Stem cell transcription factor SOX2 in synovial sarcoma and other soft tissue tumors

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By MSc. Hebatalla Zayed

Born on 20.09.1981 in Dubai, United Arab Emirates

Reviewers:

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2.

3.

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Abbreviations

AEG	:	Anophthalmia-esophageal-genital
AJC	:	American Joint Committee
BAF	:	Brahma-associated factor
ChIPSeq	:	Chromatin immunoprecipitation sequencing
CSC	:	Cancer stem cell
EMA	:	Epithelial membrane antigen
EMT	:	Epithelial mesenchymal transition
ES	:	Embryonic stem
EZH2	:	Enhancer of zeste homologue 2
FAP	:	Familial adenomatous polyposis
FISH	:	Fluorescence in situ hybridization
FNCLCC	:	Fédération Nationale des Centres de Lutte Contre le Cancer
H3K27me3	:	Trimethylated histone
HV8	:	Herpesvirus 8
IPSCs	:	Induced pluripotent stem cells
MPNST	:	Malignant peripheral nerve sheath tumor
RAAS	:	Radiation associated angiosarcoma
RT-PCR	:	Reverse transcriptase polymerase chain reaction
SCC	:	Squamous cell carcinoma
SCLC	:	Small-cell lung cancer
SEER	:	Surveillance, Epidemiology and End Results
SRY-box 2	:	Sex determining region Y-box 2
SS	:	Synovial sarcoma
STT	:	Soft tissue tumors
TMA	:	Tissue microarrays

1. SUMMARY

Background

Soft tissue tumors, as an important oncology domain, entail different entities and are challenging regarding diagnosis and therapy. Here, we focused on synovial sarcoma alongside with other soft tissue tumors and specifically analyzed the expression and amplification of the gene SOX2 which has the full name SRY (sex determining region Y)-box2. SOX2 is a transcription factor responsible for the pluripotency of undifferentiated embryonic stem cells, promoting cellular proliferation and promoting invasion, migration and metastases in melanoma and other tumors. We also tested Anti-Histone H3-trimethyl K27 (H3K27me3) expression in SOX2 positive cases in an attempt to correlate SOX2 gene expression with the posttranslational protein modification H3K27me3, both of which having been associated with stemness features of cancer cells.

Methodology

In our study, we included all samples (n=60) of synovial sarcoma at the Friedrich-Schiller University hospital of Jena (Germany) between January 2013 and December 2015 in a retrospective observational manner. We excluded cases whose histopathological material was not available anymore in the institute (n=6) and cases (n=4) whose paraffin block was not optimal for further investigation. Clinicopathological and Immunohistochemical analysis were performed by our institutional pathology team according to standard diagnostic protocols e.g. using antibodies against EMA, Bcl2, PanCK, CK7, CD34, Ki67 and S100. Molecular confirmation of the diagnosis was routinely performed by FISH and/or PCR to detect the t(x;18) translocation. We also employed tissue microarrays of different soft tissue tumors to compare the expression in synovial sarcoma with other sarcoma entities. Results were collected, tabulated and statistically analyzed.

Results

About 60 % of all synovial sarcoma cases were positive to Sox2. Meanwhile out of 343 soft tissue tumors, varying from nodular fasciitis to undifferentiated pleomorphic sarcoma, only 13 cases (3.8%) were Sox2 positive. Of these, 11 (84.6%) were

undifferentiated high grade pleomorphic sarcoma. Out of 35 Sox2 positive synovial sarcoma cases, 25 (71, 4%) were H3K27me3 positive and 10 (28, 6%) were negative. SOX2 amplification was not detectable in 6 randomly chosen synovial sarcoma cases showing SOX2 protein expression.

Conclusion

Sox2, a principal stem-cell transcription factor, is evidently involved in the tumorigenesis of many tumor entities. In soft tissue tumors, however, expression is largely restricted to synovial sarcoma. Immunohistochemical detection of SOX2 can thus help in the diagnostic challenge in differentiating synovial sarcoma from other soft tissue tumors. H3K27me3 was also found to be positive in the majority of Sox2-positive synovial sarcomas and this correlates with the idea that these tumors might have a pluripotent cell population as tumor-initiating cells. We think that this finding supports the hypothesis of synovial sarcoma as product of pluripotent mesenchymal stem cell populations rather than being derived from mutations in terminally differentiated cells. The results may add to the diagnostic scheme for synovial sarcoma diagnosis. Moreover, Sox2 might reveal a molecular approach in cancer treatment, namely by targeting epigenetic modulators that seems to play a role in SOX2 gene regulation.

