Molecular pathology in bone and soft tissue tumors: a multifunctional key for diagnosis and prediction

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Chapter 1

General introduction

Molecular pathology in bone and soft tissue tumors

Bone and soft tissue tumors are rare tumors arising within mesenchymal tissues. They comprise a spectrum of different disease entities with broad clinical and biological diversity. For the majority of these tumors, the proposed cell-of-origin is still heavily under debate and it has been hypothesized that either different committed cell types or multipotent cells such as mesenchymal stem/progenitor cells (MSCs) with the capacity to differentiate along the chondroblastic, osteogenic, adipocytic and myogenic lineage are involved. Currently, bone and soft tissue sarcomas are categorized into different disease entities according to their morphological resemblance to normal tissue using the World Health Organization (WHO) classification of 2020, for instance adipocytes, smooth muscle cells, fibroblasts and endothelial cells [1]. While morphological and immunophenotypic findings are commonly combined for diagnostic purposes, genetic assessment has become increasingly important for the diagnostics of bone and soft tissue tumors compared to epithelial tumors. Since a significant number of bone and soft tissue tumors has shown to harbor recurrent genetic alterations [2], molecular genetic characterization could be applied for diagnostic confirmation and can provide new insights to predict treatment response and prognosis.

The molecular background of an increasing number of bone and soft tissue tumors has been elucidated, allowing the rough classification into tumors with complex and simple karyotypes as a conceptual framework. Sarcomas with complex karyotypes lack specific alterations detected so far, and therefore these alterations cannot be employed as a diagnostic aid. This stands in contrast to tumors with a simple karyotype. This group can be subdivided into tumors that carry somatic gene mutations (e.g., *IDH1/IDH2* in enchondroma), tumors with more or less specific amplifications (e.g., *CDK4/MDM2* in low-grade intramedullary osteosarcoma), and tumors with specific translocations. Although some alterations were shown to be disease-defining such as *CIC* in *CIC*-rearranged sarcomas, others are not necessarily confined to one tumor entity, including the *ETV6-NTRK3* fusion. This well-known fusion was first described in infantile fibrosarcoma and subsequently also in a broad spectrum of epithelial and non-epithelial tumors including mesoblastic nephroma, secretory carcinoma of the breast and salivary gland, acute myeloid leukemia, inflammatory myofibroblastic tumor, gastrointestinal stromal tumor and papillary thyroid carcinoma (**Figure 1**) [3, 4]. Therefore, classification of tumors based on their genetic profile solely could result in incorrect diagnosis and genetics should always be integrated with the clinical, morphological, and immunohistochemical findings ideally by an expert pathologist. Previous studies have stressed the importance of expert pathology for soft tissue tumors by demonstrating minor and major diagnostic discrepancy in respectively 15.7-35% and 8-10.9% of the cases reviewed for a second opinion, resulting in different treatment decisions in a part of the cases [5, 6].

Besides the expertise of pathologists, the access to diagnostic tools such as immunohistochemistry and molecular analysis is essential. Italiano and colleagues have shown that the application of molecular diagnostics led to the modification of the final pathological diagnosis in 14% of the cases and the primary management and prognosis in most of these cases [7]. In the Netherlands, the medical treatment of bone sarcomas is centralized in four academic reference centers (Amsterdam UMC, LUMC, Radboud UMC and UMCG) where 87% of the patients underwent surgery between 2017-2018. It has been shown that management of patients with sarcoma and mesenchymal tumors of the intermediate category within a network of reference centers is associated with an improved quality of surgical management and reduced risk of relapse and mortality since the quality of initial surgery is a major prognostic factor for recurrence-free survival and overall survival [8-10]. Therefore, for soft tissue sarcoma, more awareness of the existence of such reference centers should be created among physicians to improve the management of soft tissue sarcoma, as only half of the patients were diagnosed and treated in one of the six reference centers [11].

