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

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Chapter 2

Molecular pathology of bone tumors

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Abstract

Although classic histomorphology is the cornerstone of bone tumor diagnostics, this field has rapidly evolved since the advance of new molecular techniques. The identification of novel genetic alterations in bone tumors has led to more insight into the genetic background of these tumors, which resulted in a more prominent role of molecular pathology in daily practice. Numerous studies have been conducted in the last decades and illustrated that based on molecular alterations, bone tumors are roughly divided into tumors with simple and complex karyotypes. The first group can be further divided into tumors that carry specific translocations, somatic gene mutations and those with more or less specific amplifications. On the other hand, sarcomas with complex karyotypes usually lack specific alterations. Many techniques are available for the detection of recurrent genetic alterations, now also including immunohistochemistry, and this review will focus on routinely performed assays in molecular diagnostics. Subsequently, tumor classes with distinct genetic abnormalities will be discussed and illustrated by more specific examples, and the usefulness of the molecular pathology in routine diagnostics will be highlighted.

Introduction

Bone tumors encompass a broad group of benign and malignant neoplasms and are considered as difficult to diagnose for pathologists. Clinically relevant bone tumors, especially bone sarcomas, are rare and distinction based on classic histomorphology can be challenging, due to the overlapping morphology. Furthermore, traditional immunohistochemistry, to identify the line of differentiation, is less valuable in discriminating different bone tumors in comparison with soft tissue tumors. Fortunately, in the last decades dramatic advances in molecular techniques led to the discovery of many genetic abnormalities in bone tumors. These findings have provided insights in tumorigenesis of bone tumors, leading to the molecular subclassification of bone tumors roughly into two categories, as a conceptual framework. The first group of tumors shows simple karyotypes and includes tumors with translocations, and tumors with specific gene mutations and/or amplifications. These specific recurrent genetic alterations can change transcription, cause altered signaling, or alter gene function. The second category consists of tumors with complex karyotypes, lacking any specific alterations. In addition, the identification of molecular alterations has been instrumental in the understanding of molecular pathways relevant in tumorigenesis and resulted in the merge of molecular assays in a diagnostic setting. The further development of mutation specific immunohistochemistry has greatly improved bone tumor diagnostics. This review aims at providing an overview of molecular assays currently used in the diagnosis of bone tumors. Subsequently, the molecular classification of bone tumors will be discussed combined with the altered molecular pathways relevant in tumorigenesis. Furthermore, the translation of specific molecular alterations to clinical practice relevant in the differential diagnosis of several bone tumors will be illustrated.

Molecular techniques in bone tumor pathology

Decalcification of bone tumors

One of the main challenges in the evaluation of genetic alterations in bone tumors has been the requirement of DNA or RNA from these lesions for molecular analysis. As decalcification is essential for adequate histological evaluation of bone tissue, the DNA and RNA isolated from formalin-fixed, paraffin-embedded (FFPE) bone tumors is often degraded. The availability of frozen tumor tissue is ideal but not often the reality. Among the acid based agents, strong mineral and weaker organic acids cause respectively more or less damage to DNA and RNA by hydrolysis and fragmentation of the nucleotides. Although nitric acid and other strong mineral acids are rapid in action, severe damage to the nucleotides has made molecular testing impossible which is in contrast to weaker acids, such as formic acids [1]. Another group consists of chelating agents of which ethylenediaminetetracetic acid (EDTA)

is the most gentle agent that takes up calcium ions, suitable for decalcification [1, 2]. However, a major limitation has been the relatively long processing time, which makes the use of EDTA for routine histological evaluation less appropriate. Therefore, weaker organic agents are used in routine practice as an intermediate solution prior to further molecular analysis, and the resulting degradation of nucleic acids strongly influences possibilities for additional molecular testing, especially in the absence of frozen tumor tissue.

Fluorescence in-situ hybridization (FISH)

FISH has been a commonly used technique that utilizes the presence of DNA within histological slides as the target for hybridization. The probes, incorporated with fluorophore-coupled nucleotides, anneal to the complementary sequence, which results in the visualization of the genes of interest. Since interphase FISH can be easily applied to FFPE tissue, this had led to its wide use in daily practice, though depending on the availability of (commercial) probes. Although hydrolysis of DNA due to decalcification can prompt serious problems for FISH analysis, it has been shown that limited acid decalcification in 5% formic acid can preserve DNA sufficient for FISH [3].

When focusing on the diagnosis of bone tumors, FISH is widely used for detection of translocations utilizing both split-apart and fusion probes. In case of promiscuous genes, split-apart probes, flanking the gene of interest, are usually the first choice, as for instance *EWSR1* in Ewing sarcoma. In case both partners are recurrent, fusion probes can also be used, for instance for epithelioid hemangioendothelioma classically fusing *WWTR1* to *CAMTA1* [4]. When using split-apart probes for promiscuous genes such as *EWSR1* or *FUS* one should always realize that the fusion partner will not be revealed, which may be essential for establishing the correct diagnosis. For instance, the differential diagnosis of "round cell tumors" includes, amongst many others, Ewing sarcoma, myoepithelial tumor and extraskeletal myxoid chondrosarcoma (**Table 1**), all of which can show areas with round cell morphology and overlapping immunohistochemical findings.