Zusammenfassung

Hintergrund der Studie:

Weichteiltumoren, eine wichtige onkologische Domäne, setzen sich aus unterschiedliche Entitäten zusammen und sind diagnostisch und therapeutisch schwierig. Hier konzentrierten wir uns auf das Synovialsarkom, wie auch andere Weichteiltumore, und analysierten speziell die Expression und Amplifikation des Gens SOX2, das mit vollem Namen SRY (sex determining region Y)-Box2 heisst. SOX2 ist ein Stammzell-Transkriptionsfaktor, der für die Pluripotenz undifferenzierter embryonaler Stammzellen verantwortlich ist und die Zellproliferation, Invasion, Migration und Metastasen bei Melanomen und anderen Tumoren fördert. Daneben testeten wir die Anti-Histon-H3-Trimethyl-K27 (H3K27me3)-Expression in den Sox2-positiven Fällen, um die SOX2 Expression mit der posttranslationalen Proteinmodifikation H3K27me3 zu korrelieren, beide Marker wurden mit Stamzellcharakteristika von Krebszellen assoziiert.

Methodik:

In unserer Studie wurden alle Synovialsarkome (n = 60) zwischen Januar 2013 und Dezember 2015 an der Friedrich-Schiller-Universitätsklinik in Jena (Deutschland) retrospektiv betrachtet. Wir schlossen Fälle aus, deren histopathologisches Material nicht mehr im Institut (n = 6) verfügbar oder deren Paraffinblöcke für eine weitere Analyse ungenügend waren (n=4). Klinisch-pathologische und immunhistochemische Analysen wurden von qualifizierten Mitarbeitern des Institut für Pathologie nach Standard-Diagnoseprotokollen etwa unter Verwendung von Antikörpern gegen EMA, Bcl2, PanCK, CK7, CD34, Ki67 und S100 durchgeführt. Die molekulare Bestätigung der Diagnose erfolgte routinemäßig durch FISH und/oder PCR-Analyse, um die t(x;18) Translokation nachzuweisen. Wir haben in dieser Studie auch Gewebe-Microarrays verschiedener Weichteiltumoren eingesetzt, um die Expression in Synovialsarkomen und anderen Sarkom-Entitäten zu vergleichen. Die Ergebnisse wurden gesammelt, tabelliert und statistisch untersucht.

Ergebnisse

Etwa 60% aller Fälle von Synovialsarkomen waren positiv für Sox2. Von 343 Weichteiltumoren, die von der nodulären Fasziitis bis zu undifferenziertem pleomorphem Sarkom reichten, waren nur 13 Fälle (3,8%) Sox2-positiv. Von diesen waren 11 (84,6%) undifferenzierte hochgradige pleomorphe Sarkome. Von 35 Sox2-positiven Synovialsarkomen waren 25 (71, 4%) H3K27me3-positiv und 10 (28, 6%) negativ. Eine SOX2 Amplifikation war nicht nachweisbar in 6 zufällig ausgewählten Synovialsarkomen mit SOX2 Expression.

Schlussfolgerungen

Sox2, ein Hauptstammzelltranskriptionsfaktor, ist offensichtlich an der Tumorigenese vieler Tumorentitäten beteiligt. In Weichtumoren ist die Expression jedoch weitgehend beschränkt auf Synovialsarkome. Der immunhistochemische SOX2 Nachweis kann damit hilfreich sein in der bisweilen schwierigen differenzialdiagnostischen Abgrenzung dieser Entität von anderen Weichteiltumoren. H3K27me3 war ebenfalls nachweisbar in den meisten Sox2-positiven Synovialsarkomtumoren und korreliert damit mit der Vorstellung, dass diese Tumoren eine pluripotente mesenchymale Stammzell-Population als tumorinitiierende Zellen aufweisen könnten. Wir denken, dass dieser Befund die Hypothese stützt, dass das Synovialsarkom eher ein Produkt pluripotenter mesenchymaler Stammzellpopulationen ist und nicht auf die Mutation terminal differenzierter Zellen zurückgeht. Die Ergebnisse könnten das diagnostische Schema für Synovialsarkome bereichern. Darüberhinaus könnte sich SOX2 als ein molekularer Ansatz in der Krebstherapie entpuppten in dem Sinne einer zielgerichteten Beeinflussung epigenetischer Modulatoren, die offenbar bei der Genregulation von SOX2 eine wichtige Rolle spielen.

2. INTRODUCTION

2.1 Stem cells and embryonic stem cells

Stem cells are characterized by unlimited self-renewal and they also have the capacity to differentiate into virtually all tissue types (Takashi et al. 2007; Takashi and Yamanaka 2006). Increasing effort is put in the improvement of stem cell transplantation therapies to revert the damage that is done by diseases such as Alzheimer's and Parkinson's diseases. In other closely related fields like spinal cord injury, important progress has been made using stem cells to treat patients (Pen et al. 2016).