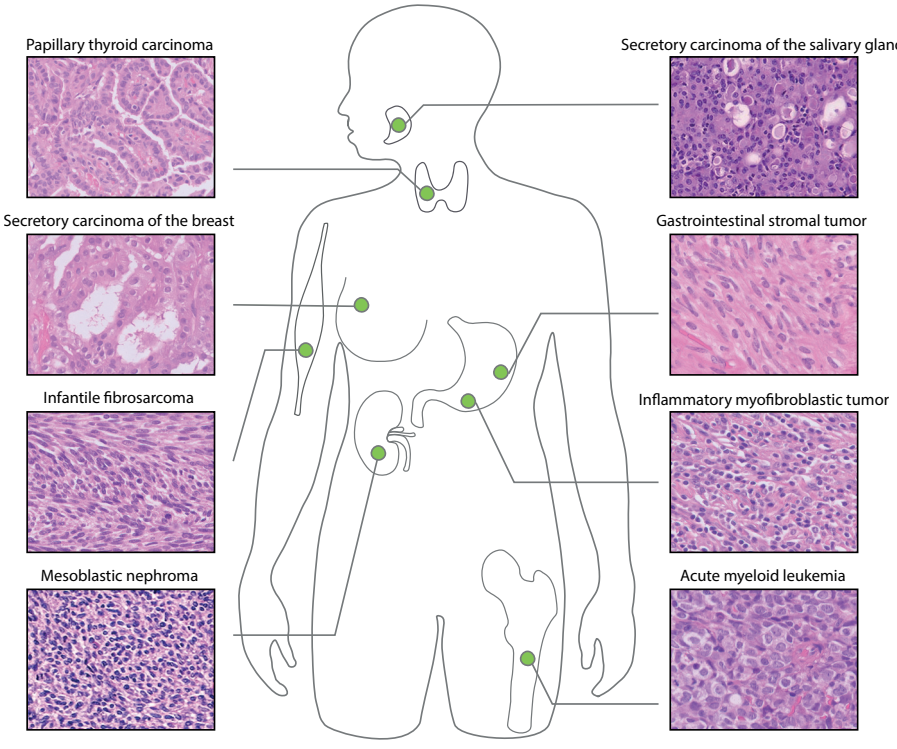

Figure 1. *ETV6-NTRK3* fusions are present in a diverse range of epithelial and non-epithelial tumors with variable morphology.

Techniques to detect genetic alterations

Fluorescence in-situ hybridization (FISH)

FISH can be employed to detect chromosomal abnormalities such as a translocation or an amplification of a certain gene utilizing the presence of DNA within a histological slide as the target for hybridization *in-situ* [12]. Specific probes incorporated with fluorophore-coupled nucleotides anneal to the complementary sequences, resulting in the visualization of the gene of interest. Over the past decades, FISH has been widely applied as a powerful and simple tool to detect chromosomal numerical and structural changes such as monosomies, trisomies, gene amplifications, deletions and translocations.

In case of the detection of translocations, the application of fusion or split-apart probes depends on the gene of interest. The driver event of aneurysmal bone cyst and nodular fasciitis is the presence of a *USP6* translocation [13, 14], but this promiscuous gene is known to have numerous fusion partners for which a split-apart probe set would be the first choice for detection. Since dermatofibrosarcoma protuberans harbors a *COL1A1-PDGFB* fusion in virtually all cases, a fusion probe set would be a good choice [15]. When using the *EWSR1* split-apart probe, one of the most commonly used FISH probes, one should be aware that *EWSR1* fusions can be seen in a large spectrum of benign and malignant tumors, and that identifying the fusion partner in certain cases is essential for the correct diagnosis. In this circumstance, an alternative method for translocation detection that identifies the fusion partner, such as next-generation sequencing, should be considered.

Next-generation sequencing (NGS)

With advances in molecular technologies, NGS allows parallel DNA sequencing on a massive scale replacing conventional methods such as reverse transcription polymerase chain reaction (PCR) and traditional Sanger sequencing. Targeted NGS is already commonly applied for detection of mutations in bone and soft tissue tumors diagnostics. More recently, this has also been implemented as a novel method for translocation detection using an anchored multiplex PCR (AMP) to prepare the library [16], for which both FFPE and fresh frozen tissue can be used. Since this technique utilizes both specific and universal primers, it circumvents the required knowledge of both fusion partners for translocation detection [16].

Immunohistochemistry as a surrogate marker for molecular alterations

In the past few years, great progress has been made in the translation of genetic alterations into antibodies for immunohistochemistry. Traditionally, immunohistochemistry detects the line of differentiation, such as myogenin for skeletal muscle and SATB2 for osteoblastic differentiation. Although the implementation of immu-

nohistochemistry has shown to be beneficial, the limitations should be considered when making a diagnosis. Most traditional markers show limited specificity, as many antigens are expressed by more than one tumor type.

