Role of Next-Generation Sequencing as a Diagnostic Tool for the Evaluation of Bone and Soft-Tissue Tumors

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Abstract

Bone and soft-tissue tumors are in general rare. Diagnosing these tumors is challenging based on the significant number of different tumor entities, the rareness of these tumors, and the considerable morphological heterogeneity which can be found within a single tumor entity. Considering that more than half of the described soft-tissue tumors and approximately 25% of the bone tumors harbor recurrent genetic alterations, the use of auxiliary molecular examinations should be strongly considered. Molecular analyses are important to confirm the diagnosis, to guide treatment, to provide information about prognosis, and to allow patient recruitment for basket trials based on the molecular signature of a tumor. In addition, novel molecular alterations detected by next-generation sequencing (NGS) obtain further insights into the pathogenesis of these rare tumors and allow a more detailed genetic classification. Based on our single-center results of NGS using the Ion AmpliSeq Cancer Hotspot Panel v2 and the Ion AmpliSeq Comprehensive Cancer Panel (Thermo Fisher Scientific) for mutational analyses as well as

the Archer FusionPlex Sarcoma Kit (ArcherDX, Inc) to detect gene fusions in 26 genes since early 2016, we have experienced NGS as a very sensitive method to detect genetic alterations. In our experience, the use of the Archer FusionPlex Sarcoma Kit is superior to fluorescent in situ hybridization as an auxiliary tool in the routine workup of soft-tissue and bone tumors.

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Introduction

Bone and soft-tissue tumors are in general rare tumors. Malignant bone tumors account for only 0.2% of all malignancies, and soft-tissue sarcomas account for less than 1% of adult solid malignant tumors but add up to 20% of pediatric solid malignancies. Although patients with bone and soft-tissue sarcomas are commonly referred to academic centers where specialized physicians and supplementary molecular diagnostic methods are available, treatment of patients with these rare tumors is challenging. An interdisciplinary team of sarcoma specialists including orthopedic surgeons, radiologists, oncologists, pathologists, and radiation therapists are necessary to provide best care. A precise diagnosis has become

an integral part in more disease entities and guides personalized treatment of sarcoma patients. More than 117 different soft-tissue tumors and 58 bone tumors are defined in the recent WHO classification of bone and soft-tissue tumors [1]. The diagnosis of these tumors is challenging based on the significant number of different tumor entities, the rareness of these tumors, as well as the considerable morphological heterogeneity which can be found within a single tumor entity. In addition, limited biopsy material is complicating the precise pathological workup. Considering that more than half of the described soft-tissue tumors harbor recurrent genetic alterations, auxiliary molecular examinations should be strongly considered. Molecular analyses are important to confirm the given diagnosis based on morphology and immunohistochemistry (IHC) with an “independent” method, to guide treatment, to provide information about prognosis, as well as to allow patient recruitment for basket trials based on the molecular signature of a tumor. The number of new tumor entities described especially in soft-tissue tumors with defined molecular changes is still growing, and known tumor entities get better defined by the detection of recurrent molecular changes. Over the last years, evidence accumulated that the number of different fluorescent in situ hybridization (FISH) probes a laboratory needs to have for the adequate workup of soft-tissue tumors is rapidly increasing. However, based on the rareness of some tumor entities, commercially available FISH probes are not always available, which emphasizes the need for alternative molecular methods.

FISH is one of the most common auxiliary techniques used for the detection of chromosomal abnormalities. We routinely employ FISH analyses for soft-tissue tumors using commercially available dual-color fusion, break-apart, and amplification probes from ZytoVision GmbH to investigate chromosomal aberrations. Samples are evaluated according to the guidelines of Reference Laboratory at the Cleveland Clinic (for break-apart probes, optimal samples have a minimum of 40 tumor cells for analysis). In a positive test, $\geq 10\%$ cells show a break-apart configuration of the 2 probes included in each of the individual assays, and in a negative test, $<10\%$ break-apart signals are identified [2]. In case of MDM2 amplification, an MDM2/CEP12 ratio >2.0 is indicative of gene amplification [3, 4]. However, defined cutoff levels for the evaluation of FISH for soft-tissue tumors and studies on interobserver variability for the interpretation of FISH examinations in soft-tissue tumors do not exist to the best of our knowledge. We especially experienced problems in examining FISH analyses of external speci-

mens or limited material and when alternative fixatives were used. Using the EWSR1 break-apart probe, one of the most commonly used FISH probes, one should be aware that EWSR1 gene rearrangement can be seen in a large spectrum of benign and highly malignant tumors. Therefore, it is always necessary to corroborate FISH results with morphology and IHC. In addition, conflicting FISH results need to be confirmed by alternative techniques (real-time polymerase chain reaction [RT-PCR] or next-generation sequencing [NGS]). One should also keep in mind that in cases of SMARCB1-related deletions, unbalanced abnormalities in EWSR1 can be detected by FISH [5]. To determine not only the EWSR1 break-apart but also the EWSR1 fusion partner, at least 2 FISH examinations have to be performed. The necessity to have more than the commercially available spectrum of FISH probes as a referral center as well as the fact that is necessary to detect not only the break-apart but also the fusion partner increases the turn-around time and the cost factor. Therefore, we searched for alternatives and started to use NGS.

We are experienced in NGS using the Ion AmpliSeq Cancer Hotspot Panel v2 and the Ion AmpliSeq Comprehensive Cancer Panel (Thermo Fisher Scientific) for mutational analyses for several years as well as the Archer FusionPlex Sarcoma Kit (ArcherDX, Inc.) to detect gene fusions in 26 genes since early 2016. In our opinion, NGS is a very sensitive method to detect genetic alterations. Detection of fusion genes is a difficult task as break points are usually located in introns, which renders sequencing of the genomic DNA unfeasible. Instead, fusion analysis usually starts from the RNA where the exons of fusion products are in direct succession. The Archer FusionPlex Sarcoma Panel interrogates fusions involving 26 genes commonly altered in soft-tissue sarcomas from as little as 250 ng total RNA. In the Archer Fusion work flow, a single-sided PCR product is generated from cDNA starting in the exons of genes included in the assay (gene-specific primer). After initial elongation of the first strand, a linker sequence is ligated to the end of the PCR strand, which can then be used to amplify the PCR product. This reaction is highly multiplexed to allow the analysis of the 127 exons of the 26 genes. Sequencing of the PCR product can then be applied to reveal the fusion partner and thus allows to detect all gene fusions involving these exons, even novel fusions not yet known in the literature. The whole work flow is well automated and can be performed in 5 working days. The core biopsy bone specimens usually contain enough nucleic acids for molecular analysis. The regular decalcifying process with formic acid means a