Embryonic stem (ES) cells are pluripotent stem cells derived from the inner cell mass of the early stage blastocyst (Yu &Thomson 2008). Self-renewal is crucial to stem cell function, because it is required to persist for the life-time of the animal. Moreover, whereas stem cells from different organs may vary in their developmental potential, all stem cells must self-renew and regulate the relative balance between self-renewal and differentiation (Tannishtha et al. 2001). ES cells possess the capacity of unlimited self-renewal while maintaining pluripotency. Their ability to differentiate into all cell types of the three embryonic germ layers makes them interesting candidates for cell replacement therapies and has led to the identification of three core transcription factors that are essential for maintenance of ES cells: Oct4, Sox2 and Nanog (Chen & Daley 2008). Many stem cell-specific transcription factors, including the pluripotency transcription factors, Oct4, Nanog and Sox2 function in combinatorial complexes to regulate the expression of loci, which are involved in embryonic stem (ES) cell pluripotency and cellular differentiation (Kashyap et al. 2009).

Considerable effort has also been invested in attempts to dedifferentiate somatic cells towards pluripotency, a strategy that could be used for personalized regenerative medicine. One approach is to virally induce exogenous expression of transcription factors forming induced pluripotent stem cells (Johansson et al. 2010).

As well as the experimental induction of pluripotency is done by somatic cell nuclear transfer (Byrne et al. 2007), nuclear programming/cell fusion experiments (Lluis

et al. 2008), and most recently by retroviral introduction of the four critical genes, now sometimes referred to as Yamanaka factors: Oct4, Sox2, Klf4 and c-Myc or a combination of Oct4, Sox2, Nanog and Lin28 (Yu et al. 2007). This technique has permitted the reprogramming of multiple distinct mouse and human differentiated cell types to yield induced pluripotent stem (iPS) cells (Yamanaka et al. 2008). These iPS cells are similar to embryonic stem (ES) cells in morphology, proliferation and capacity to form teratomas (Takahashi et al. 2006).

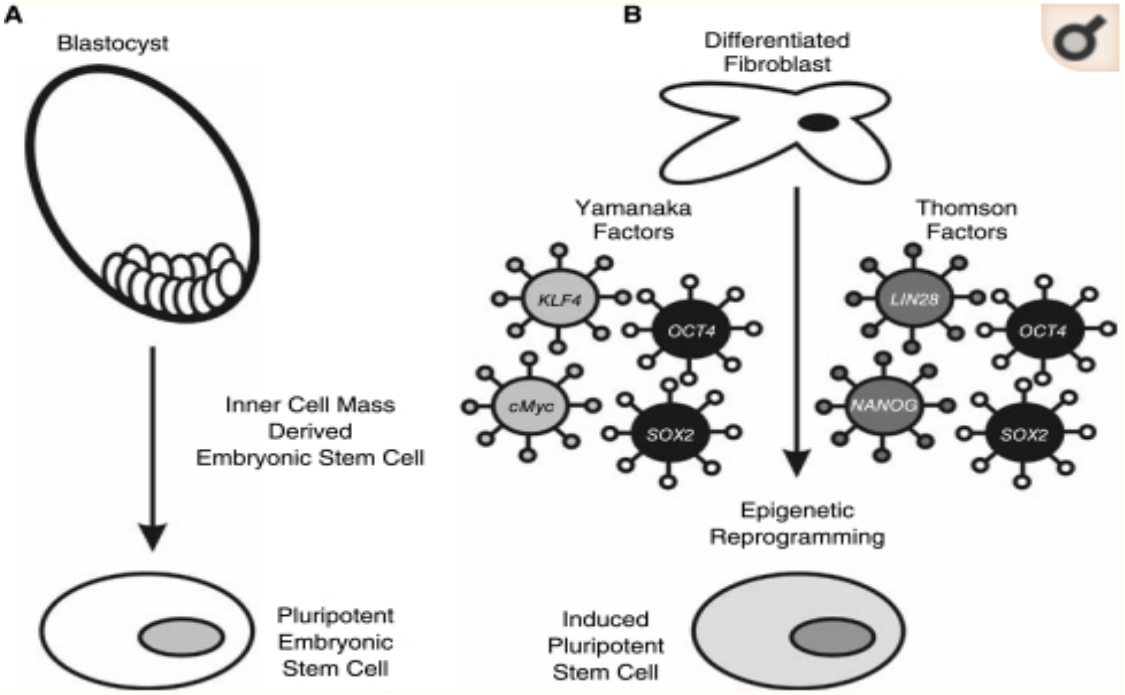

Fig 1: Pluripotent stem cells can be derived from cells isolated from the inner cell mass of early stage blastocysts (A) or experimentally derived by epigenetic reprogramming of differentiated adult cell types (B). Greatest reprogramming efficiency is achieved when combinations of 4 factors, *OCT4, SOX2, c-MYC*, and *KLF4*, or *OCT4, SOX2, NANOG*, and *LIN28* genes are introduced into the differentiated cell. However, OCT4 and SOX2 appear critically required to induce pluripotency (Kashyap et al. 2009).