Additional use of only split-apart probes for *EWSR1* will not solve this, as all three entities can harbor translocations involving this gene, while other specific molecular assays for translocation detection that do reveal the fusion partner will. Members of the ETS-family are involved in translocations in Ewing sarcoma [5], while *EWSR1* is frequently translocated with either *POU5F1*, *PBX1* or *ZNF444* in myoepithelial tumors [6] and with *NR4A3* in extraskeletal myxoid chondrosarcoma [7]. FISH is also used for amplification detection, for example in parosteal osteosarcoma, which is characterized by amplification of the 12q13-15 region including, amongst others, the *MDM2* and *CDK4* genes [8, 9].

Despite the sensitivity of FISH, this assay is not the primary choice in tumors with a high amount of non-tumor cells. For instance, in aneurysmal bone cyst (ABC) *USP6* rearrangements are restricted to the neoplastic spindle cells and are not found in the other ABC cell components, such as the multinucleated giant cells and metaplastic bone associated osteoblasts. Since the reported percentage of neoplastic cells varies from 7% to 82% [10], this can lead to false negative results in samples with a low percentage of tumor cells.

The interpretation of FISH signals can sometimes be difficult due to cutting artefacts and unusual patterns, such as loss of one of the split signals or presence of numerous 5' centromeric signals or 3' telomeric signals. In one study of 135 bone and soft tissue tumors, FISH was performed for the detection of *EWSR1* rearrangements. Besides the usual fused and split paired signals, in respectively 16% and 24% of the nuclei atypical break apart patterns were observed in both rearranged (n=39; 56%) and non-rearranged (n=30; 45%) cases [11]. In combination with borderline levels of rearrangements around the cut-off, this can pose potential problems of misinterpretation. In some cases, atypical FISH patterns such as amplification of the 5' signal might be an indication for a certain tumor type, as this can be exemplified for *EWSR1-NFATc2* rearranged tumors, where amplification of the fusion gene has been observed in all described cases [12].

Table 1. Molecular alterations in "round cell tumors" of bone

Entity	Molecular alteration
Ewing sarcoma	*EWSR1/FUS*-ETS family members
EWSR1-non-ETS rearranged round cell sarcomas	*EWSR1-NFATc2* *EWSR1-SP3* *EWSR1-SMARCA5* *EWSR1-PATZ1*
CIC-rearranged round cell sarcomas	*CIC-DUX4* *CIC-FOXO4* *CIC-NUTM1*
BCOR-rearranged round cell sarcomas	*BCOR-CCNB3* *BCOR-MAML3* *ZC3H7B-BCOR*
Non-Hodgkin lymphoma/leukemia	Depending on subtype
Synovial sarcoma	*SS18-SSX*
Mesenchymal chondrosarcoma	*HEY1-NCOA2*
Extraskeletal myxoid chondrosarcoma	*EWSR1-NR4A3* *TAF15-NR4A3* *TFG-NR4A3* *TCF12-NR4A3*
Myoepithelial tumor	*EWSR1-POU5F1* *EWSR1-PBX1* *EWSR1-ZNF444*

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is a very sensitive technique for the detection of pathogenic translocations, even in case of only small amounts of tumor cells. Since a fusion gene is formed, the presence of its fusion transcript confirms the translocation. From a technical point of view primer design can be challenging, especially when applied to RNA isolated from FFPE tissue, as fusion genes can show different breakpoints, which might result in multiple chimera transcripts. In addition, some tumors harbor alternating fusion partners, exemplified by Ewing sarcoma. Although in approximately 85% of the tumors an *EWSR1-FLI1* fusion is present, the fusion of *EWSR1* with other members of the ETS family has been illustrated in addition to the rare substitution of *EWSR1* by *FUS* [13]. Fortunately, the availability of a multiplex primer set in one PCR reaction could overcome this problem. Due to the incompatibility with decalcified material and the advances of new molecular techniques, RT-PCR is slowly being expelled in both research setting and routine practice.

Next-generation sequencing (NGS)

Sequencing is the process of determining the precise order of nucleotides within a DNA molecule. While Sanger sequencing has been widely used in the past, NGS using different platforms allows high-throughput sequencing with production of enormous data [14]. This has led to many NGS-based studies, resulting in the unravelling of the molecular landscape of diverse bone tumors. With the use of whole genome sequencing rearrangements of *GRM1* were revealed in chondromyxoid fibroma [15] as well as distinct point mutations in *H3F3A* and *H3F3B* in respectively giant cell tumor of bone and chondroblastoma [16]. Furthermore, using whole transcriptome sequencing *FOS* rearrangements were identified in epithelioid hemangioma of bone [17, 18] and a *WWTR1-CAMTA1* fusion was found in epithelioid hemangioendothelioma (**Table 2**) [19]. The latter findings are useful in the differential diagnosis of vascular tumors, as morphology can show much overlap and distinction is necessary given the differences in biological potential and clinical course.

