebalanceerde translocaties en inversies alsmede puntmutaties in het DNA, niet gedetecteerd worden middels array-CGH. Meerdere recente studies van specifieke entititeiten binnen de vaattumoren, zoals bijvoorbeeld het epitheloid haemangioendothelioom, pseudomyogeen haemangioendothelioom en zelfs het epitheloid haemangioom, hebben specifieke gebalanceerde translocaties in deze tumoren aangetoond. De Nieuwe Generatie Sequencing-technologie (Next Generation Sequencing, NGS) zou daarmee ook zeer interessant zijn voor toepassing op angiosarcomen van het bot: hoofdzakelijk om te bestuderen of specifieke genetische veranderingen aanwezig zijn in angiosarcomen van het bot en indien dit het geval is, of deze genetische veranderingen een verband tonen met de morfologie en het klinisch beloop. Daarnaast zouden specifieke genetische veranderingen opheldering kunnen geven omtrent de tumorgenese en daaruit zouden eventuele nieuwe therapeutische strategiëen kunnen voortvloeien. Tot op heden is deze techniek echter niet geschikt voor (ontkalkt) materiaal verkregen uit formaline gefixeerd en in paraffine ingebed weefsel.

Op basis van genomisch onderzoek van één angiosarcoom dat op meerdere plaatsen in de voet gelokaliseerd is, kunnen we in dit proefschrift geen cytogenetisch zichtbare gebalanceerde herschikking aantonen (Hoofdstuk 5). Recente studies hebben echter identieke veranderingen aangetoond in multifocale vaatlesies binnen één patiënt. Alle onderzochte tumoren van een epitheloid haemangioendothelioom bij een zelfde patiënt bevatten een translocatie met identiek breekpunt[8], en meerdere enchondromen en spoelcellige goedaardige vaatafwijkingen (haemangiomen) in patienten met het Maffucci syndroom bevatten allen de R132C hotspot mutatie[7]. Deze bevindingen ondersteunen de gedachte van een klonale aandoening en

impliceren dat de tumor nodi eerder uitzaaiingen zijn, dan gelijktijdig ontstane tumorklonen[8]. NGS testen van meerdere tumorstalen van een zelfde patient zou in dit opzicht erg interessant en behulpzaam zijn in de ontrafeling van de klonale evolutie van deze lesies.

Hoewel het nog steeds niet duidelijk is of angiosarcomen van het bot nu daadwerkelijk verschillend zijn van angiosarcomen van de weke delen, of dat deze tumoren behoudens het verschil in lokalisatie toch als één groep beschouwd dienen te worden, hebben we in **Hoofdstuk 6** aangetoond dat op basis van de array-CGH resultaten geen evident genetisch verschil aantoonbaar is tussen beide groepen. Op basis van het immuunhistochemisch onderzoek verricht in hoofdstuk 4 is er echter wel een duidelijk verschil in eiwitexpressie tussen beide groepen. We hebben aangetoond dat TGF-beta route actiever is in angiosarcomen van het bot, waarbij de PI3K/Akt route actief is in zowel angiosarcomen van het bot als de weke delen. Aangezien deze tumoren een gelijkwaardige morfologie en genetisch profiel hebben, kan het verschil in eiwitexpressie en activatie van verschillende routes veroorzaakt worden door epigenetische veranderingen of door verschillen in tumor-omgeving. Deze verschillen hebben mogelijk ook therapeutische implicaties. Om dit nader te onderzoeken is er behoefte aan goede *in vitro* modellen en is het wenselijk om een cellijn afkomstig van een angiosarcoom van het bot te ontwikkelen.