Table 1. Classification of bone and soft-tissue tumors according to molecular genetic categories

<i>Tumors with a simple genome</i>	
Chimeric transcription factor or transcriptional deregulation	ABC, BSNS, DSRCT, EH, EHE, EMC, ES, ELS, LGFMS, LPF-NT, MCS, PEComa, PmHE, SEF, SFT, SS
Deregulated signaling	DFSP, GIST, IMT
Epigenetic deregulation	Chondroblastoma, GCTB, myoepithelial carcinoma, rhabdoid tumor, SS
Expression of oncometabolite via epigenetic deregulation	Chondrosarcoma
<i>Tumors with a complex genome</i>	
Copy number alterations	LMS, low-grade OS, myxofibrosarcoma, postradiation angiosarcoma, UPS, WD/DDLS
Highly complex karyotype	Conventional osteosarcoma

ABC, aneurysmal bone cyst; BSNS, biphenotypic sinonasal sarcoma; DFSP, dermatofibrosarcoma protuberans; DSRCT, desmoplastic small round cell tumor; EH, epithelioid hemangioma; EHE, epithelioid hemangioendothelioma; ELS, Ewing-like sarcoma; EMC, extraskeletal myxoid chondrosarcoma; ES, Ewing sarcoma; GCTB, giant cell tumor of the bone; GIST, gastrointestinal stromal tumor; IMT, inflammatory myofibroblastic tumor; LGFMS, low-grade fibromyxoid sarcoma; LMS, leiomyosarcoma; LPF-NT, lipofibromatosis-like neural tumor; MCS, mesenchymal chondrosarcoma; OS, osteosarcoma; PEComa, perivascular epithelioid cell neoplasm; PmHE, pseudomyogenic hemangioendothelioma; SEF, sclerosing epithelioid fibrosarcoma; SFT, solitary fibrous tumor; SS, synovial sarcoma; UPS, undifferentiated pleomorphic sarcoma; WD/DDLS, well-differentiated/dedifferentiated liposarcoma.

qualitative limitation for molecular examinations, but materials decalcified by EDTA are suitable for both FISH and NGS investigations. Using NGS, a highly sensitive method to detect genetic changes, a high number of novel genetic aberrations have been described in several bone and soft-tissue tumors. These findings can help to precisely diagnose these tumors and to obtain further insight into the pathogenesis of these rare tumors and will further allow a more detailed genetic classification of soft-tissue and bone tumors. In addition, comprehensive knowledge of underlying genetic changes will provide the basis for the development of targeted therapies, may define prognostic markers, and may help patients to participate in basket trials.

The following article is focusing on our single-center experience with NGS in the field of bone and soft-tissue tumors. We will summarize known molecular alterations in bone and soft-tissue tumors and will focus on the detection of recurrent molecular changes using NGS as a reliable method to detect fusions/mutations in the routine workup of these tumors.

Selected tumor entities where NGS analysis facilitates the diagnosis will be discussed.

Overview

In general, molecular classification divides soft-tissue sarcomas into two main categories: (i) sarcomas with specific genetic alterations (fusions, mutations, amplifications, and loss of a tumor suppressor gene) and (ii) sarcomas with complex karyotypic abnormalities (e.g. myxofibrosarcomas, leiomyosarcomas, and pleomorphic sarcomas) [6]. The molecular classification of herein-described bone and soft-tissue tumors is listed in Table 1 [7].

Selected Bone Tumors with Recurrent Genetic Alterations That Can Facilitate a Precise Diagnosis

Chondroblastoma/Giant Cell Tumor of the Bone/Aneurysmal Bone Cyst

Chondroblastoma is a primary, intermediate, rarely metastasizing bone tumor according to the recent WHO classification. It typically affects people in the second decade of life and is located at the epiphysis of long bones. Histologically, the tumor is composed of chondroblasts with abundant pink cytoplasm, chicken wire matrix with

pink-bluish chondroid differentiation, and numerous osteoclast-type giant cells.

Giant cell tumor of the long bones (GCTB) is another primary, intermediate, rarely metastasizing bone tumor. This tumor occurs usually in individuals aged 20–40 years and also has epiphyseal location sometimes spreading to the metaphysis. Microscopically, GCTB is composed of multinucleated osteoclast-like giant cells mixed with uniform mononuclear cells and foamy macrophages. Although its histological appearance can be worrisome with mononuclear, spindle cells, frequent mitoses, and intravascular spreading, these are not signs of malignancy.

These two lesions can be a diagnostic challenge on small biopsy specimens. IHC can be helpful but shows less specificity. Chondroblastomas can show positivity for S100 and SOX9, and specific, but weak and very limited, focal positivity for DOG1 [8–10]. In contrast, diffuse p63 positivity can support the diagnosis of giant cell tumor [11]. At least in our experience, IHC is of limited help in difficult cases. However, recent advances in molecular pathology using NGS demonstrated K36M mutations in the H3F3B gene in 95% of chondroblastomas and H3F3A mutations in 92% of giant cell tumors of the bones [12, 13]. Both genes encode for histone H3.3 [14]. Also, in the rare variant of GCTB with symplastic/pseudoanaplastic changes which can mimic primary sarcoma or sarcomatous transformation, the same mutation as in conventional GCTB has been demonstrated [15]. Therefore, in our experience, mutational analysis using NGS is a very helpful technique to distinguish these tumor entities based on limited material.

Another differential diagnosis that could be considered in the context of giant cell-rich lesions is aneurysmal bone cyst (ABC). This lesion occurs usually in individuals younger than 30 years in the metaphysis of the bone. It has also been described in the soft tissue. Histologically, the solid variant of ABC can closely mimic giant cell tumor of the bones. To be able to differentiate primary ABC from giant cell tumor of the bones, mutational analysis demonstrating a H3F3A mutation or NGS demonstrating a USP6 fusion can facilitate the diagnosis [16]. Especially the use of a USP6 FISH probe can be very challenging as only a limited number of tumor cells demonstrate the USP6 translocation. Therefore, the interobserver variability is high, at least in our institution, and, in addition, diagnosis is complicated by a described range of USP6 rearrangement cells of 7–82% [17]. Therefore, NGS using the Archer FusionPlex Sarcoma Kit is favorable to FISH analysis. In addition, in rare cases of telangiectatic osteosarcomas (OS) which can also mimic ABC, NGS might be of help in selected cases.