Although whole genome and whole exome sequencing as well as whole transcriptome sequencing are widely used as a research tool, it is not yet feasible to sequence large numbers of complex genomes in their entirety in routine practice. Therefore targeted enrichment strategies are used during library preparations, so only portions of the genome of interest are sequenced. This strategy can be used for the detection of specific pathogenic somatic hotspot mutations, such as *IDH1/IDH2* mutations in central chondrosarcoma [20], *GNAS* in fibrous dysplasia [21] and *BRAF* in Langerhans cell histiocytosis [22]. For the detection of translocations an innovative targeted enrichment method termed anchored multiplex PCR (AMP) has been developed [23]. This method is admissible for FFPE material and utilizes both gene specific adapters and universal adapters, resulting in detection of gene

rearrangements without prior knowledge of the fusion partners [23]. The usefulness of this method in routine diagnostics was recently evaluated for bone and soft tissue tumors, and it showed superior results compared to RT-PCR and FISH [24]. Also, this method led to the discovery of novel fusion partners for *USP6* in primary aneurysmal bone cyst [25] and nodular fasciitis [26]. Its limitation is that novel fusions involving genes outside the selectively captured gene regions will not be revealed.

Immunohistochemistry

Until relatively recently, the additional value of immunohistochemistry in bone tumors was rather limited in daily practice, and most tests lacked specificity. With the identification of novel specific recurrent alterations, for which mutation specific antibodies were developed, this mutation specific immunohistochemistry is rapidly being introduced in the field. Immunohistochemistry is a relatively easy and inexpensive technique, which even can be applied to severely decalcified material. For example, nuclear staining of CAMTA1 can be used as a diagnostic marker for classic epithelioid hemangioendothelioma [27], while nuclear TFE3 staining can suggest the diagnosis of a rare morphological variant of epithelioid hemangioendothelioma, characterized by a YAP1-TFE3 fusion [28]. Specific antibodies for detection of distinct point mutations are available for giant cell tumors of bone (H3F3A G34W) [29], and chondroblastoma (H3F3B K36M) (**Figure 1**) [30]. In cartilaginous tumors the *IDH1* R132H mutation can be detected using immunohistochemistry [31], although this specific mutation is much less common in comparison to the R132C mutation, for which no commercial antibody exists [32].

Molecular mechanisms in bone tumors

There are fifty-eight different histological subtypes of bone tumors according to the WHO classification of bone and soft tissue tumors [33]. At the molecular level, they can be roughly divided into two groups (**Table 2**, **Figure 2**). One group contains tumors with a relatively simple karyotype with specific translocations, mutations or amplifications. Chromosomal translocations in bone tumors represent an early event in tumorigenesis. There are three molecular mechanisms by which translocations cause tumorigenesis: 1. formation of a chimeric gene, of which the fusion transcript acts as an aberrant transcription factor (e.g., *EWSR1-FLI1*), causing transcriptional deregulation; 2. promoter swap, in which a promoter of a gene that is normally highly expressed in bone, is fused to the coding sequence of another gene (e.g., *USP6*) causing its upregulation and thereby altered signaling; and 3. disruption of a specific gene, causing inactivation or altered function of the gene (e.g., *FOS*). Likewise, specific hotspot mutations can change the transcriptional program when occurring in genes causing epigenetic changes (e.g., *H3F3A/B, IDH1/2*). Moreover, hotspot mutations or amplifications can also cause aberrant signaling by activating oncogenes (e.g., *GNAS, BRAF, MDM2*).

Table 2. Molecular subclassification of bone tumors

Mechanism	Examples	Molecular alteration
Simple karyotype		
• Deregulated transcription		
Translocations resulting in chimeric transcription factors	Ewing sarcoma	*EWSR1-FLI1* *EWSR1-ERG* *EWSR1-ETV1* *EWSR1-ETV4* *FUS-FEV* *FUS-ERG*
	Epithelioid hemangioendothelioma	*WWTR1-CAMTA1*
Mutations causing epigenetic changes	Chondroblastoma	*H3F3B*
	Giant cell tumor of bone	*H3F3A*
	Enchondroma	*IDH1/IDH2*
• Altered function		
Translocations resulting in an altered structure of the protein	Epithelioid hemangioma	*FOS*-deletion through various fusion partners
	Osteoblastoma	
• Altered signaling		
Translocations resulting in gene overexpression	Aneurysmal bone cyst	*CDH11-USP6* *TRAP150-USP6* *ZNF9-USP6* *OMD-USP6* *COL1A1-USP6* *E1F1-USP6* *RUNX2-USP6* *PAFA1BA-USP6* *CTNNB-USP6* *SEC31A-USP6* *FOSL2-USP6* *STAT3-USP6*
	Chondromyxoid fibroma	*COL12A1-GRM1* *TBL1XR1-GRM1* *BCLAF1-GRM1*
	Atypical epithelioid hemangioma	*ZFP36-FOSB*
Amplifications / gains	Low-grade (parosteal and intramedullary) osteosarcoma	12q13-15, including *MDM2* and *CDK4*
Activating mutations	Fibrous dysplasia	*GNAS*
	Langerhans cell histiocytosis	*BRAF, ARAF, MAP2K1* and *MAP3K1*
Complex karyotypes		
Multistep progression	Chondrosarcoma	Several non-specific alterations
Chromothripsis	Osteosarcoma	Several non-specific alterations
	Chordoma	Several non-specific alterations