The highest efficiencies of induced pluripotency are achieved when all four factors (Yamanaka factors) were utilized; however c-Myc and Klf4 have been shown to be dispensable for somatic cell reprogramming to pluripotency under specific culture conditions (Nakagawa et al. 2008). Specifically, the histone deacetylase inhibitor valproic acid (VPA) both enhances the efficiency of iPS derivation by the combined four factors and permits the derivation of iPS cells using just Oct4 and Sox2 (Huangfu

et al. 2008). These studies indicate that Oct4 and Sox2 are critical factors required for maintaining self-renewal and pluripotency of mouse and human stem cells (Guo G et al. 2009).

Two major studies have demonstrated that Oct4, Nanog and Sox2 share a substantial fraction of target genes and in fact, co-occupy genes in both mouse and human ES cells (Boyer et al. 2005). As reported by Boyer and colleagues, these genes occupy collectively about 10 % of the promoters in the human genome. About half of the promoter regions bound by Oct4 were also bound by Sox2 and 90% of these doubly bound genes were in turn bound by Nanog (Boyer et al. 2008). Moreover, the OCT4, SOX2 and NANOG-binding sites were in close proximity, further confirming that the proteins work in concert (Loh et al. 2006).

Oct4, Nanog, Sox2 and a number of associated transcription factor proteins activate and maintain the expression of genes involved in self-renewal, while simultaneously repressing genes that mediate differentiation (Wu et al. 2006). Thus Oct4, Nanog and Sox2 form a self-reinforcing and intricately connected network that preserves ES cell character (Yang et al. 2008).

2.2 Induced pluripotent stem cells and the Yamanaka Factors

In 2006, Shinya Yamanaka was the first to successfully reprogram cells using four distinct factors, thereby generating induced pluripotent stem cells (iPSCs) from terminally differentiated fibroblasts. IPSCs can be established by the over expression of four key transcription factors: Oct4, Klf4, Sox2 and c-Myc (OSKM) (Takashi et al. 2007; Takashi and Yamanaka 2006). One of the major advantages of iPSCs is that they can be made autologous and can provide a sufficient quantity of cells by culturing, making the use of other stem cell sources unnecessary (Pen et al. 2016).

Reprogramming of any somatic cell type can be achieved by initiating several synergistic processes. In the process of reprogramming, induced pluripotency elicits several transcription waves driven by c-Myc/Klf4 and Oct4/Sox2/Klf4. The expression levels of distinct pluripotency genes (alkaline phosphatase (AP), stage specific embryonic antigen (SSEA), Nanog and Oct4) increase step wise (Brambrink et al.

2008), and upon achieving stable pluripotency levels, their DNA methylation patterns are changed (Polo et al. 2012). Nonetheless, the exact mechanisms of reprogramming still remain unclear. Obviously the reprogramming factors reactivate an endogenous pluripotency circuitry by re-inducing the cells' capacity for unlimited growth without inducing genetic alterations, as it is frequently observed in cancer (Polo et al. 2012).

It has been demonstrated that abbreviated reprogramming factor expression pattern results in dysplasia and tumor formation in vivo, thus suggesting that OKSM has an impact on epigenetic changes that are substantially involved in the regulation of cell growth and tumorigenesis (Ohnishi et al. 2014). This observation is corroborated by the fact that iPSCs form teratomas upon implantation in vivo (Magnuson et al. 1982). Of note, human iPSCs develop teratomas more efficiently and faster than human embryonic stem cells (Gutierrez-Aranda et al.2010; Avior et al. 2015).