Cartilaginous Tumors

In general, cartilage-forming tumors are common. More than 60% of benign bone tumors are cartilage-forming tumors. Amongst the malignant bone tumors, chondrosarcomas (CS) following OS are the second most common tumor entities and account for approximately 10–20% of all malignant primary bone tumors. Under the age of 30 years, the vast majority of cartilaginous tumors are benign, including enchondroma, chondroblastoma, osteochondroma, and chondromyxoid fibroma. In the age group between 30 and 40 years, beside enchondromas and osteochondromas, atypical cartilaginous tumor (ACT)/CS G1 and rarely central/peripheral CS are described. Over the age of 40 years, the most common cartilaginous tumor is ACT/CS G1, followed by high-grade CS and mesenchymal CS. Dedifferentiated CS can develop in a wide age range, but usually the patients are older than 50 years. Ollier disease and Maffucci syndrome are related to multiple enchondromas, and these patients show an increased risk to develop CS in younger age. Multiple osteochondroma is a genetic disease with autosomal dominant heritage. The lifetime risk of a malignant transformation in multiple osteochondroma is low (<1%). Molecular changes in EXT1/EXT2 genes are known in osteochondromas as well as in secondary peripheral CS arising from osteochondromas. Multiple osteochondroma affects hedgehog signaling and upregulation of BCL2 [18].

Initially, IDH1 or IDH2 mutations were described in 81% of patients with Ollier disease and 77% of patients with Maffucci syndrome [19]. However, IDH1 or IDH2 mutations can also be detected in sporadic cartilaginous tumors in both central and periosteal enchondromas (21% of all cases) and in central CS (G1–2: 13%, G3: 0%) including dedifferentiated central CS (4%). In contrast, no IDH mutations are observed in osteochondromas and peripheral CS [20]. Identification of IDH1/IDH2 mutations with NGS can be a useful tool in the diagnosis especially of dedifferentiated CS with limited G1 component and biopsy material exclusively obtained from the dedifferentiated component, a component with tremendous morphological heterogeneity [19–21]. In addition, IDH mutational analysis can also be very helpful to distinguish CS and chondroblastic OS since OS do not harbor somatic mutations in IDH1/IDH2 [22].

Moreover, detection of genetic changes in CS using NGS could also have clinical-translational relevance and improve diagnosis [23]. Speetjens et al. [24] summarized the current clinical trials in CS patients. Several alterations in different pathways were recognized in CS, in-

cluding alterations in IDH, hedgehog signaling, retinoblastoma protein, and p53. In part, these alterations might be the basis for selective targeted treatment modalities [24, 25].

Low-Grade Central/Parosteal/Periosteal OS and Fibrous Dysplasia

Low-grade OSs are rare tumors usually occurring in the third decade of life. They occur in different anatomic locations. Low-grade central OSs are usually located in the long bones, parosteal OS arises predominantly on the surface of the distal posterior femur, and periosteal OS arises typically in the diaphysis or dia-metaphysis as a sessile lesion. Fibrous dysplasia (FD), a benign medullary lesion in one or more bones (e.g., craniofacial bones and the femur) usually occurring in children and adults can histologically mimic low-grade OS [1]. Differential diagnosis between low-grade central OS and FD may be challenging based on pure morphology. Demonstration of MDM2 and CDK4 overexpression in low-grade OS using IHC/FISH is very helpful [26]. In contrast, FD, which is negative for MDM2 and CDK4, commonly demonstrates mutations in the GNAS gene, while all types of low-grade OS lack the GNAS mutation. To confirm the diagnosis of FD with GNAS mutation analysis may help to find a precise diagnosis [27].

Soft-Tissue Tumors

In this review, we are focusing only on subgroups of soft-tissue tumors to highlight the necessity of molecular investigation for a precise diagnosis and guidance of personalized therapy.

We will provide a summary about the diagnostic process of small round cell tumors (i), followed by perivascular epithelioid cell neoplasms (ii), subgroups of vascular tumors (iii), myxoid soft-tissue tumors (iv), and finally we summarize the diagnostic features of two recently described new entities (v).

Small Round Cell Tumors

Small round cell tumors represent a group of malignant neoplasms sharing histologically similar features. They are composed of small round cells with round nuclei containing fine chromatin and scanty clear or eosinophilic cytoplasm. Tumors within this category commonly occur in children and young adults. Tumors that should be considered in the differential diagnosis of small round cell tumors include: Ewing sarcoma (ES), Ewing-like sar-

Box 1. Specific translocations in small round cell tumors

Ewing sarcoma-specific translocations:		
t(11;22)(q24;q12)	EWSR1-FLI1	(85%)
t(21;22)(q22;q12)	EWSR1-ERG	
t(7;22)(q22;q12)	EWSR1-ETV1	
t(17;22)(q12;q12)	EWSR1-ETV4	
t(2;22)(q33;q12)	EWSR1-FEV	
t(16;21)(p11;q22)	FUS-ERG	
t(2;16)(q35;p11)	FUS/FEV	
Ewing-like sarcoma-specific translocations:		
t(6;22)(p21;q12)	EWSR1-POU5F1	
inv(22)(q12)	EWSR1-ZSG (ZNF278)	
t(4;22)(q31;q12)	EWSR1-SMARCA	
t(2;22)(q31;q12)	EWSR1-SP3	
t(1;22)(p36.1;q12)	EWS-ZNF278	
t(4;19)(q35;q3)	CIC-DUX4	
inv(X)(p11.4p11.22)	BCOR/CCNB3	
Complex ring chromosome with amplification of the translocated segments EWSR1-NFATc2		

comas (ELS), lymphomas, desmoplastic small round cell tumor, neuroblastoma, Wilms tumor, alveolar rhabdomyosarcoma, poorly differentiated monophasic synovial sarcoma, mesenchymal CS, melanoma, small-cell OS, and undifferentiated sarcoma with round cell morphology.

ES, ELS, and undifferentiated sarcoma with round cell morphology share the same histological features but harbor different specific translocations. Specific translocations in ES and ELS are listed in Box 1 [1, 28–30].