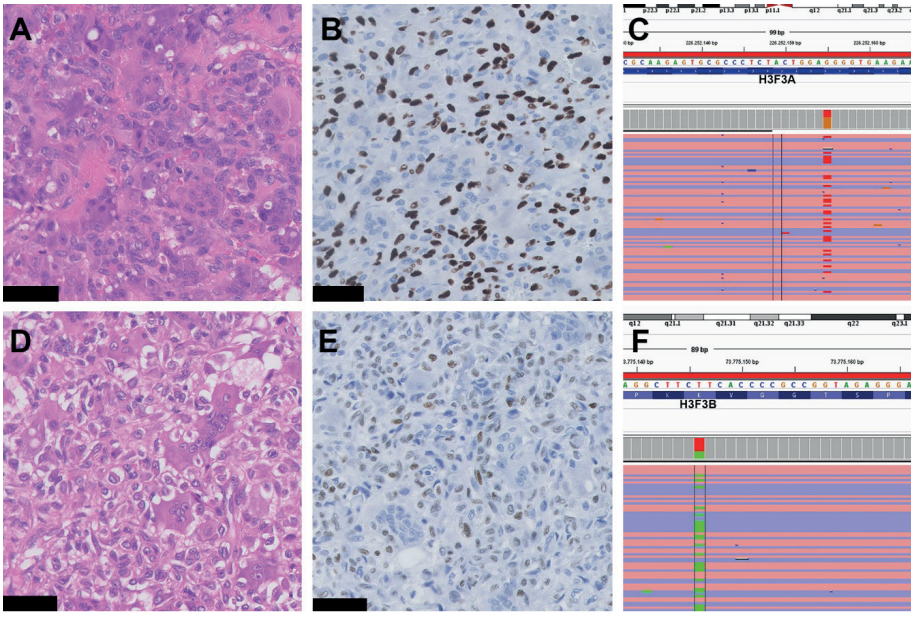

Figure 1. Giant cell tumor of bone (H&E), showing mononuclear cells admixed with giant cells (**A**). Nuclear staining with H3F3 G34W mutant antibody, highlighting the mutant mononuclear cells, while the giant cells are negative (**B**). Next-generation sequencing (NGS) data showing the point mutation in *H3F3A* c.103G>T, p.Gly34Trp (**C**). Chondroblastoma (H&E) showing small chondroblast-like cells admixed with giant cells, and focal matrix deposition. Note the morphological overlap with giant cell tumor of bone (**D**). Nuclear staining with H3F3 K36M mutant antibody staining the mutant chondroblast-like tumor cells (**E**). NGS data showing the point mutation in *H3F3B* c.110A>T, p.Lys36Met (**F**). Each scale bar corresponds to 50 µm.

The other group of bone tumors harbor a complex karyotype with non-specific multiple molecular alterations (**Table 2**). This complex genome can be achieved gradually during tumor progression (e.g., multistep progression model in chondrosarcoma) or can arise in one single cell division (e.g., chromothripsis in osteosarcoma). This section will discuss these different molecular mechanisms involved in bone tumorigenesis illustrated by their prototypical examples.

Simple genome, reciprocal translocation with deregulated transcription: Ewing sarcoma

Formation of a chimeric gene has been extensively studied in Ewing sarcoma, a morphologically small, round cell sarcoma, which has been characterized by recurrent balanced translocations involving *EWSR1* or *FUS* with members of the transcription factor family ETS (**Figure 2A** and **2B**). Both *EWSR1* and *FUS* genes encode RNA-binding proteins with similar amino-acid sequences and are considered as members of the TET family. In approximately 85% of the Ewing

sarcomas, the N-terminal part of EWSR1, with a highly potent transactivation domain, fuses together with the C-terminal segment of FLI1 that encodes the DNA-binding domain [34]. In the remaining cases, fusion of *EWSR1* with other members of the ETS family (e.g., *ERG*, *ETV1*, *ETV4* or *FEV*) has been described in addition to the rare substitution of *EWSR1* by *FUS* [13]. The chimeric gene causes deregulated transcription, as the EWSR1-ETS fusion protein acts as an aberrant transcription factor [5]. Its target genes were shown to be involved in sustaining proliferative signaling (upregulation of *PDGF-C*, *CCDN1*, *c-MYC*) and angiogenesis (VEGF), the evasion of growth suppressors (downregulation of TGF-β and cyclin-dependent kinase inhibitors) and apoptosis (downregulation of *IGFBP-3*), replicative immortality (upregulation of *hTERT*), invasion and metastasis (matrix metalloproteinases) [5], all well-known hallmarks of cancer.

