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**Author:** Verbeke, Sofie Lieve Jozef  
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Summary and future perspectives
Introduction

Chapter 1 emphasizes the need for more specific morphological, immunohistochemical and/or molecular tools to support the classification of the different vascular tumours of bone. Because vascular tumours of soft tissue are more common and more easily accessible for research, these have been better delineated at the morphological as well as molecular level over the past years. Vascular tumours of bone need decalcification for adequate routine histological analysis, which has a severe negative impact on DNA quality. It is still unclear whether vascular tumours of bone are comparable to their soft tissue counterparts simply differing in location, or whether they should be regarded as different entities with their own molecular profile and clinical behaviour.

Chapter 2 summarizes all different histological types of vascular tumours of bone based on radiological imaging, histology and their genetic profile, as far as available, before the WHO classification 2013. Based on the available literature a classification scheme was proposed, which was later on adopted in the 2013 WHO classification.

Characterization of angiosarcoma of bone based on morphology

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

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

In Chapter 4 we characterize angiosarcomas of bone based on protein expression of a large panel of oncogenes, tumour-suppressor genes, and signalling molecules. In addition, hotspot mutation analysis of PIK3CA, KRAS and BRAF was performed. In this thesis we demonstrate
that in more than half of the angiosarcomas of bone the Rb pathway is disrupted, either by loss of expression of CDKN2A or by overexpression of Cyclin D1. Unlike angiosarcoma of soft tissue, the TP53 pathway seems of no importance in angiosarcoma of bone. Disruption of the Rb pathway would suggest that angiosarcomas belong to the category of sarcomas with complex genetic profiles. In addition, we demonstrate that the TGF-beta pathway is highly active in angiosarcoma of bone and may play a role in tumourigenesis. This finding also suggests therapeutic options for angiosarcoma of bone, since inhibitors of the TGF-beta pathway are available. We also demonstrate that the PI3K/Akt pathway is active in angiosarcoma of bone and soft tissue, but differences in protein expression suggest different mechanisms of activation. In angiosarcoma of bone this could be explained by the decreased expression of PTEN in nearly half of the tumour samples, compared to 7% in angiosarcoma of soft tissue. Although a single case concerning an angiosarcoma of the liver has been reported to contain a PTEN gene mutation, it is so far unclear whether PTEN mutations are present in angiosarcoma of bone. It is known that genetic aberrations in the PI3K/Akt pathway, such as PIK3CA mutations, have the potential to cause malignancies. The possible role of PI3K/Akt pathway in angiosarcoma of bone provides a rationale for the use of PI3K/Akt pathway inhibitors although future studies are needed to reveal its value. In angiosarcoma of soft tissue the tyrosine kinase receptor KIT is overexpressed in 90%, compared to only 17% in angiosarcoma of bone. Although there is one report of a very good response to imatinib (Glivec) in angiosarcoma of soft tissue, no KIT or PDGFRA mutations have been detected in angiosarcoma of soft tissue.

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

Formic acid based decalcification which is routinely used in daily diagnostics in most pathology labs for tissues containing bone decreases the quality of the DNA by degrading it. The length of DNA fragments obtained from these samples are often less than 150–200 base pairs, hampering molecular testing. In order to characterize our retrospective series of angiosarcomas of bone, mostly consisting of decalcified FFPE blocks, we had to circumvent this technical hurdle. We therefore first optimized and validated the array CGH technique on various heavily decalcified formalin-fixed, paraffin embedded bone tumours of multiple institutions in Chapter 5. In this thesis we use oligonucleotide based array chips containing reporter elements of ~60 base pairs. Because enzymatic labelling can cause further fragmentation of the DNA, two different labelling techniques are tested. The first is a direct chemical labelling (Universal Linkage System) without further fragmentation of the DNA, while the second method is a commercially available random primer labelling kit especially developed for formalin-fixed, paraffin embedded tissue derived DNA. Both labelling methods show a good reproducibility and show similar results by using 500ng starting material. Furthermore, the DNA concentrations are measured by two independent methods: absorption-based DNA concentration measurements and ethidium bromide stained gel-imaging. We demonstrate that the estimation of the DNA concentration is more important for successful testing than the quality (fragment size) of DNA obtained from formalin-fixed, paraffin embedded tissue.
After optimization, in Chapter 6 we applied this technique to create genomic profiles of the first large series of angiosarcomas of bone in comparison to a small group of angiosarcomas of soft tissue. In this study we demonstrate no evident molecular genetic difference between angiosarcoma of bone and soft tissue. In contrast, we identify two groups of angiosarcomas: angiosarcomas with a complex genetic profile and a second group of angiosarcomas with only few genetic aberrations. Although previous results in this thesis indicate a possible role of the Retinoblastoma (Rb) pathway and/or TP53 pathway, here we show no correlation between deregulation of the TP53/Rb pathway and a complex karyotype. One angiosarcoma of soft tissue demonstrates the presence of MYC amplification, without any history of radiation therapy or chronic lymphedema, and a high level of amplification at region 5q, containing the FLT4 and MAPK9 (JNK2) gene. We show high protein expression of MAPK9 (JNK2) in the vast majority of angiosarcomas (bone and soft tissue), irrespective of whether genomic amplification of this region was present or not. A possible role of JNK2 has not been described yet. However, it is known that TGF-beta can activate JNK2. Thus we hypothesize that the presence of JNK2 expression can be explained by the high expression of TGF-beta, which we demonstrate previously in Chapter 4. Another angiosarcoma of bone shows an amplicon at region 1p36, amongst others containing SKI (Sloan Kettering Institute proto-oncoprotein). SKI is not only involved in many signalling pathways (its most well known function is to negatively regulate TGF-beta signalling), but can also promote cancer progression and is highly expressed in different human solid tumours. Here we demonstrate high expression of SKI in both angiosarcoma of bone and soft tissue. Although previous reports suggest that overexpression of SKI plays a role in tumour growth and angiogenesis, its exact role in angiogenesis still remains unclear. Although the threshold for recurrent aberrations (25%) is not reached within our series, three angiosarcomas show a high level of amplification of chromosome 2q and 17q. High level amplification of 17q23 has been described in many tumour types, and especially in breast carcinomas it is suggested to be associated with tumour progression and poor prognosis. To date, high level amplification of 2q32-34 has not been described yet.