For the diagnosis of ES, expanded IHC testing is necessary to exclude the mentioned differential diagnoses, and, in addition, molecular testing is mandatory. Tumors lacking the mentioned genetic changes should not be called ES. A group of tumors sharing morphological similarities to ES, however, that lack the typical genetic changes has been further subclassified in the last years. About two-thirds of EWSR1-negative small blue round cell tumors (SBRCT) are associated with CIC-DUX4-related fusions, whereas another small subset shows either BCOR-CCNB3 X-chromosomal paracentric inversion or a BCOR-MAML3 or the alternative ZC3H7B-BCOR fusion.

The CIC-DUX4 fusion-related tumors are morphologically characterized by tumor cells in large fragments or sheet-like growth. Tumor cells have ovoid-to-round hyperchromatic nuclei, occasionally prominent nucleoli, and moderate eosinophilic or clear cytoplasm, and are

Table 2. Distinguishing features in small blue round cell tumors with clinical relevance

Tumors	HE staining	IHC	Molecular analysis	(Molecular) histology-based therapy ¹
Ewing sarcoma		CD99+	Archer panel NGS	IGF 1R inhibitor with cixutumumab and mTOR inhibitor, lurbinectedin robatumumab, dalotuzumab +/- ridaforolimus <i>Mutations in PTPRD, GRB10, KRAS</i>
Ewing-like sarcoma		CD99+WT1+/- CCNB+/-BCOR+/-	Archer panel NGS	IGF 1R inhibitor with cixutumumab and mTOR inhibitor
Neuroblastoma		CD99+CD56+	FISH N-MYC amplification	
Rhabdomyosarcoma		Desmin+MYF4+ Myogenin+PAX5+/-	Archer panel NGS	
Undifferentiated small cell sarcoma		CD99+/-	Archer panel NGS	
Synovial sarcoma		TLE1+CK+(focal) EMA+(focal)	Archer panel NGS	
Lymphoma/leukemia		CD45+(-) CD79a+ Other markers: depending on subtype	Depending on subtype	
Small cell osteosarcoma	Osteoid			
Mesenchymal chondrosarcoma	Cartilage	CD99+/-	Archer panel NGS	
Desmoplastic small round cell tumor	Desmoplasia	CD99+(-) FLI1+ Desmin+/- CK+/- NSE+/-	Archer panel NGS	Pazopanib, sunitinib, IGF-1R inhibitor with cixutumumab and mTOR inhibitor <i>Mutations in PTPRD, GRB10, KRAS</i>

¹ Potential therapy targets are shown in italics.

embedded in a fibrous stroma. In some cases, myxoid stroma was also observed. Geographic tumor necrosis is also frequently found. In comparison with ES, round cell sarcoma with CIC-DUX4 fusion demonstrates more cytological atypia [31]. The BCOR-CCNB3-positive tumors preferentially occur in children and in the bone in contrast to alternative BCOR-rearranged SBRCT, which develop later, in young adults, with a variable anatomic distribution. Histologically, BCOR-rearranged tumors often contain spindle cell areas and show a histological overlap with poorly differentiated synovial sarcoma [32]. In contrast, areas of spindle cells arranged in fascicles are usually not present in CIC-DUX4-related SBRCT. These tumors also contain tumor cells with vesicular nuclei, with distinct, often enlarged nucleoli, but lacking a higher degree of heterogeneity, which is typical for other tumors in the ES family [33]. IHC may facilitate the diagnosis of ELS, e.g., WT1 positivity in CIC-DUX4-associated sarcomas [33–35] or CCNB3 or BCOR staining to distinguish BCOR-CCNB3-associated sarcomas from other ELS [36,

37]. Further specific immunostaining to differentiate SBRCT is listed in Table 2.

The differential diagnosis in the ES and ELS group is challenging based on the morphological variability and limited specific IHC markers. NGS is, therefore, a powerful tool to classify the group of ES and ELS based on their underlying molecular changes [33].

Desmoplastic small round cell tumor is a highly malignant mesenchymal tumor. Its peak incidence is in the third decade of life, but with a wide age range from the first to the fifth decade. A striking male predominance is observed. The majority develops in the abdominal cavity. Beside containing small round cells, these tumors usually demonstrate prominent stromal desmoplasia. Multiphenotypic differentiation with expression of epithelial, muscular and neural markers is well known. Staining with antibodies against CK, EMA, desmin (dot-like), and NSE may vary in extent. The demonstration of a recurrent translocation t(11;22)(p13;q12) resulting in EWSR1-WT1 fusion is typical for this tumor entity.

Fig. 1. Histological and immunohistochemical features of small round cell tumors. **a** Ewing sarcoma. **b** CIC-DUX4 translocation-associated Ewing-like sarcoma. **c** Desmoplastic small round cell tumor. **d** Solid alveolar rhabdomyosarcoma.

Mesenchymal CS is a malignant tumor with biphasic pattern, containing undifferentiated round cells and well-differentiated hyaline cartilage. It can occur at any age but preferentially occurs in the second and the third decade. Besides widespread skeletal distribution, extra-skeletal involvement with meningeal origin is well known. Recently, two fusion genes were described in mesenchymal CS: the HEY1-NCOA2 fusion and the IRF2BP2-CDX1 fusion [38, 39]. Because of the overlapping morphological features in meningeal hemangiopericytoma and mesenchymal CS, identification of the HEY1-NCOA2 fusion, which is not present in meningeal hemangiopericytoma, is inevitable for the diagnosis of mesenchymal CS [40].

In addition to its diagnostic role, NGS may confirm the presence of mutations and translocations, which are potential targets of molecular-based personalized treatment options in ES or desmoplastic small round cell tumor (Table 2) [41–50]. Histological and IHC features of SBRCT are shown in Figure 1. Other SBRCT subsets mentioned above are not described in more detail in this issue.

