Chapter 8

Summary and future perspectives
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8.1 Molecular genetics and cytogenetics to classify myxoid tumours of soft tissue

An increasing number of (cyto-) genetic aberrations have been identified in (myxoid) soft tissue tumours during the last decade. This gives a. insight in their underlying biology, b. often provides tools for differential diagnosis, prediction of clinical outcome and potential clues for more targeted therapy (1-3). Chapter 2 gives an overview of the myxoid tumours of soft tissue recognized and their characteristic molecular/cytogenetic aberrations. Roughly, sarcomas can be divided in two major groups: (a) sarcomas with relatively "simple" karyotypes showing specific genetic alterations, such as balanced translocations in about 15% in general, and reaching up till 95% of myxoid liposarcoma with t(12;16) leading to the formation of the FUS/DDIT3 fusion gene or point mutations in a single gene like KIT/PDGFR in GIST (4) and (b) sarcomas with non-specific gene alterations and very complex karyotypes with structural and numerical aberrations, e.g. myxofibrosarcoma (5). Cytogenetic data on myxofibrosarcoma were scarce until now with only isolated cases described in the literature. In chapter 3 we describe a large serie of karyotyped myxofibrosarcoma showing that upon recurrence, there is an increase in tumour-grade (5); as well as, upon increase in grade, myxofibrosarcomas show an increase in non-tumourtype-specific cytogenetic aberrations. Our data suggests that myxofibrosarcoma arises and progresses by genetic instability via a multistep tumour progression model, which is estimated to be the case in about 50% of soft tissue sarcomas (6). The differential diagnosis of myxoid tumours of soft tissue can be difficult especially in biopsies and when presenting as an intramuscular tumour. An example of this is the differential diagnosis between low-grade myxofibrosarcoma and intramuscular myxoma (especially its cellular variant, a.k.a. cellular myxoma) (7, 8). In chapter 5 we show that karyotyping can be helpful to distinguish both entities as intramuscular myxoma (including cellular myxoma) often show no cytogenetic aberrations, whereas low-grade myxofibrosarcoma does (9). However, (molecular) karyotyping is a technique which is not routinely available in most pathology labs, is laborious and has a limited success rate. Therefore, we investigated the potential role of direct mutational analysis of GNAS1 activating mutations which is a more accessible technique and more easily to incorporate in pathology laboratory practice. GNAS1 activating mutations (codon 201 and 227) have previously been described in fibrous dysplasia, a benign bone tumour that can be associated with intramuscular myxoma in the Mazabraud- and McCune-Albright syndromes (10). Scarse cases of intramuscular myxoma in both syndromatic as well as non-syndromatic context showed activating mutations in GNAS1 (11). We confirmed the presence of these mutations in 50% of intramuscular myxoma cases, in a larger series including its cellular variant, which has been confirmed in a similar study (12). We could not detect these mutations in low-
grade myxofibrosarcoma. GNAS1 codon 201/227 mutation analysis is thus a helpful and specific (though not very sensitive) method to distinguish intramuscular myxoma from low-grade myxofibrosarcoma. Activating mutations in GNAS1 lead to consecutive activation of cAMP, increased expression of c-Fos and subsequent transcription of genes involved in cell cycle regulation (13). We confirmed high expression of c-Fos on both the protein and the mRNA level in intramuscular myxoma as well as in low-grade myxofibrosarcoma. Thus immunohistochemistry for c-Fos is not a discriminatory diagnostical adjunct to differentiate between both entities. KRAS codon 12/13 and TP53 mutations were previously shown to be involved in sarcomatogenesis (14). We could not detect mutations in these genes in low-grade myxofibrosarcoma, nor in intramuscular myxoma.

Interestingly, previous immunohistochemical studies showed p53 overexpression in myxofibrosarcoma upon increase in grade, suggesting that TP53 mutations are a relatively late event in the tumourigenesis of myxofibrosarcoma (15). Because of its role in guarding cell cycle regulation, TP53 mutations probably contribute to genomic instability in myxofibrosarcoma, reflected in the increase in non-specific cytogenetic aberrations in higher grades (grade 2 and 3) whereas low-grade (grade 1) myxofibrosarcoma usually has a normal karyotype or only slight cytogenetic aberrations (5).