In routine diagnostics detection of one of these specific recurrent translocations found in Ewing sarcoma can be of help in distinguishing it from other "round cell tumors", as these tumors do not harbor this translocation (**Table 1**) [6, 7, 12, 35-37]. However, the diagnosis should always be made within the appropriate clinical, histomorphological and immunohistochemical context, as for instance acute myeloid leukemia also can harbor a *FUS-ERG* translocation [38].

Simple genome, reciprocal translocation with overexpression: aneurysmal bone cyst (ABC), chondromyxoid fibroma and atypical epithelioid hemangioma

Altered signaling can be caused by the exchange of regulatory control elements without affecting the coding sequence of the target gene, for instance by promoter swapping. Primary aneurysmal bone cysts are locally aggressive and rapidly growing cystic bone tumors. The first recurrent chromosomal translocation described in this tumor involved the promoter region of the osteoblast cadherin gene *CDH11* and the entire coding sequence of the ubiquitin-specific protease *USP6*, resulting in overexpression of *USP6* due to juxtaposition to the highly active *CHD11* promoter [39]. Subsequently, several alternative fusion partners were found for *USP6*, substituting the promoter region of *USP6* [40]. The role of *USP6* in ABC has been slowly elucidated, as studies have shown that *USP6* induces matrix metalloproteinase production via activation of NF-κB, leading to osteolysis, inflammation and high degree of vascularization, all morphological features of ABC [41].

It has been noticed that the solid variant of ABC, lacking prominent cystic formation, showed identical morphological features as giant cell lesions of the small bones [42] and giant cell reparative granuloma of the facial bones, skull and jaw [43]. After the discovery of *USP6* rearrangements in ABC a follow-up study illustrated the pathogenetic relationship between ABC and giant cell lesions of the small bones,