Perivascular Epithelioid Cell Neoplasms

Perivascular epithelioid cell neoplasms (PEComa) are a family of rare mesenchymal tumors with perivascular epithelioid cell differentiation expressing myo-melanocytic markers. The PEComa family includes angiomyolipoma, clear-cell “sugar” tumor of the lung, and lymphan-

giroleiomyomatosis. A subset of tumors with identical histological features occurs in the soft tissue or visceral sites. Most commonly, they arise in the retroperitoneal or pelvic region as well as in the uterus or in the gastrointestinal tract. These tumors occur in all age groups, but they are most common in young to middle-aged adults. Microscopically, PEComas are composed of epithelioid cells with abundant eosinophilic or clear cytoplasm arranged in small nests surrounded by capillary vessels. They typically express HMB45, Melan-A, MITF, SMA, and desmin. The distinction between “benign” and “malignant” tumors is based on mitotic activity, necrosis, marked nuclear atypia, and pleomorphism. Although most PEComa harbor loss-of-function mutations in TSC1/TSC2, a small subset carries TFE3 gene rearrangement [1]. SFPQ/PSF-TFE3 gene fusions were previously reported by Tanaka et al. [51]. In addition, a novel DVL2-TFE3 gene fusion was also described. Based on a recent publication, PEComa with a TFE3 gene abnormality shows strong TFE3 and cathepsin K immunoreactivity and weak-to-strong reactivity for HMB45 [52–54]. The diagnosis of PEComa is usually based on HE-stained slides combined with IHC. However, identification of the underlying genetic alterations could be necessary to assess the prognosis and to determine the best treatment option. In a clinical follow-up study, Agaram et al. [55] demonstrated that PEComa patients with TFE3 rearrangement may have a better prognosis. In patients with TSC1/2 mutation, mTOR inhibitors have proven to be effective [56, 57]. Especially in patients suffering from malignant/metastatic PEComa, NGS may provide further insight regarding potential treatment strategies.

Vascular Tumors

In the last years, tremendous efforts in the classification of vascular tumors have been achieved by detecting recurrent genetic changes. The group of vascular tumors contains several entities showing endothelial differentiation with diverse patterns in bone and soft tissue. This article is restricted to vascular lesions where molecular analysis is of diagnostic and/or prognostic relevance.

Epithelioid Hemangioma. In contrast to epithelioid hemangioma (EH) of the soft tissue, EH of the bone is a locally aggressive tumor occurring in patients with a wide age range. The tumors are usually located in long tubular bones or affect flat bones, vertebrae, or small bones of the hands. Regional multifocal forms can occur. Histologically, these tumors show a lobular architecture with typically small vessels at the periphery. Besides solid sheets of epithelioid tumor cells, vascular structures lined with ep-

ithelioid cells are observed. Small foci of necrosis and fascicles of proliferating spindle cells may be seen, and nuclear atypia can be present [1]. In biopsy material, where the architecture is sometimes hard to evaluate, the distinction between EH and malignant vascular tumors may be challenging. Recently, FOS rearrangement has been described in an EH subset, presumably in correlation with morphological appearance and the involved site. Huang et al. [58] have demonstrated FOS rearrangement in a third of EH, with increased prevalence in intraosseous (59% of intraosseous EH) and cellular EH (70% of all FOS-rearranged EH). In a study by Antonescu et al. [59], FOSB rearrangement was observed in 20% of EH. Morphologically, tumors with FOSB rearrangement contained solid areas, increased cellularity, and nuclear pleomorphism. Fusion-negative EH were less cellular, had less solid components, and lacked cytological atypia helping to distinguish EH with atypia from angiosarcoma (AS) by detecting FOSB rearrangement. Molecular investigation of FOS gene rearrangement, if present, can significantly facilitate the diagnosis of EH, especially in cases with nuclear atypia. However, the absence of a FOS gene rearrangement, on the other hand, does not exclude EH, because this genetic change is only seen in up to one third of cases. Therefore, in the absence of FOS rearrangement, the distinction between EH with cytological atypia and other malignant vascular tumors remains still challenging.

Pseudomyogenic Hemangioendothelioma. Pseudomyogenic hemangioendothelioma is a rarely metastasizing vascular tumor occurring most commonly in young male adults. This tumor occurs usually in the extremities; in approximately 50% of cases, it can be located multicentric in different tissue planes. The tumor is histologically composed of plump spindle cells with abundant eosinophilic cytoplasm mimicking myoid or epithelioid differentiation, which is arranged in sheets and fascicles. The tumor cells express keratin AE1/AE3, FLI1, and ERG, and 30% of the tumors show focal positivity for SMA. Staining for CK-MNF116, EMA, S100, CD34, and desmin is negative in the tumor cells. FOSB rearrangement is also described in subsets of pseudomyogenic hemangioendotheliomas [60–62].

Epithelioid Hemangioendothelioma. Epithelioid hemangioendothelioma (EHE) is a malignant vascular neoplasm that develops mostly after the second decade of life. It arises usually in the soft part of the extremities in angiocentric location. Intraosseous EHE occurs mostly in the long tubular bones. The tumor is composed of epithelioid endothelial cells embedded in myxohyaline stroma

growing in an angiocentric distribution. Besides WWTR1-CAMTA1 rearrangement in EHE subsets, YAP1-TFE3 rearrangement is noted [1, 63, 64]. IHC identification of CAMTA expression [65] as well as IHC demonstration of TFE3 can be also helpful to differentiate EHE from AS.

Postradiation AS. AS is a malignant vascular tumor of the elderly. Most often, AS arises from the deep soft tissue of the extremities, but intraosseous tumors with wide skeletal distribution are also described. AS subsets are associated with previous irradiation [1]. Radiotherapy-associated vascular proliferation covers a range of benign and malignant tumors. Atypical vascular lesions (AVL) are macroscopically composed of small papules with discoloration. Usually, they develop short after radiation therapy (median 3 years). AVL are located in the superficial or mid-dermis, and, in a few cases, AVL extend to the deep dermis, without subcutaneous infiltration. It is a well-circumscribed, but unencapsulated lesion composed of anastomosing vascular channels. The endothelial cell nuclei are hyperchromatic with prominent hobnailing. Cytological atypia, mitosis, necrosis, or multilayering of endothelial cells are not present. Vascular channels are often more compressed in the deeper counterpart. AVL show morphological overlap with well-differentiated AS, but so far their clinical behavior was reported to be benign. AS shows microscopically evident signs of malignancy, atypical cells with prominent nucleoli, mitoses, papillary endothelial hyperplasia, and “blood lakes.” Infiltration into the subcutis and dissection of dermal collagen are also present. Well-differentiated AS can also contain AVL-resembling areas with anastomosing vascular channels and focal endothelial hobnailing without cytological atypia or multilayering. It is challenging to differentiate these two lesions in HE-stained slides of a small biopsy [66]. Mentzel et al. [67] have found MYC amplification in postradiation cutaneous AS, whereas control cases and cases of atypical vascular proliferation after radiotherapy were negative for MYC. Using IHC, strong positive nuclear staining for MYC was seen in cases of postradiation cutaneous AS, in contrast to atypical vascular proliferations. Cornejo et al. [68] confirmed that detection of MYC protein expression by IHC and MYC gene amplification by FISH are valuable to distinguish AVL from postradiation AS. Moreover, MYC immunostaining is helpful to control resection margins [67].