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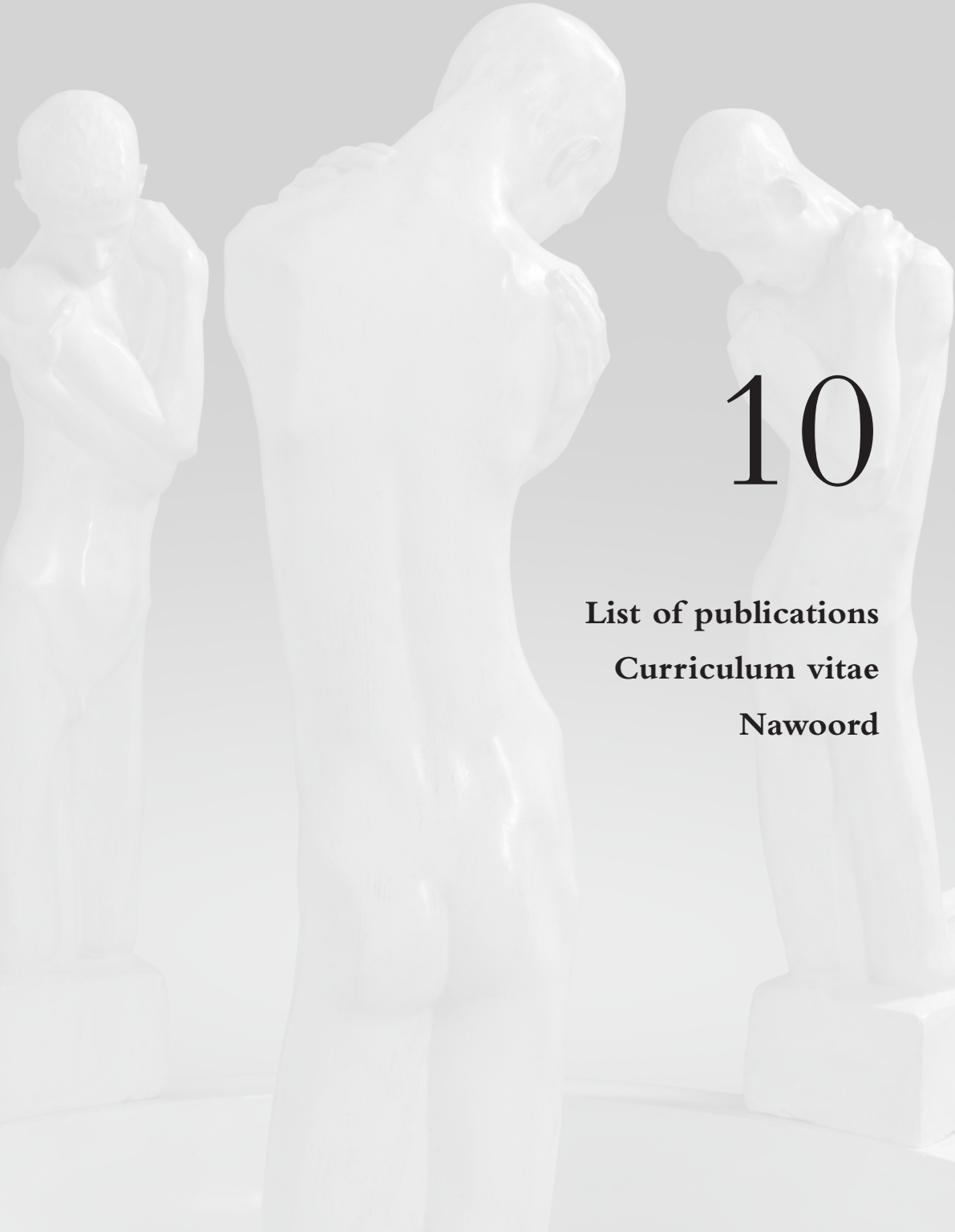

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List of publications

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Curriculum Vitae

Sofie Verbeke werd geboren op 28 september 1977 te Gent (België). Na het behalen van het diploma algemeen secundair onderwijs aan de Visitatie Humaniora te Gent (België), begon zij in 1995 aan de studie Geneeskunde aan de Universiteit Gent (België). Na het behalen van haar artsendiploma in 2003, trad zij op 1 augustus 2003 in dienst als assistent in opleiding (AIO) bij de afdeling Pathologie van het Leids Universitair Medisch Centrum te Leiden (opleider: Prof. Dr. Gert-Jan Fleuren). In 2007 werd een KWF Onderzoeksbeurs voor arts-assistenten behaald waarmee zij vanaf oktober 2007 gedurende een jaar onderzoek verrichtte in de onderzoeksgroep van Prof. Dr. P.C.W. Hogendoorn onder begeleiding van Prof. Dr. J.V.M.G. Bovée. Het onderzoek dat werd verricht, is beschreven in dit proefschrift en betreft de classificatie van de in het bot gelokaliseerde vaattumoren gerelateerd aan de histologisch en genetische karakteristieken. Vanaf september 2010 tot 2012 was zij staflid patholoog op de afdeling Pathologische Anatomie (diensthoofd: Prof. Dr. Patrick Pauwels) in het Universitair Ziekenhuis Antwerpen (UZA) te Edegem (België). Vanaf oktober 2012 tot augustus 2014 was zij als staflid patholoog werkzaam op de afdeling Pathologie van het Universitair Medisch Centrum te Utrecht (afdelingshoofd: Prof. Dr. Paul van Diest). Heden is zij werkzaam als patholoog op de afdeling Anatomo-Pathologie (diensthoofd: Dr. Sabine Declercq) van het ZNA Middelheim te Antwerpen (België).

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Nawoord

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