8.2 Constitution of myxoid extracellular matrix depends on type and grade of tumours

Myxoid tumours of soft tissue are, per definition, characterized by their myxoid ("jelly") extracellular matrix. In chapter 2, we describe how the concept of "myxoid" evolved over the years and still its exact constitution has not been elucidated, 150 years after Virchow introduced the term "myxoid" (16). In this chapter we give an overview of the literature of the high- and low-abundant constituents which have been reported to be present in the myxoid extracellular matrix in both reactive lesions and in tumours (17). We discuss their potential role and conclude that myxoid changes are not tumour specific at all since they can be found in tumours of both mesenchymal and epithelial origin (both benign as well as malignant). In chapter 4 we showed that next to glycosaminoglycans, serum proteins (IgGs and albumin) are major components of the myxoid extracellular matrix. With classical Alcian Blue staining using the CEC (critical electrolyte concentration) method, we demonstrated that the relative amount of these glycosaminoglycans in the myxoid extracellular matrix depends on histological type and grade of the tumour and how this might relate to their different clinical behavior (18). In chapter 5 we studied the myxoid extracellular matrix by liquid chromatography mass spectrometry, a more sophisticated technique not applied before in these tumours. We confirmed the abundant presence of serum proteins and collagens in the myxoid.
extracellular matrix. Above that, we showed that low-grade myxofibrosarcoma contains certain small leucine rich proteins, such as biglycan, decorin, lumican, which we could not detect in intramuscular myxoma (9). We confirmed that decorin, collagen I, VI, XII and XIV were significantly overexpressed in low-grade myxofibrosarcoma (both on the protein and at the mRNA level) compared to intramuscular myxoma. Based on these results we argue that immunohistochemistry for decorin might be a helpful tool in the differential diagnosis between these two entities. Our findings are also interesting from a conceptional point of view, on one hand showing that the myxoid extracellular matrix is characterized by the presence of certain molecules and peptides (such as serum proteins, glycosaminoglycans, collagens etc.). On the other hand, the myxoid extracellular matrix (such as in intramuscular myxoma) is characterized by low expression of decorin and collagens which are important for the well-structured formation of the extracellular matrix (19). This paucity of structural proteins/molecules suggests that a myxoid histology reflects a merely improper organization of the extracellular matrix. In chapter 6 we show that direct profiling of tissue slides by imaging mass spectrometry, is an elegant and robust method to classify myxoid tumours of soft tissue (myxofibrosarcoma and myxoid liposarcoma) according to tumour-type and tumour-grade. Low-grade myxofibrosarcoma is characterized by a multinodular growth pattern on both the macroscopical and microscopical level. Using imaging mass spectrometry, we demonstrate that these histologically identical nodules express different peptides/proteins and thus display intratumour heterogeneity on the biochemical level. We hypothesize that this might be the reflection of clonal selection upon tumour progression in myxofibrosarcoma. With the same technique, we demonstrate that myxoid liposarcoma shows a transition in lipid profiles with decreased fatty acid content upon increase in grade, whereas phosphocholines were predominantly detected in the higher grades. Interestingly, these findings were reported in a different independent group of myxoid liposarcoma by ex vivo NMR spectroscopy (20-22). We speculate that these differences reflect the genetic changes occurring upon tumour progression especially in relation to the role of peroxisome proliferator-activated receptor γ (PPARγ), a key player in adipocytic differentiation. Hereby we illustrate how imaging mass spectrometry can form a bridge between the molecular genetics and the morphological features characteristic of myxoid liposarcoma.

8.3 New therapeutic strategies for myxoid liposarcoma patients with advanced disease

In chapter 5 we describe that activating mutations in exon 201 and 227 of the GNAS1 gene were present in intramuscular myxoma but not in low-grade myxofibrosarcoma. These mutations subsequently lead to consecutive activation of c-AMP and downstream transcription of c-Fos. From a theoretical point of view, targeting this pathway would be an option for targeted therapy, as these upstream activating mutations are highly
specific (despite its low sensitivity of 50%). However, intramuscular myxoma is a benign tumour and curative surgery suffices with no need for (neo) adjuvant therapy (23). In contrast to intramuscular myxoma, myxoid liposarcoma is a malignant soft tissue tumour metastasizing in about 30-80% of cases (24). In this respect, identification of new targets in treatment of liposarcomas makes especially sense because therapeutic options for patients with advanced/inoperable disease are rather limited. Chemotherapeutical options are restricted to ifosfamide and anthracyclins, which have only response rates of 20-40% although trabectidin (ET-743, Yondelis®) has recently shown activity in phase I and II trials and retrospective series (25-28). In chapter 6 we illustrate by imaging mass spectrometry that upon increase in grade, myxoid liposarcomas shows a decrease in the content of fatty acids, which is probably the effect of deceased signaling of PPARγ. Active signaling of the transcriptional activator PPARγ does not only play a key role in adipogenesis but also leads to cell cycle arrest (29, 30). In this perspective, PPARγ signaling might offer a promising target in the treatment of (myxoid) liposarcoma although activation of this pathway by PPARγ agonists (rosiglitazone) in patients with advanced disease was not conclusive (31, 32).