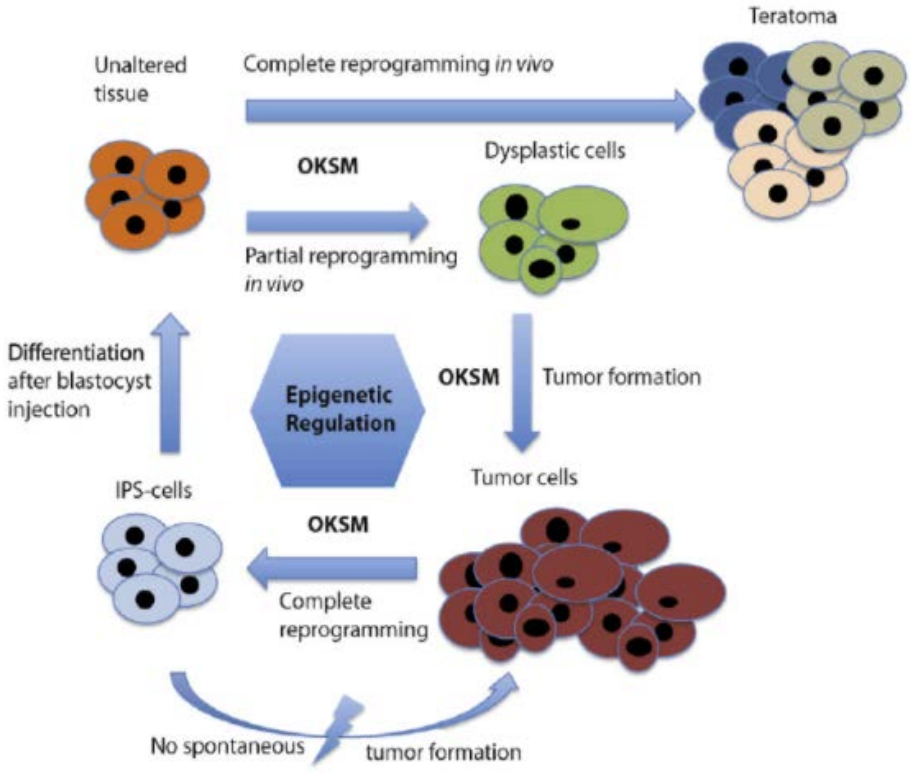

Fig 2: In vivo overexpression of the four pluripotency factors OCT4, c-MYC, KLF4 and SOX2 (OSKM) leads to epigenetic changes resulting in dysplasia. Extension of OSKM overexpression subsequently drives tumor formation. Both dysplasia and tumor formation result from an incomplete reprogramming process. Cells derived from these tumors can be fully reprogrammed towards unaltered iPSCs that do not have tendencies to re-initiate tumorigenesis after blastocyst injection. In case of complete in vivo reprogramming, teratoma formation becomes apparent. Figure is adapted from Ohnishi et al. (2014).

Several studies have assigned the OKSM factors to tumorigenesis. Abad et al. were the first to successfully reprogram in vivo by transiently inducing OSKM, resulting in teratomas formation, and detection of fully reprogrammed cells in various tissue types. The presence of the niche in vivo even allowed superior reprogramming to the totipotent state (Abad et al. 2013). Intriguingly, further studies showed that partial or incomplete reprogramming induced particular tumor types in vivo (Ohnishi et al. 2014).

More importantly, up regulation of these proteins was associated closely with tumor metastases and poor prognosis in various human malignancies including prostate cancer, lung adenocarcinoma, gliomas, rectal cancer, gastric carcinoma and oral squamous cell carcinoma (Chiou et al. 2010; Guo et al. 2011; Matsuoka et al. 2012).

The mesenchymal phenotypic changes by increased motility and invasiveness of epithelial tumor cells are known as epithelial-mesenchymal transition (EMT). EMT is defined by the loss of epithelial morphology and the acquisition of a mesenchymal phenotype, which is initially found to be a central program in early embryonic morphogenesis (Lim et al. 2012).

In a few years, evidence has mounted for EMT as the key means through which cancer cells acquire more highly mobile potentials to migrate and metastasize to distant sites during tumor progression (Scheel and Weinberg 2011). E-cadherin, a classical cadherin from the cadherin superfamily, is required for maintaining epithelial cell plasticity. N-cadherin, known as an important member of the cadherin family that mediates calcium-dependent adhesion, is normally expressed in mesenchymal cells. Loss of E-cadherin and increased N-cadherin expression (E/N cadherin switch) is now defined as a major hallmark of EMT (Nakajima et al. 2004; Werling et al. 2011).

Over the past few years, accumulating data has demonstrated that EMT correlates closely with the acquisition of stem cells-like properties in cancer cells (Polyak et al. 2009; Sarkar et al. 2012).

Luo et al. showed that overexpression of Sox2, Oct4 and Nanog were significantly associated with high expression of N-cadherin, but adversely with low E-cadherin expression (except SOX2). Additionally, overexpression of these proteins

correlated strongly with the expression of Snail, a central transcription factor as E-cadherin repressor (Luo et al. 2013).

In the kidney, OKSM-induced tumors bear features of a Wilm's tumor, a common pediatric cancer. Interestingly, these tumors only display epigenetic alterations, as indicated by global changes in their DNA methylation patterns (Müller et al. 2016).

Interestingly, reprogramming of OKSM-induced tumors resulted in non-tumorigenic iPSCs that contributed to regular organ formation upon subsequent differentiation in vivo. This indicates that reprogramming with the Yamanaka factors primarily leads to epigenetic alterations, generating a "cancer-poised" but not yet "cancer-committed" state (Ohnishi et al. 2014).