Myxoid Soft-Tissue Tumors

Myxoid soft-tissue tumors represent a wide group of tumors with benign to malignant behavior. In the following paragraph, we will focus on tumors where molecular

Box 2. Summary of myxoid soft-tissue neoplasms with molecular diagnostic relevance

Benign tumors
Nodular fasciitis
Intermediate tumors
Myxoinflammatory fibroblastic sarcoma/tumor
Dermatofibrosarcoma protuberans
Solitary fibrous tumor
Malignant tumors
Myxofibrosarcoma
Low-grade fibromyxoid sarcoma
Myxoid liposarcoma
Extraskeletal myxoid chondrosarcoma
Myoepithelial carcinoma
Myxoid synovial sarcoma

examination is a useful diagnostic tool. A summary of these lesions is given in Box 2.

Myxoinflammatory Fibroblastic Sarcomas/Tumors. They are locally aggressive tumors usual occurring at acral sites most commonly in the distal extremities and typically occur in middle-aged people [1]. Myxoinflammatory fibroblastic sarcoma/tumor is composed of moderately cellular nodules with basophilic myxoid matrix alternating with fibrohyaline matrix. They are composed of 3 distinct types of tumor cells: large Reed-Sternberg or virocyte-like cells, spindle-to-epithelioid mononuclear tumor cells, and so-called “pseudolipoblasts” with abundant vacuolated myxoid basophilic cytoplasm. Hallmark of the lesion is the prominent mixed acute and chronic inflammatory cell infiltrate rich in eosinophils, neutrophils, lymphocytes, and plasma cells. Diagnosis is based on HE morphology while IHC is of limited use. To exclude inflammatory conditions or other myxoid soft-tissue tumors, NGS can be performed to demonstrate a MGEA5-TGFBR3 fusion [69].

Myxofibrosarcoma. Myxofibrosarcoma is one of the most common sarcomas in elderly patients, usually arising in the limbs. Histologically, the hallmarks of this tumor are curvilinear blood vessels with accentuated atypical spindle cells in a myxoid background. Hypercellular solid sheets and fascicles of spindle as well as pleomorphic tumor cells may be present in variable ratio in correlation with histological grade. Because of the lack of specific immunostaining and specific genetic alterations, the diagnosis is mainly based on HE morphology and differential diagnoses need to be excluded. Especially in low-grade tumors, a myxoid variant of dermatofibrosarcoma

Fig. 2. Myxoid liposarcoma, nested variant. HE. **a** $\times 10$. **b** $\times 40$.

protuberans (DFSP), low-grade fibromyxoid sarcoma (LGFMS), or a cellular myxoma are included in the differential diagnosis. The demonstration of a PDGFRB-COL1A1, FUS-CREB3L1, and FUS-CREB3L2 fusion, or a mutation in the GNAS gene can facilitate the diagnosis of the mentioned differential diagnoses.

Low-Grade Fibromyxoid Sarcoma. LGFMS is a low-grade malignant sarcoma with morphological overlap especially with benign mesenchymal lesions. Most of the reported cases affect young adults, and it is typically located in the proximal extremities or trunk. Although this is a low-malignant sarcoma, a long-term follow-up is required due to the occurrence of late metastases. LGFMS and a subset of sclerosing epithelioid fibrosarcomas harbor the same chromosomal translocation with FUS-CREB3L2 or FUS-CREB3L1 rearrangement. There are several other tumors with recurrent translocations, involving the FUS gene, e.g., myxoid liposarcoma or angiomatoid fibrous histiocytoma [70, 71]. Detection of MUC4 expression by IHC is a very powerful tool to confirm the diagnosis of LGFMS. The presence of MUC4 expression is also observed in sclerosing epithelioid fibrosarcoma,

Fig. 3. Myxoid liposarcoma. FISH showing a CHOP break-apart signal (arrows).

but MUC4 staining does not predict the presence of FUS rearrangement, as only 40% of MUC4-positive sclerosing epithelioid fibrosarcomas show FUS rearrangement [72, 73].

Myxoid Liposarcoma. It accounts for 20% of liposarcomas and typically arises in the deep soft tissue of the extremities of children and adolescents. Microscopically, low-grade myxoid liposarcoma is composed of monomorph round-to-ovoid cells in a myxoid background with arborizing “chicken wire” capillaries. Various morphological patterns, e.g., traditional myxoid, traditional round cell, pulmonary edema-like, lipoblast-rich, island, lipomatous, stromal hyalinization, cord-like, nested (Fig. 2), chondroid metaplasia, and hemangiopericytoma-like patterns, have been described [74]. The lack of specific IHC markers as well as the morphological heterogeneity underlines the use of FISH (Fig. 3) or NGS to demonstrate FUS-DDIT3 or EWSR1-DDIT3 fusions in this tumor entity.

Dermatofibrosarcoma Protuberans. DFSP is a low-grade, locally aggressive fibrohistiocytic tumor with recurrent COL1A1-PDGF fusion. It develops in young to

middle-aged adults typically superficial in the trunk or proximal extremities. Usually, DFSP shows a typical morphological pattern of a hypercellular tumor composed of uniform spindle cells arranged in a storiform pattern and infiltrates the subcutaneous adipose tissue in a so-called “honeycomb pattern.” The diffuse CD34 positivity is very helpful in diagnosing DFSP [1]. The diagnosis of the myxoid variant of DFSP, however, may be challenging. As neoadjuvant imatinib mesylate may be of benefit in unresectable tumors or in reducing tumor size, molecular testing for a COL1A1-PDGF fusion to confirm the diagnosis as well as to predict therapy response should be performed [71].