More importantly, both benign and malignant neoplasms may have aberrant antigen expression, and no markers or marker combinations are available to distinguish between benign and malignant lesions [17]. Immunohistochemistry is commonly used for the diagnosis of most soft tissue neoplasms [18, 19], but their application was limited for bone tumors until more recently [20]. The identification of novel specific recurrent alterations has allowed the specific detection of these proteins, using immunohistochemical testing in selected cases (**Table 1**) [17], allowing the incorporation of a fast and cost-effective method for diagnostics. For example, immunohistochemical detection of the mutant protein for H3F3A G34W in giant cell tumor of the bone and H3F3B K36M in chondroblastoma has found its way in routine diagnostics. Also, the *IDH* R132H mutation can be detected using a specific antibody. However, this mutation is, in contrast to glioblastoma, rare in chondrosarcoma where R132C is the most frequent mutation [21], which cannot be detected using immunohistochemistry [22]. Moreover, fusions can lead to overexpression of proteins due to loss of a part of the gene (e.g., FOS) or due to promoter swapping (e.g., FOSB) [23-26], both of which can be detected immunohistochemically. Another possibility is the generation of a chimeric protein, leading to a new or altered function of a protein. In solitary fibrous tumors, the nuclear localization signal of *NAB2* is retained in the *NAB2-STAT6* fusion gene, leading to an aberrant concentration of STAT6 in the nucleus. In these cases, immunohistochemistry can serve as a surrogate for the detection of the fusion gene [27].

Table 1. Immunohistochemistry as a surrogate marker

Disease entity	Immunohistochemistry
Giant cell tumor of bone	H3F3A G34W
Chondroblastoma	H3F3A/B K36M
Osteoid osteoma	FOS
Osteoblastoma	FOS
Cementoblastoma	FOS
Epithelioid hemangioma	FOS and FOSB
Pseudomyogenic hemangioendothelioma	FOSB
Epithelioid hemangioendothelioma	Nuclear CAMTA1, TFE3
Conventional chondrosarcoma	IDH R132H (<30%)
BCOR-CCNB3 sarcoma	CCNB3
Inflammatory myofibroblastic tumor	ALK, ROS1
Alveolar soft part sarcoma	TFE3
Solitary fibrous tumor	Nuclear STAT6

Molecular pathology to increase the accuracy of diagnostics

With the acceleration of molecular techniques, resulting in the discovery of genetic alterations in bone and soft tissue tumors, our understanding of the underlying biology has been enriched. Moreover, molecular alterations were proven to be a powerful diagnostic aid, leading to the incorporation of genetic findings to aid diagnostic decision-making as an integral part of the diagnostic work-up. In 2016, for the first time, molecular parameters were used in the WHO classification of central nervous system tumors in addition to histology to define many tumor entities, thereby breaking with the century-old principle of nomenclature solely based on microscopy. Since this update, a much more precise diagnosis of especially diffuse glioma and embryonal central nerve system tumors has been achieved [28]. In parallel, this shift towards integration of molecular findings in the designation of tumor entities is also reflected in the latest WHO classification of soft tissue and bone tumors, published in 2020. In this edition, advances in molecular characterization have driven further refinements in classification, sometimes so far-reaching that some entities are defined by their genetic alteration. For example, molecular diagnostics have allowed the recognition of unique clinical, pathologic, and genomic characteristics of patients and tumors, illustrated by the entity *CIC*-rearranged sarcoma, which was not long ago referred to as an Ewing-like sarcoma. *CIC*-rearranged sarcomas occur most commonly in soft tissues of young adults and have a wide spectrum of morphology including round, epithelioid and spindle cells. They are associated with an aggressive clinical course, with an inferior overall survival compared to Ewing sarcoma. These emerging molecular and clinical data have supported the classification of *CIC*-rearranged tumors as an independent molecular and clinical subset of small blue round cell tumors distinct from Ewing sarcoma [29].