**Haemangiopericytoma of bone: a true entity?**

Since the 2002 World Health Organisation Classification of Tumours of Soft Tissue and Bone, haemangiopericytoma of soft tissue is no longer recognized as a separate entity and it has been accepted that these lesions should be classified as solitary fibrous tumour, monophasic synovial sarcoma, and (infantile) myofibromatosis or myofibroblastic lesions. In Chapter 7 we again exploit parallels between vascular tumours of bone by comparison with their soft tissue counterpart, by studying a relatively large series of cases previously diagnosed as “haemangiopericytoma of bone”. Based on histological review, two principal morphological patterns are recognized: one group of six tumours demonstrate a patternless architecture and varying cellularity with monomorphic tumour cells with bland nuclei that do not overlap and have a limited amount of pale eosinophilic cytoplasm with indistinct cell borders, consistent with the morphology of solitary fibrous tumours. A second group of three tumours are characterized by a fascicular arrangement of uniform non-pleomorphic spindle cells with a sparse amount...
of cytoplasm, nuclear overlap and indistinct cell borders, consistent with the morphology of synovial sarcoma. These histological findings could in nearly all cases be confirmed by either immunohistochemistry and/or molecular testing by SS18-FISH. At the time of evaluation of these tumours, no specific genetic aberration and/or specific immunohistochemical marker was known to support the diagnosis of solitary fibrous tumour. Recently, a specific NAB2-STAT6 gene fusion product has been described in solitary fibrous tumour of soft tissue and brain. Moreover, an antibody directed against STAT6 is commercially available and proven useful to support the diagnosis of solitary fibrous tumour, as due to the fusion with NAB2, STAT6 which is normally localized in the cytoplasm, translocates to the nucleus. In this thesis, we confirm that similar to its soft tissue counterpart, haemangiopericytoma in bone also merely represents a growth pattern rather than a true entity. Therefore, STAT6 immunohistochemistry and/or SS18 FISH should be used in the diagnostic work-up of spindle cell tumours of bone displaying a haemangiopericytomatosus vascular pattern.

**Future perspective**

In this thesis we delineated the most malignant part of vascular tumours of bone, more specific angiosarcoma of bone. These are extremely rare tumours for which the collaboration with multiple different institutions is needed. Due to the partnership within the EuroBoNeT consortium, a European Commission granted Network of Excellence for studying pathology and genetics of bone tumours that stimulates and promotes the multicentric collaboration within Europe, this research was possible. Although we could identify in Chapter 3 three high-risk histological parameters that correlated with poor prognosis, not all angiosarcomas exhibit all three histological features and a small portion of these tumours have a different, even better, clinical course. To date, however, there is no evidence or supporting data that low-grade angiosarcoma or haemangioendothelioma of bone is a distinct or truly existing entity. Array-CGH analysis, performed in Chapter 6, could only identify two subgroups of angiosarcoma (with or without a complex genetic profile) which did not correlate with prognosis. Therefore, the results of the array-CGH do not provide diagnostic or prognostic markers that can assist in the classification of angiosarcoma of bone or that can distinguish angiosarcoma of bone with a poor prognosis (2-years survival 0%) from angiosarcoma of bone with a slightly better prognosis (5-years survival 33%). However, balanced genomic rearrangements, such as balanced translocations and inversions, and point mutations in the DNA are not detected by array-CGH. More recent molecular studies of distinct vascular entities, such as epithelioid haemangioendothelioma, pseudomyogenic haemangioendothelioma and even epithelioid haemangioma, have shown the presence of a specific balanced translocation in a vast majority of these tumours. In this perspective, Next Generation Sequencing (NGS) of angiosarcoma of bone would be very interesting; mainly to investigate whether recurrent genetic alterations are present in angiosarcoma of bone and if so, whether these alterations correlate with morphology and clinical outcome. Moreover, these genetic alterations could
elucidate the process of tumourigenesis and subsequently lead to new and better therapeutic options. However to date, this molecular procedure is not optimized for (decalcified) formalin-fixed, paraffin embedded material.

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

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

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