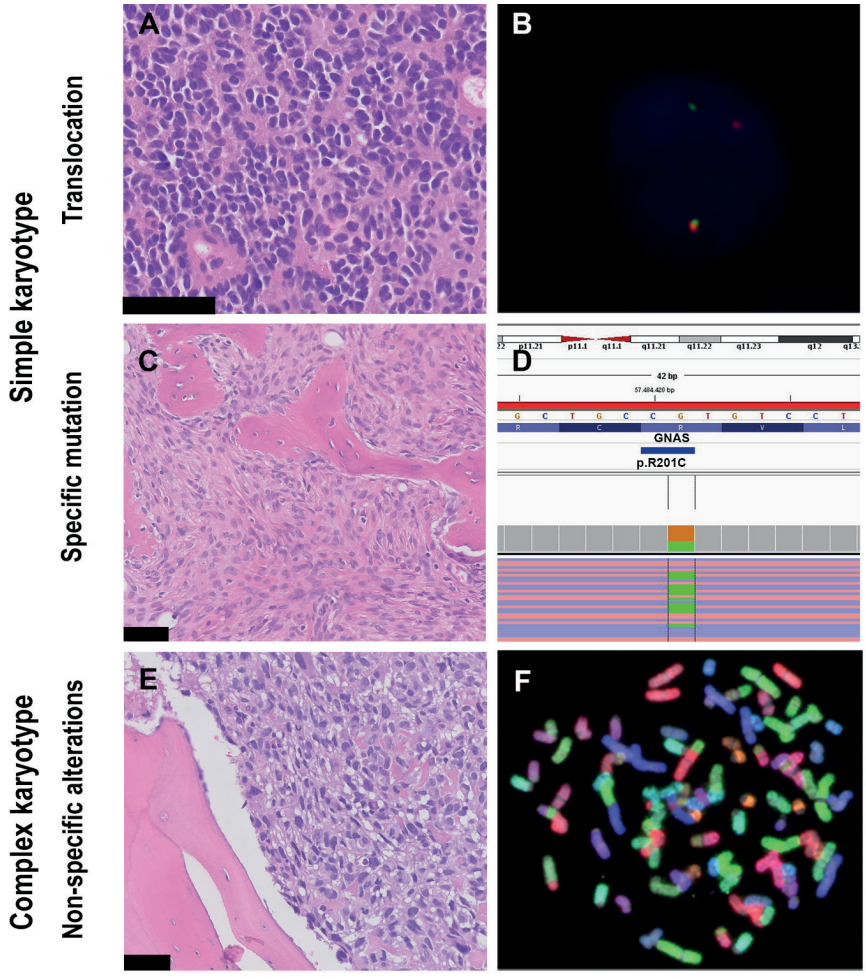

Figure 2. As a conceptual framework, molecular alterations of bone tumors can be divided into two categories: tumors with a simple or complex karyotype. Simple karyotypes include recurrent translocations, such as predominantly seen in round cell tumors (Table 1). The prototypical example is Ewing sarcoma (**A**), harboring fusions with mostly *EWSR1* and ETS-family members, as can be demonstrated using fluorescence *in-situ* hybridization (FISH) with split-apart probes flanking *EWSR1* showing distantly located red-green signals, indicating the presence of a break in the *EWSR1* locus (**B**). Bone tumors carrying specific point mutations also fall into the category of simple karyotypes. This can be exemplified by fibrous dysplasia, which shows bland fibroblastic spindle cells with areas of woven bone on H&E staining (**C**). Next-generation sequencing (NGS) can be used to demonstrate the specific point mutation in *GNAS* c.602G>A, p. Arg201His (**D**). Tumors with complex karyotypes lack specific alterations that can be used in routine diagnostics. A prototypical example includes conventional osteosarcoma, which shows on H&E staining atypical hyperchromatic cells producing tumor osteoid (**E**). COBRA-FISH illustrates a complex chaotic karyotype, with numerous regions of amplification and deletions, combined with many translocations (**F**). Each scale bar corresponds to 50 µm.

as the latter also harbor translocations involving *USP6*. Translocations were absent in giant cell reparative granuloma of the facial bones, skull and jaw, suggesting a different pathogenesis [44]. For pathologists the differential diagnosis of ABC can be difficult, as it can also present as a solid mass and resemble numerous osteoclast-like giant cell containing bone tumors (e.g, chondroblastoma and giant cell tumor of bone) with overlapping morphological features. Although classic histomorphology remains at the cornerstone of the diagnosis, identifying the specific *USP6* rearrangement can be of help in distinguishing ABC from its mimics.

A promotor swap has also been identified to underlie the development of chondromyxoid fibroma, a very rare locally aggressive bone tumor, causing upregulation of *GRM1* [15]. It is so far unknown how overexpression of *GRM1* causes bone tumor formation. Another example includes the *ZFP36-FOSB* fusion in atypical epithelioid hemangioma, causing upregulation of FOSB, as the DNA binding domain of FOSB remains intact and only 7 amino acids of ZFP36 are retained in the chimeric protein [45].

Simple genome, reciprocal translocation with altered protein structure: epithelioid hemangioma and osteoblastoma

In the past, it was widely assumed that truncation of a protein would result in loss of function. Recently, different translocations all involving *FOS* were identified in 59%-72% of epithelioid hemangioma of the bone [17, 18], a vascular neoplasm with well-formed vascular channels lined by epithelioid endothelial cells. The *FOS* gene is a member of the FOS family and encodes a transcription factor that can form dimers with members of the Jun family. These form the main components of the activating protein-1 (AP-1) complex, which regulates numerous processes relevant in tumorigenesis. All translocations found in epithelioid hemangioma of the bone affected the 3' end of the *FOS* gene, leading to a mutant protein that lacks a highly conserved helix consisting of the C-terminal four amino acids of FOS. This mutant protein is indispensable for fast, ubiquitin-independent FOS degradation, and as FOS stimulates endothelial sprouting, disrupted degradation of mutant FOS protein could account for the abnormal vessel growth in epithelioid hemangioma [17, 46]. Also, recurrent rearrangements of *FOS* and its paralogue *FOSB* were recently found in osteoblastoma. Whereas the *FOSB* translocation led to substitution of the promoter, tumorigenesis in *FOS* translocated osteoblastoma is probably stimulated by the same mechanism of disrupted degradation of FOS [47].

Simple genome, specific mutation causing epigenetic changes: chondroblastoma, giant cell tumor of bone and enchondroma

Specific gene mutations are only described in a few bone tumors of which p.Lys36Met alteration in *H3F3B*, has been recently found in the majority of

chondroblastomas. Interestingly, alterations in exclusively *H3F3A* have been seen in giant cell tumor of bone, leading to mainly p.Gly34Trp [16]. Both are locally aggressive bone tumors containing giant cells and affecting relatively young patients, but with different morphology and clinical behavior. These genes are located on respectively chromosomes 17 and 1, and both encode histon H3.3 proteins with identical amino acid sequence [48]. In chondroblastoma, the specific p.Lys36Met alteration causes epigenetic changes by reducing *H3K36* methylation. It thereby promotes tumorigenesis due to possible alterations of several cancer-related processes including colony formation, apoptosis and chondrocyte differentiation [49]. These mutations have rapidly made their way into routine diagnostics, either by mutation detection using Sanger sequencing [50] or targeted NGS [51]. Sensitivity depends on the technique used, ranging from ~70% using conventional Sanger sequencing to ~100% using the much more sensitive targeted NGS, which is caused by the relatively high amount of non-mutated cells (e.g., the giant cells) in these tumors. More recently, the use of mutation specific H3F3A G34W and H3F3B K36M antibodies for immunohistochemistry have shown great potential to strongly improve diagnostic accuracy for giant cell containing bone tumors (**Figure 1**) [29, 30] .