2.3 Cancer and cancer stem cells

Similar to normal tissues, cancer compromise heterogeneous cell populations with distinct phenotypes, functions and gene expression profiles (Marte 2013). The phenotypic characteristics of some cancer cells, particularly of poorly differentiated to undifferentiated tumors have been found to be quite similar to undifferentiated embryonic cells (Curry et al. 2015).

A tumor can be viewed as an aberrant organ initiated by a tumorigenic cancer cell that acquired the capacity for indefinite proliferation through accumulated mutations (Reya et al. 2001). If one views a tumor as an abnormal organ, then the principles of normal stem cell biology can be applied to understand better how tumors develop (Kummermehr 2001). Both normal stem cells and tumorigenic cells have extensive proliferative potential and the ability to give rise to new (normal and abnormal) tissues. Both tumors and normal tissues are composed of heterogeneous combinations of cells, with different phenotypic characteristics and different proliferative potentials (Nowell 1986).

Because most tumors have a clonal origin (Fearon et al. 1987), tumorigenic cancer cells must give rise to phenotypically diverse progeny, including cancer cells with indefinite proliferative potential, as well as cancer cells with limited or no proliferative potential. This suggests that tumorigenic cancer cells undergo processes

that are analogous to the self-renewal and differentiation of normal stem cells (Reya et al. 2001).

Both normal stem cells and tumorigenic cells give rise to phenotypically heterogeneous cells that exhibit various degrees of differentiation. Thus, tumorigenic cells can be thought of as cancer stem cells that undergo an aberrant and poorly regulated process of organogenesis analogous to what normal stem cells do (Sell et al. 1994)

The cancer stem cell (CSC) hypothesis posits that tumors may be initiated and maintained by a subset of cells that maintain or acquire stem-cell properties and that each tumor contains a small subpopulation of cells that have the ability to differentiate into multiple cell lineages and self-renew (Jordan et al. 2006; Reya et al. 2001). Cancer stem cells or cancer stem-like cells have been identified in several solid tumor types such as breast cancer and colon cancer (Al-Hajj et al. 2003) This subpopulation is closely associated not only with carcinogenesis, but also with recurrence and metastasis of tumors (Jordan CT et al. 2006).

Small numbers of stem cells are believed to exist in most if not all adult tissues (Blanpain et al. 2009). Adult stem cells can evade the stringent genetic controls of their normal pathways of cellular differentiation and proliferation and give rise to cancer. Cancer stem/initiating cells have been defined as a subset of cancer cells that have the exclusive ability of self-renewal and cause the heterogeneous lineages of cancer cells that comprise the tumor (Hill et al. 2007).

These cancer stem cells are implicated in cancer initiation, malignant potential, metastatic progression, and in the post treatment recurrence of many human cancer types (Dalerba et al. 2008). Stem cell-specific proteins, including Oct4, Nanog and Zfp42/Rex1 are implicated in some cancers (Chiou et al. 2008). Histologically poorly differentiated tumors showed preferential overexpression of genes normally enriched in ES cells. Activation targets of Nanog, Oct4, Sox2 and c-Myc are more frequently overexpressed in poorly differentiated tumors than in well-differentiated tumors (Ben-Porath et al. 2008). It appears that the genes active in both ES cells and cancer stem cells are controlled by a few master regulatory genes (Wong et al. 2008)

2.4 Sox2

SRY(sex determining region Y)-box2, also known as Sox2, is a transcription factor that is essential for maintaining self-renewal or pluripotency of undifferentiated embryonic stem cells and plays a critical role in maintenance of embryonic and neural stem cells (Rizzino 2009). The Sox2 gene is located on chromosome 3q26.3-q27, belongs to the SoxB1 group and encodes for a protein consisting of 317 amino acids (Collignon et al. 1996). Sox2 research thus far has heavily emphasized its crucial role in stem cell maintenance, lineage fate determinant and as a necessary factor to reprogram somatic cells back towards pluripotency (Takahshi and Yamanaka 2006).

Varying levels of SOX2 affect embryonic stem cells' fate of differentiation. SOX2 inhibits differentiation into mesoderm germ layer and promotes differentiation into neural ectoderm germ layer (Thomson et al. 2011). A study conducted in Milano, Italy showed, through the development of a knockout model, that deficiency of SOX2 results in neural malformations and eventually fetal death, further underlying SOX2's vital role in embryonic development (Ferri et al. 2004).

In addition to its fundamental role in the maintenance of embryonic stem cells, SOX2 is important during embryonic development of gastrointestinal organs: it is expressed in the developing foregut and gastric epithelium. SOX2 co-localizes with p63 in the basal layer of the esophagus and is critical in the maintenance of the stratified squamous epithelium; however it is down regulated in intestinal metaplasia of the stomach and esophagus (Long and Hornick. 2009).