Extraskelatal Myxoid CS/Myoepithelial Carcinoma. Extraskelatal myxoid CS (EMC) is a malignant tumor of unknown origin occurring in adults, usually in the deep soft tissue of the proximal extremities or limb girdles. Morphologically EMC is a multinodular tumor displaying cords of uniform tumor cells in an abundant myxoid matrix. Myoepithelial tumors of the soft tissue are rare. Histologically, these tumors show a wide morphological spectrum and are similar to their salivary gland counterparts. EMC and myoepithelial carcinoma demonstrate morphological similarities and an overlap in the IHC profile. Therefore, demonstrating specific reciprocal translocations, involving the NR4A3 gene with the fusion partners EWSR1, TAF15, TCF12, and TGF, can support the diagnosis of EMC. In contrast, loss of SMARCB1/INI1 is a typical alteration in the majority of myoepithelial carcinomas [75–78]. Taking into account that SMARCB1/INI1 and EWSR1 genes are located close to each other on chromosome 22, larger SMARCB1 deletions may encompass the EWSR1 locus, which can lead to misinterpretation by FISH analysis. This reveals the necessity of NGS in significant subgroups of tumors with EWSR rearrangement [5]. Especially in myxoid soft-tissue tumors, NGS can facilitate a precise diagnosis especially on biopsy material by demonstrating recurrent gene fusions or mutations [79].

Recently Described New Entities of the Soft Tissue

Lipofibromatosis-Like Neural Tumor. The group of pediatric fibroblastic and myofibroblastic tumors contains a wide spectrum of entities; histologically, features can overlap with sarcomas, such as infantile fibrosarcoma or spindle-cell rhabdomyosarcoma. Among the superficial lesions, lipofibromatosis (LPF), composed of adipose tissue and fibroblastic elements, has so far been diagnosed as infantile fibromatosis or fibrous hamartoma of infancy. LPF-like neural tumor (NT) affects young

children and develops usually in the soft tissue of the trunk or distal extremities. Local recurrence is often due to the highly infiltrative growth pattern with subsequent incomplete resection. Immunohistochemically, these tumors are CD34 and SMA positive and stain negative for other markers. Recently, a tumor with similarities on morphological examination located in a wide anatomic distribution has been described with variable cytological atypia and at least focal-positive staining for S100. This tumor is composed of plump spindle cells arranged in fascicles infiltrating the subcutaneous adipose tissue. The tumor cells show eosinophilic cytoplasm and fusiform nuclei with mild hyperchromasia. Mitotic activity is low (<2/10 HPF), and necrosis has not been reported. Beside S100 expression, CD34 and variable SMA expression has been reported. No staining with antibodies against desmin, GFAP, STAT6, HMB45, Melan-A, and SOX10 was reported. Agaram et al. [80] reported a TPR-NTRK1 or TPM3-NTRK1 gene fusion in 71% of cases. Furthermore, the performed NTRK1 immunostaining was positive only in NTRK1-rearranged S100-positive LPF-NT but negative in classic LPF. These results suggest that NTRK1 oncogenic activation through gene fusion defines a novel and distinct entity, provisionally named LPF-NT; expanded IHC analysis including NTRK1, S100, CD34, CK, and myogenic markers is helpful to diagnose this tumor in addition with molecular diagnostics.

PAX3 Fusion-Positive Biphenotypic Sinonasal Sarcoma. PAX3 fusion positive biphenotypic sinonasal sarcoma (BSNS) is a recently described, low-grade sarcoma developing in the sinonasal region, mostly in the nasal cav-

(Footnote to Table 3.)

ABC, aneurysmal bone cyst; AFH, angiomatoid fibrous histiocytoma; AS, angiosarcoma; ASPS, alveolar soft part sarcoma; BSNS, biphenotypic sinonasal sarcoma; cc., carcinoma; CCS, clear-cell sarcoma; DF, deep-type fibromatosis; DFSP, dermatofibrosarcoma protuberans; EH, epithelioid hemangioma; EHE, epithelioid hemangioendothelioma; EMC, extraskelatal myxoid chondrosarcoma; ES, epithelioid sarcoma; f, focal; GCTB, giant cell tumor of the bone; GIST, gastrointestinal stromal tumor; IMT, inflammatory myofibroblastic tumor; LGFMS, low-grade fibromyxoid sarcoma; LMS, leiomyosarcoma; LPF-NT, lipofibromatosis-like neuronal tumor; MFS, myxofibrosarcoma; MIFS, myxoinflammatory fibroblastic sarcoma; MLS, myxoid liposarcoma; MPNST, malignant peripheral nerve sheath tumor; OS, osteosarcoma; PEComa, perivascular epithelioid-cell tumor; PmHE, pseudomyogenic hemangioendothelioma; RMS, rhabdomyosarcoma; SEF, sclerosing epithelioid fibrosarcoma; SFT, solitary fibrous tumor; SMA, smooth muscle actin; SS, synovial sarcoma; TSGCT, tenosynovial giant cell tumor.

¹ Fusions are in normal font; mutations, amplifications, overexpression, and loss of functions are in italics. ² Potential therapy targets are in italics.

Table 3. Differential diagnostic guidelines of bone and soft-tissue neoplasms according to genetic alterations and therapeutic relevance