A well-known diagnostic challenge where molecular diagnostics are indispensable, is when malignant small blue round cells are observed on light microscopic evaluation. The differential diagnosis is broad and includes a tumor of epithelial, mesenchymal or lymphoreticular origin. Although the differential diagnosis can be narrowed by immunohistochemistry, the profile is not always specific for a certain entity, especially in the group of sarcomas. In these cases, identification of a specific molecular alteration is needed to establish the correct diagnosis (**Chapter 2**). Also, identification of *FOS* or *FOSB* rearrangements in osteoid osteoma, osteoblastoma and cementoblastoma, three bone tumors with overlapping morphology, could be helpful in the differential diagnosis which most importantly includes high-grade osteosarcoma [23, 30-32]. Although the detection of genetic alterations is promising for a more accurate classification of older and new disease entities, the application of molecular pathology is complex and comes with several challenges (e.g., the need of capital investment, bioinformatic hardware, experts).

Molecular pathology to predict prognosis

Besides the important role for the assessment of molecular alterations in the diagnosis in bone and soft tissue tumors, molecular markers are also used as a prognostic marker, though less frequently compared to certain epithelial cancers. For instance, in alveolar rhabdomyosarcoma, the specific fusion type is of prognostic value. In these tumors, *FOXO1* is fused with either *PAX3* or *PAX7*, of which the former fusion partner is correlated with a worse prognosis [33]. In addition, secondary genetic alterations such as *TP53* alterations and homozygous deletion of *p16/p14ARF* were shown to be present in a quarter of Ewing sarcoma cases, defining a subset of tumors with highly aggressive behavior and poor chemotherapy response [34].

Molecular pathology to predict response

Most of the driver changes in bone and soft tissue tumors involve transcription factors, which are not directly targetable yet. However, the discovery of receptor or ligands activating alterations has provided new precision medicine-based therapy in a few bone and soft tissue tumors (**Table 2**) [35, 36]. A well-known example is the identification of an activating *KIT* or *PDGFRA* mutation in gastrointestinal stromal tumors (GIST), which could be effectively inhibited by small-molecule kinase inhibitors such as Imatinib. The susceptibility for therapy also tightly corresponds to the specific type of mutation as demonstrated by exon 11 *KIT* mutations which are sensitive to Imatinib, while the most common *PDGFRA* mutation in GIST (D842V in exon 18) is resistant to this drug [37, 38].

Another immediate implication for precision medicine accounts for the above-mentioned targetable gene fusion involving *NTRK* [39]. In general, this fusion is found at a high frequency among specific rare cancer types (e.g., secretory breast carcinoma and infantile fibrosarcoma) and at a low frequency across more common cancers [39], for which a traditional disease-specific study is not feasible owing to insufficient patient enrollment. Particular interest in this gene has been raised since clinical trials have shifted away from site-of-origin and histological subtype-specific designs and more towards tumor agnostic basket trials [40]. These trials are designed to test therapies targeted towards specific molecular mechanisms irrespective of the histotype, allowing novel therapeutic options in rare tumors, especially in sarcoma where therapeutic options are often limited. Response to Trk inhibition was observed in different trials, in a histology-agnostic fashion and regardless of fusion type or upstream partner [39], leading to an increased demand for *NTRK* testing in advanced cancer patients. Since it is not feasible nor cost-effective to test all sarcoma patients, a three-tiered diagnostic algorithm has been proposed for prioritization of *NTRK* fusions testing according to the likelihood of finding a fusion [41]. These fusions can be detected using a variety of methods. Pan-Trk immunohistochemistry has been studied as a surrogate marker for *NTRK* fusions,

with variable sensitivity and specificity among different tumors. For example, specificity of 100% was seen for carcinoma of the colon and lung, though decreased sensitivity in breast carcinoma (82%) and salivary gland carcinoma (52%) and poor sensitivity and specificity in sarcoma were observed [51]. While targeted DNA-based next-generation sequencing is not of the first choice due to large intronic regions in *NTRK2* and *NTRK3* fusions, different commercial assays for targeted RNA-based NGS are currently available allowing the detection of *NTRK* fusions. However, one should be aware that non-oncogenic aberrant *NTRK* rearrangements (passenger alterations) that do not yield constitutively active fusion proteins could be found. RNA expression data or expression of Trk, at the protein level, could be helpful for differentiation between driver and passenger alterations, which will be helpful in the prediction of response to Trk therapy [39, 41].