During the last decade, with the unraveling of (aberrant) cell signaling pathways in many cancers, small molecule targeting has been shown to be a promising therapeutic approach (33). This more “rationale” based targeting of cancer cells by (more or less) specifically inhibiting pathways involved in tumourigenesis has been shown to be effective, such as in translocation driven sarcomas and hematological malignancies as well tumours driven by activating mutations, such as in lung cancer and GIST (34). In chapter 7, we showed by in vitro kinome profiling and pathway analysis that Src and NF-kB pathway are active in myxoid liposarcoma cells. We were able to block these pathways by their respective inhibitors dasatinib® and 4,5,6,7-tetrabromobenzotriazole. This led in the case of 4,5,6,7-tetrabromobenzotriazole to a significant decrease in cell growth probably by induction of apoptosis. Interestingly, administration of both drugs had an addidative effect. Our results open perspectives to the development of new therapeutic strategies in the treatment of metastatic, or irresectable myxoid liposarcomas. For myxofibrosarcoma, no direct clues for targeted therapy were obtained, mainly hampered by the lack of myxofibrosarcoma cell lines for performing functional experiments.

### 8.4 Future view

The results as described in this thesis have provided a more profound understanding of the biology of myxoid tumours of soft tissue. With a wide array of techniques ranging from classical Alcian Blue to kinome analysis and imaging and liquid chromatography mass spectrometry of tissue samples, we analyzed myxoid tumours of soft tissue in...
more depth. We showed that the myxoid extracellular matrix differs in composition according to tumour-type and tumour-grade, and how these differences might affect the biology/development of various myxoid tumours of soft tissue. We showed that for the study of the proteome of these tumours, mass spectrometry contributes to a more profound knowledge of high-and low abundant constituents of the myxoid extracellular matrix. An advantage of mass spectrometry is the simultaneous analysis of numerous proteins at the same time without a priori knowledge of these proteins (35). Imaging mass spectrometry adds spatial information of the identified molecules, which is especially interesting as the importance of spatial (next to temporal) protein expression is essential in many (inter) cellular processes both in physiological and pathological conditions (such as cancer) (35). Imaging mass spectrometry allows direct mass spectrometric analysis of tissue sections, which makes trypsinization -essential for subsequent tandem MS/MS experiments- rather challenging and hampers direct identification of m/z values of interest. Future challenge is to overcome this problem, as well as increasing the spatial resolution (yet up to 50 micrometer) to the level of the individual cell, without losing the quality of the spectra. The possibility to use not only frozen tissue samples but also (molecular cross-linked) formalin fixed paraffin embedded tissue would substantially expand its application and possibility. Another future challenge will be the analysis and integration of the huge amounts of datasets generated by (imaging) mass spectrometry. Especially in this respect, the field of mass spectrometry can learn a lot from the hurdles which had to be (and have been taken) in the field of genomics. Reserving substantial time, money and effort for data analysis in any mass spectrometry experiment is crucial and should be well considered beforehand.

Aberrant cell signaling has been shown to play a pivotal role in many cancers (33, 36). As cell signaling is predominantly regulated at the posttranslational level (thus at the level of the protein), proteomic analysis is not only complementary but even crucial for proper understanding the biology of cancer (37). Understanding the exact molecular pathways activated in intramuscular myxoma offer specific targets for molecular diagnostics. This has resulted in the incorporation of direct GNAS1 mutation analysis in the molecular diagnostic amendatory section of the department of pathology. So albeit without implications for targeted therapy with small inhibitory molecules, correct (molecular) classification by GNAS1 mutation analysis has a direct clinical application as it is helpful in differentiating intramuscular myxoma from low-grade myxofibrosarcoma (and thus whether free resection margins are required). In myxofibrosarcoma we could not detect any tumour specific karyotypic aberrations which might however be unrevealed by more detailed genomic screens in the future. Functional studies to validate potential molecular targets in myxofibrosarcoma are hampered by the lack of myxofibrosarcoma cell lines which are available for many other sarcomas. By such experiments, such as in vitro kinome analysis, we were able
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to identify new treatment options for myxoid liposarcoma patients with advanced disease. This is especially relevant as conventional chemotherapeutic options in these patients are rather limited. Pathway analysis uncovers the mechanisms behind tumour biology and may offer potential treatment targets. In vitro blocking of kinases can also be achieved excellently, and sometimes more specifically with RNA interference. Given the way that kinase-inhibitors and RNAi have transformed basic research and the unprecedented speed with which they have reached the clinic, the near future promises to be exciting (38).

References