Another specific mutation causing epigenetic changes is described for enchondroma, a benign cartilage tumor in the medullary cavity. Heterozygous mutations in *IDH1* and *IDH2* are present in half of the solitary enchondromas, and in about 80%-90% of individuals with Ollier disease (multiple enchondromatosis) [31, 52]. Mutations in *IDH1* and *IDH2* cause the enzyme isocitrate dehydrogenase to acquire new enzymatic activity converting α-ketoglutarate to D-2-hydroxyglutate (D2HG), an oncometabolite which accumulates in IDH mutant cells. D2HG inhibits α-KG-dependent enzymes involved in epigenetic regulation, collagen synthesis and cell signaling [53]. Furthermore, it has been shown that mutant IDH or D2HG causes persistence of hypertrophic chondrocytes, giving rise to rests of growth-plate cells that persist in the bone as enchondroma [54] and that increased levels of D2HG promote chondrogenic over osteogenic differentiation, causing development of benign cartilaginous tumors [55].

Simple genome, mutations causing altered signaling: fibrous dysplasia and Langerhans cell histiocytosis

In fibrous dysplasia activating mutations in *GNAS* are present in up to 93% of the cases [21] (**Figure 2C** and **2D**). This gene is located on chromosome 20 and encodes the α-subunit of the stimulatory G-protein (GSα). In the basal state, stimulatory G-Protein (GS) is a heterodimer composed of GDP-bound GSα and a βγ heterodimer. After activation of the ligand bound receptor, replacement of GDP with GTP results in dissociation of GTP-bound GSα from βγ heterodimer and leads to coupling of hormone receptors to adenylyl cyclase required for intracellular cAMP generation. This mechanism is turned off by an intrinsic GTPase activity, which hydrolyses

bound GTP to GDP. *GNAS* mutations interfere with this GTP-ase inactivation mechanism, resulting in prolonged activation of GSα and its downstream effectors [56]. *GNAS* mutations are also present in McCune Albright syndrome, characterized by polyostotic fibrous dysplasia, café au lait pigmentation and endocrinopathies and non-skeletal isolated endocrine lesions. This suggests a spectrum of phenotypic expression of the same basic disorder, likely reflecting different patterns of somatic mosaicism [57].

In daily practice, identification of *GNAS* mutations with targeted NGS is of diagnostic value in the differential diagnosis of fibrous dysplasia, which includes osteofibrous dysplasia, ossifying fibroma and low-grade fibroblastic osteosarcoma. Whereas morphological findings may show overlap, no *GNAS* mutations were found in ossifying fibroma and osteofibrous dysplasia [58] and the percentage of *GNAS* mutations in low-grade fibroblastic osteosarcoma seems low to absent [59, 60].

Another lesion that can present in bone and is caused by altered signaling is Langerhans cell histiocytosis, a rare proliferative disorder of epidermal antigen-presenting cells, admixed with inflammatory cells. In ~50%, an oncogenic *BRAF* V600E mutation is present [22]. *BRAF* V600E mutations can also be found in Erdheim-Chester disease, a xanthogranulomatous histiocytosis involving numerous tissues, including bone [61]. *BRAF* is a proto-oncogene within the RAS-RAF-MEK-ERK pathway that involves the regulation of proliferation, differentiation and cell survival [62]. The mutation converts BRAF into its active conformation that leads to direct phosphorylation and activation of MEK and the downstream signaling pathway [63]. In the remaining 50% of the cases, other genes involved in the same pathway can be mutated, including *ARAF*, *MAP2K1* and *MAP3K1* [64].

Simple genome, amplification causing oncogene activation: parosteal and low-grade intramedullary osteosarcoma

Non-specific amplifications are often found in bone tumors and usually involve the long arm of chromosome 12 (12q13-15), including, amongst others, the proto-oncogenes *CDK4* and *MDM2*. *CDK4* encodes for cyclin-dependent kinase 4, which leads to phosphorylation and thereby inactivation of *RB1*, whereas MDM2 protein targets p53 for ubiquitination and degradation by the proteasome [65, 66]. Although these events are also seen in some other sarcomas, it can be used to diagnose parosteal and low-grade intramedullary osteosarcomas. These tumors are characterized by a simple karyotype with supernumerary ring chromosomes comprising amplification of *CDK4* and *MDM2* [8, 9], whereas their morphological mimickers (e.g., non-ossifying fibroma, desmoplastic fibroma and fibrous dysplasia) do not harbor these amplifications. Therefore, immunohistochemical staining for MDM2 and CDK4, or FISH, were shown to be of additional value as an auxiliary diagnostic tool in this differential diagnosis [67].

Complex genome, multistep progression: chondrosarcoma

The multistep progression model has been well known for epithelial cancers, especially colorectal cancer, and shows the malignant transformation of a normal cell through sequential accumulation of multiple genetic changes [68]. Normal cells are continuously exposed to internal and external mutagens, leading to DNA damage. Although most of the damage is repaired, a small fraction may be converted into fixed changes that eventually lead to activation of several oncogenes and the loss of tumor suppressor genes [69]. The multistep development of cancer with accumulation of genetic changes will result in the gradual acquisition of the hallmarks of cancer [70].