Likewise, several molecular biomarkers are used in attempts to predict response to immune checkpoint inhibition (ICI). Blocking of the PD-1 pathway, which normally represses Th1 and cytotoxic response in the presence of its ligands, reinvigorates antitumor response, which has resulted in impressive successes in the treatment of different cancers [52, 53]. It has specifically been noticed that immune checkpoint inhibition has remarkable efficacy in tumors with a high tumor mutation burden, due to the production of more neo-antigens that might be recognized by the immune system and thereby eliciting an anti-tumor response. Where the use of immune checkpoint inhibition was previously limited to mismatch repair-deficient colon carcinoma, this finding has accelerated approval of immune checkpoint inhibition therapy as a therapy option for any mismatch repair-deficient tumor [54]. This ultimately illustrates the key aspects of genome-driven oncology, where the oncology landscape is shaped by efforts to understand molecular changes underlying cancers and attempts to target these molecular changes.

Table 2. Examples of molecular targeted therapy in soft tissue tumors

Tumor type	Target	Drug	Reference
Gastrointestinal stromal tumor	KIT, PDGFRA	Imatinib, other TKI's	Corless, *et al.* [42]
Dermatofibrosarcoma protuberans	COL1A1-PDGFB	Imatinib	Rutkowski, *et al.* [43]
Tenosynovial giant cell tumor	COL6A3-CSF1	CSF1R inhibitor	Cassier, *et al.* [44]
	Other CSF1 rearrangement		Gelderblom, *et al.* [45]
Inflammatory myofibroblastic tumor	ALK and ROS1 fusion	Crizotinib	Lovly, *et al.* [46]
	NTRK fusion	Imatinib	Schoffski, *et al.* [47]
			Butrynski, *et al.* [48]
Infantile fibrosarcoma	ETV6-NTRK3	Larotrectinib	Laetsch, *et al.* [49]
Liposarcoma	CDK4	Palbociclib	Dickson *et al.* [50]

Aim and outline of this thesis

The aim of this thesis is to study how molecular alterations can be employed as a tool, from a diagnostic and predictive perspective, in mesenchymal tumors. Since the interest for the molecular background of bone and soft tissue tumors has rapidly evolved, it has brought more insight into tumorigenesis and has provided opportunities to improve diagnostic accuracy and predict response to treatment.
Chapter 2 provides a general overview of the molecular assays used in the diagnosis of bone tumors and defines the classification of these tumors from a genetic point of view. The corresponding altered molecular pathways in several bone tumors are reviewed and translation of specific molecular alterations to clinical practice is discussed. In **chapter 3**, the utility of anchored multiplex PCR for NGS as a novel technique for translocation detection is studied by comparing it to previously used single gene molecular tests. While molecular analyses are increasingly applied in clinical pathology, their utility to improve diagnostic accuracy, understand tumorigenesis and refine the classification of bone tumors has been demonstrated in chapters 4-6. In **chapter 4,** a series of conventional chondrosarcomas with peculiar clear cell change is described. *IDH1* mutation analysis is used to compare both components to investigate whether these lesions should be considered as conventional chondrosarcoma or as clear cell chondrosarcoma. The distinction is of crucial importance, as treatment and prognosis differ significantly. In **chapter 5,** we focus on the *FOS* translocation driven tumors osteoid osteoma and osteoblastoma. In this chapter, the utility of FOS immunohistochemistry is studied as a diagnostic marker in both osteoid osteoma and osteoblastoma and their differential diagnosis. Since cementoblastoma shows striking morphological resemblance to osteoblastoma, in **chapter 6** presence of similar *FOS* rearrangements is studied by FISH and FOS immunohistochemistry. **Chapters 7** and **8** focus on the predictive aspects of molecular pathology. In **chapter 7** the frequency of mismatch repair (MMR) deficiency is evaluated across the different bone and soft tissue tumors in order to elucidate in which specific patient population and in which tumor type MMR deficiency might be encountered, thereby providing pathologists guidance for MMR testing in bone and soft tissue cancers. Since durable disease control in many patients has been described in advanced-stage *NTRK* fusion-positive cancers, in **chapter 8** we explore the frequency of *NTRK* fusions in a large series of bone tumors on tissue microarrays using pan-Trk immunohistochemistry, as literature on this subject is sparse. Finally, in **chapter 9** results of the studies are summarized and discussed, with an outlook to the future.

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