In neurogenesis, SOX2 is expressed throughout developing cells in the neural tube as well as in proliferating central nervous system progenitors (Graham et al. 2003). Cells expressing SOX2 are capable of both producing cells identical to themselves and differentiated neural cell types, two necessary hallmarks of stem cells (Suh et al. 2007).

In diseases, SOX2 alterations have been associated with developmental maladies, such as anophthalmia-esophageal-genital (AEG) syndrome which occurs when there is a heterozygous mutation of Sox2 that leads to abnormal development of ectodermal and endodermal tissues (Williamson et al. 2006).

Cancer is a disease characterized by determined hallmarks, some of which are: sustained proliferative signaling, activation of invasion and metastases, and evasion of cell death (Hanahan and Weinberg, 2011). The orchestration of tumor initiation and maintenance has been shown in some cancers to be driven by cancer stem cells (CSCs). These cells may acquire tumor-initiating and self-renewal properties through similar molecular mechanisms governing cellular reprogramming (Vazquez-Martin et al. 2013).

Sussman and colleagues discovered that the ubiquitin-specific protease 22 (USP22) is responsible for controlling the cellular transition from stemness towards differentiation (Sussman et al. 2013). Moreover they found USP22 represses the SOX2 promoter in order to control the embryonic stem cell transition from self-renewal to differentiation (Sussman et al. 2013). Therefore, not only is Sox2 an essential stem cell marker but its suppression is mandatory for cellular differentiation. For these reasons, Sox2 has been heavily investigated in CSCs in several cancer types (Weina and Utikal 2014)

SOX2 amplification has been found in several cancer types including glioblastoma, small-cell lung cancer (SCLC) and many forms of squamous cell carcinoma (SCC) (Rudin et al. 2012).

SOX2 has been shown to promote cellular proliferation in breast, prostate, pancreatic and cervical cancers (Herreros-Villanueva et al. 2013), evade apoptotic signals in prostate, gastric cancer and non-small cell lung carcinoma (Herreros-Villanueva et al. 2013; Chen et al. 2013) and promote invasion, migration and metastases in melanoma, colorectal glioma, gastric, ovarian cancer and hepatocellular carcinoma (Sun et al. 2013; Lou et al. 2013).

Cellular proliferation is tightly regulated by Sox2 in many cancer types. Sox2 knockdown in pancreatic cancer cells resulted in cell growth inhibition through cell cycle arrest, not apoptosis (Herreros-Villanueva et al. 2013). When Sox2 was over expressed, cell proliferation was promoted through cyclinD3 (Herreros-Villanueva et al. 2013).

Additionally, Fang and colleagues found in lung small cell carcinoma (LSCC), SOX2-silencing inhibited cellular proliferation via the up regulation of BMP4 (Fang et al. 2014). After performing chromatin immunoprecipitation and luciferase experiments, SOX2 was found to transcriptionally repress the BMP4 promoter. The authors therefore suggest that BMP4 is playing a tumor suppressor role in LSCC, while SOX2 repression of BMP4 transcription causes cell growth (Fang et al. 2014). It's also important to note that the involvement of SOX2 in cell proliferation has been controversially discussed in colorectal and gastric cancer (Liu H et al. 2013).

SOX2 also plays an important role in evading apoptotic signals. In prostate cancer, in vitro and in vivo xenograft experiments using DU145 Sox2-overexpressing cells in NOD/SCID mice revealed that SOX2 caused an increase in apoptotic resistance by decreasing store-operated calcium entry (Jia et al. 2011). Equivalently, upon silencing of SOX2 in non-small cell lung carcinoma (NSCLC) cell lines, apoptosis was induced (Chen et al. 2013)

Research has indicated that SOX2 is a novel regulator of cell invasion, migration and metastasis. For example, in melanoma, SOX2 knockdown in A2058 cells resulted in a 4.5 fold decrease in invasion in vitro (Girouard et al. 2012). Likewise in colorectal cells, SOX2 was involved in cellular migration and invasion in vitro (Han et al. 2012). This invasive phenotype was also confirmed in malignant glioma, since siRNA-mediated down regulation of SOX2 resulted in a significant decrease in migration and invasion capabilities (Alonso et al. 2011).

2.5 Soft Tissue Tumors

2.5.1 Epidemiology

The incidence of soft tissue tumors, especially the frequency of benign tumors relative to malignant ones, is nearly impossible to determine accurately. Benign soft tissue tumors outnumber malignant tumors by a wide margin (Goldblum et al. 2014). However, according to an analysis of the Surveillance, Epidemiology and End Results (SEER) database, the incidence changes with age; for children younger than 10 years of age, the annual incidence was 0.9/100,000 in children but rose to 18.2/100,000 in adults

over the age of 70 years. The most dramatic increases occurred at 30 and 70 years of age (Ferrari et al. 2011).