	Genetic alterations ¹	Immunohistochemistry	(Molecular) histology-based therapy ²
Bone tumors			
ABC	USP6-CDH11		
GCTB	<i>H3F3A</i>	p63+	
Chondroblastoma	<i>H3F3B</i>	S100+/- SOX9+ DOG1 f+/- H3F3 K36m+	
Chondrosarcoma	<i>IDH1/IDH2 mutation</i>	IDH1/IDH2 m (R132/172) f+	<i>SHH, BCL2, IDH, Rb</i>
Low-grade OS	<i>MDM2/CDK4 amplification</i>	MDM2+ CDK4+	
Fibrous dysplasia	<i>GNAS mutation</i>		
OS	<i>EZH2 overexpression in advanced cases</i>	SATB2+ EZH2+/-	
Soft-tissue tumors			
PEComa	SFPQ/PSF-TFE3 DVL2-TFE3 <i>TSC1/TSC2 mutation</i>	HMB45+ Melan-A+ SMA+ TFE3+/- Cathepsin K+ PAX8-	mTOR inhibitors
Vascular neoplasms			
EH	ZFP36-FOSB		
PmHE	SERPINE1-FOSB		
EHE	WWTR1-CAMTA1 YAP1-TFE3	CAMTA1+/- TFE3+/-	
Postradiation AS	<i>MYC amplification</i>	MYC+	Sorafenib, taxanes, gemcitabine
Myxoid neoplasms			
MIFS		Excluding	
MFS		Excluding	
LGFMS	FUS-CREB3L2 or FUS-CREB3L1	MUC4+	
MLS	FUS-DDIT3/EWSR1-DDIT3		Trabectedin
DFSP	COL1A1-PDGF	CD34+	Imatinib
EMC	NR4A3-EWSR1/TAF15/TCF12/TGF		
Myoepithelial cc.	<i>Loss of SMARCB1/INI1</i>	INI1-	
Nodular fasciitis	USP6-MYH9		
SFT	NAB2-STAT6	STAT6+	Sunitinib
Non-myxoid neoplasms			
SEF	FUS-CREB3L2	MUC4+	
IMT	EML4-ALK, TFG-ROS1, RET rearr.	ALK+/-	Crizotinib, kinase fusions
DF	<i>CTNNB1 mutation</i>	β-Catenin+	
MPNST	<i>SUZ12, EED, EGFR, IGF1R, SOX9, EYA4, TOP2A, ETV4, BIRC5</i>	S100f+ Leu7f+ MyoD1/MYF4f+/-	<i>EGFR, IGF1R, SOX9, EYA4, TOP2A, ETV4, BIRC5</i>
New entities			
LPF-NT	NTRK1-TPR/TPM3	S100+	
BSNS	PAX3-AML3/NCOA1/FOXO1 MAML3-non-PAX3	S100+ β-catenin+ SOX10+	
Others			
AFH	EWSR1-CREB1/FUS-ATF1/EWSR1-ATF1		
ASPS	ASPSR1-TFE3	TFE3+	Sunitinib, cediranib
CCS	EWSR1-CREB1/-ATF1	S100+ HMB45+ Melan-A+	
Chordoma		CK+ S100+ brachyury+	
ES	<i>Loss of INI1</i>	INI1-	
GIST	<i>KIT/PDGFR/SDHA/-B/-C/-D</i>	CD34+/- CD117+/- DOG1+/-	
Lipoblastoma	<i>PLAG1-HAS2/COL1A2/HMGA2</i>		
LMS	<i>MYOCD loss of p53/ATR</i>	SMA+ desmin+ h-caldesmon+	Pazopanib, gemcitabine, trabectedin
Alveolar RMS	FOXO1-FGFR1/PAX3-FOXO1/-FOXO4/ -NCOA1/-NCOA2/INO80D/PAX7-FOXO1	SMA+ Desmin+ MYOD1+	Crizotinib, ceritinib, palbociclib, BMS-754807, cixutumumab, R1507 anti-IGF1R antibody, vemurafenib, dabrafenib, MEK inhibitor, selumetinib, ERK2 inhibitor, EZH2 inhibitor
Sclerosing RMS	<i>RAS, FGFR4, ERBB2, PIK3CA, PI3K, FBXW7, NF1, TP53, CTNNB1, BCOR, MYOD1</i>	SMA+ desmin+ MYOD1+	
Spindle-cell lipoma	<i>Loss of RB1</i>	CD34+ RB-	Pazopanib
SS	SS18-SSX1/-SSX2/-SSX4/SS18L1-SSX1	TLE1+ CKf+ EMAf+	Imatinib, nilotinib, PLX3397, emactuzumab
TSGCT	CSF1-COL6A3/other		

ity or ethmoid sinus. There is a striking predilection for women in the fifth decade of life. Histologically, this tumor shows an infiltrative growth pattern and resembles adult fibrosarcoma or monophasic synovial sarcoma. A classic “herringbone” pattern can be identified in most cases. Despite the cellularity, only rare mitotic figures are present. A characteristic finding in the majority of these neoplasms is an epithelial proliferation. This proliferation is composed of surface-type respiratory epithelium. A prominent hemangiopericytomatous vascular pattern is also present in a subset of these tumors. BSNS typically shows a unique, biphenotypic IHC staining pattern [81]. This unique dual phenotype can be explained by the recurrent rearrangements of PAX3, a transcription factor that promotes commitment along neural and myogenic lineages. Most BSNS harbor a pathognomonic PAX3-MAML3 fusion; an alternative fusion partner gene is NCOA1. The tumors usually express at least focal S100, SMA, calponin, β -catenin, and factor XIIIa, and subset of tumors expresses focal EMA, CD34, desmin, and/or myogenin. None of the cases reacted with SOX10. An extended IHC panel including β -catenin and SOX10 as well as NGS supports the diagnosis of BSNS [82–84].

Bone and soft-tissue neoplasms are listed in Table 3 according to their genetic alterations and therapeutic relevance [85–104].

Concluding Remarks

To date, approximately 50% of the described soft-tissue tumors and 25% of bone tumors harbor recurrent genetic alterations that can be used to reach a precise diagnosis. It is most likely that using NGS more tumor entities will be characterized by recurrent genetic changes. Con-

sidering the number of newly described entities after the release of the recent WHO 2013 classification, one can suspect that the number of new tumor entities will still grow in the future. Although HE morphology in combination with IHC (and FISH) is the golden standard in the diagnosis of bone and soft-tissue tumors, NGS can be used as an independent diagnostic tool to confirm a given diagnosis and can reduce interobserver variability. In addition, classification of the tumors according to the genetic alteration will allow the development of more targeted treatment options. Taking into account that some rare bone and soft-tissue tumors show similar or the same genetic alterations as more common tumor entities, one can hope that patients suffering from these rare tumors will have the opportunity to participate in basket trials based on the genetic tumor fingerprint. NGS will hopefully provide information for new personalized treatment options and the identification of useful biomarkers.

Based on our single-center experience in NGS using the Ion AmpliSeq Cancer Hotspot Panel v2 and the Ion AmpliSeq Comprehensive Cancer Panel (Thermo Fisher Scientific) for mutational analyses as well as the Archer FusionPlex Sarcoma Kit (ArcherDX, Inc) to detect gene fusions in 26 genes since early 2016, we have experienced NGS as a very sensitive method to detect genetic alterations. In our experience, the use of the Archer FusionPlex Sarcoma Kit is superior to FISH as an auxiliary tool in the routine diagnostic approach of soft-tissue and bone tumors.

Disclosure Statement

The authors have no conflict of interest to declare.

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