In bone tumors, this multistep progression model is assumed for secondary central chondrosarcomas, which arise from an enchondroma. Progression towards a low-grade secondary central chondrosarcoma is an infrequent event in solitary enchondromas, but is significantly higher in Ollier disease [71]. Although low-grade central chondrosarcomas barely show molecular alterations besides *IDH1/IDH2* mutations, high-grade chondrosarcomas have a far more complex karyotype. Besides mutations in *COL2A1* and *TP53* [72], amplification of 12q13, which includes *MDM2* and *CDK4* and loss of 9p21 (including *CDKN2A*) are described, leading to alterations in the *TP53* and *RB1* pathway, which emphasize their role in tumor progression [73].

A substantial portion of both primary and secondary central chondrosarcoma harbors *IDH1* (R132C, R132G, R132H, R132L, R132S) or *IDH2* (R172S) mutations [20, 52]. This can be useful for example in the distinction of central chondrosarcoma from chondroblastic osteosarcoma [74]. Furthermore, another diagnostic dilemma can arise when only the high-grade non-cartilaginous sarcoma component of dedifferentiated chondrosarcoma is present in a small biopsy. This non-cartilaginous component often has the appearance of an undifferentiated pleomorphic sarcoma or an osteosarcoma that would require different treatment procedures. The presence of an *IDH1/IDH2* mutation, found in ~50% of dedifferentiated chondrosarcomas [20, 31] can be very helpful. Use of Sanger sequencing or targeted NGS seems to be of primary choice in detection of *IDH1/IDH2* mutations, as only 4~17% of the *IDH1* mutations can be identified using the specific IDH1 R132H antibody [20, 31]. Currently, there is no commercially available antibody that includes the most common (~65%) *IDH1* R132C mutation seen in central chondrosarcoma [75, 76].

Complex genome, non-specific alterations and chromothripsis: osteosarcoma

A classic example of a bone tumor which shows a complex chaotic karyotype is conventional high-grade osteosarcoma. This osteoid producing bone tumor affecting adolescents and young adults is characterized by an instable genome with numerous recurrent regions of amplification and deletions, combined with many translocations involving both oncogenes and tumor suppressor genes

(**Figure 2E** and **2F**). This is illustrated by a study that identified a total of 10,806 structural variations (SV) and 50,426 validated somatic sequence mutations in 34 pediatric osteosarcoma samples. In half of the cases hypermutable regions with the hallmarks of kataegis were identified [77]. Non-specific recurrent amplification and DNA copy number gains have been detected at several distinct chromosomal regions, such as 6p12-p21 (including *RUNX2*, relevant in terminal osteoblast differentiation) and 8q (including *MYC*) [8]. Recurrent loss is less often described and includes for example region 3q13 [78]. The most frequently altered gene is *TP53*, where besides mutations predominantly translocations were found confined to the first intron of the gene. Another frequently mutated gene is *RB1*, which plays a role in controlling cell cycle signaling [8, 77]. A more recently conducted study of 112 osteosarcomas also showed a diverse molecular landscape with a median of 38 mutations per tumor. Further analysis of the rearrangements showed three patterns of cytogenetic configurations. In 30% of the cases the genomic landscape of chromothripsis was identified [79], which is in line with a previous conducted study [80]. This phenomenon of chromothripsis was postulated as an alternative mechanism for tumor development as a counterpart for the multistep progression model [80] and is characterized by dozens to hundreds of clustered rearrangements involving only one or few chromosomes. Other features include alternating regions of copy number loss and copy number gain and aberrant stitching of chromosomes after presumed scattering of chromosomal fragments [68, 80]. Across many cancer subtypes approximately 2%-3% is characterized by chromothripsis, but in osteosarcomas and chordomas this event seems to occur more often. The second pattern, characterized by a distinct copy number pattern of chromothripsis and amplification, was found in 59% of the cases. The remaining and minority of the cases (11%) showed few or no rearrangements [79]. Although the molecular alterations in osteosarcoma are being slowly elucidated, the alterations thus far found are non-specific, and therefore at present no molecular diagnostic tools to assist histological evaluation are available.

Conclusion

The advances of molecular techniques have accelerated the unravelling of the molecular landscape of bone tumors. It has helped in understanding the mechanisms in tumorigenesis and in providing a conceptual framework to understand bone tumorigenesis. Furthermore, the many examples discussed above illustrate that specific recurrent alterations are useful as a diagnostic marker in the differential diagnosis of bone tumors that are often found difficult by pathologists. Recently, mutation specific immunohistochemical stainings have become available facilitating molecular testing and improving diagnostic accuracy. Given the rapidity of this changing field, it will be a matter of time for the discovery of other specific alterations in the remaining group of bone tumors and hopefully diagnostic tools as well as therapeutic targets will be further disclosed.

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