Soft tissue sarcomas are rare tumors, representing less than one percent of all malignant neoplasms (Katenkamp and Katenkamp 2009). Adult soft tissue tumors are not represented in the figures of the Robert Koch Institute reporting of 427 000 individuals in Germany being diagnosed with cancer in 2006 (Bertz et al. 2010). Assuming that mesenchymal malignancies constitute about one-hundredth of all cancer diagnoses (Fletcher et al. 2006), an estimated number of 4500 Germans annually develop a sarcoma.

2.5.2 Classification

Soft tissue tumors constitute a large and heterogeneous group of neoplasms (Fletcher et al. 2006). Traditionally, soft tissue sarcomas have been classified according to a histogenetic concept (e.g., fibrosarcoma as a tumor arising from fibroblasts, osteosarcoma as a tumor arising from osteoblasts, and so on). However morphologic, immunohistochemical and data from experimental animals suggest that most if not all sarcomas arise from primitive multipotential mesenchymal cells, which in the course of neoplastic transformation undergo differentiation in one or more lines (Mills et al. 1995).

The acceptance of this alternative scheme does not require a change in terminology: a liposarcoma remains as such but is now viewed not as a tumor arising from a lipoblast but as a tumor exhibiting lipoblastic differentiation (Fletcher et al. 2006).

2.5.3 Pathogenesis

The large majority of soft tissue sarcomas arise de novo rather than from malignant degeneration of preexisting benign tumors. Although the latter phenomenon may occur (as in neurofibromas), in most cases in which a given benign tumor is said to have become malignant, review of the original material showed that it was malignant from its inception (Laskin et al. 1988).

2.5.4 Environmental factors

Trauma is frequently implicated in the development of sarcomas. Much has been written in the medical and legal literature on the possible relationship between trauma and soft tissue sarcoma, but no convincing evidence has been provided for a definite cause-effect relationship between the two (Monkman et al.1974). Rare soft tissue sarcomas have been reported as arising in scar tissue following surgical procedures or thermal or acid burns, at fracture sites, and in the vicinity of plastic or metal implants, usually after a latent period of several years (Piscitelli et al. 2011).

Worth noting is that trauma, whether etiologically related to a tumor or proven to be indulged in tumor-emergence by causing specific genetic alterations, should be regarded as more than just a random co-incidence. The emergence of mutations, for example, may be favored in the proliferative state that occurs within fibroblasts in the setting of a trauma (Petersen 2017).

Phenoxyacetic acid herbicides, chlorophenols and their contaminants such as 2, 3, 7, 8-tetrachlorodibenzo-para-dioxin (dioxin) have been linked to sarcomagenesis (Zambon et al. 2007; Collins et al. 2009). A series of case-control studies from Sweden from 1979 to 1990 reported an up to six fold increased risk of soft tissue sarcoma associated with exposure to phenoxyacetic acids or chlorophenols in individuals exposed to these herbicides in agricultural or forestry work (Hardell et al. 1998). Similar reports of an increased risk of sarcoma associated with these herbicides were reported from Italy (Bertazzi et al. 2001), Great Britain and New Zealand.

The possibility of an increased incidence of sarcomas was claimed for some of the two million soldiers stationed in Vietnam between 1965 and 1970 who were exposed to Agent Orange, a defoliant that contained dioxin as a contaminant (Kramárová et al. 1998). Vinyl chloride exposure is clearly associated with the development of hepatic angiosarcoma (Sahmel et al. 2009; Sherman M et al. 2009).

Radiation exposure has been related to the development of sarcomas, but considering the frequency of radiotherapy, radiation-induced soft tissue sarcomas are quite uncommon. The incidence of post-radiation sarcoma is difficult to estimate, but

reports generally range from 0.03% to 0.8% (Mark et al. 1996; Inoue et al. 2000). Nearly all post-radiation sarcomas occur in adults, and women develop these tumors more frequently, an observation which reflects the common use of radiation for the treatment of breast and gynecologic malignancies (Weaver et al. 2009).

Although the incidence of radiation associated angiosarcoma (RAAS) is low, the number of cases reported is increasing as a function of the improved likelihood of surviving early stage breast carcinoma. Furthermore, with increased use of external beam radiation in the management of breast cancer patients, the incidence of post-radiation sarcomas may increase in the future. Long-term follow-up is needed for early disease detection (Torres et al. 2013).

Unfortunately most post radiation sarcomas are high-grade lesions and are detected at a relatively higher stage than their sporadic counterparts (Billings et al. 2004). Patients with radiation-induced sarcoma of the extremities have the best survival (approximately 30% at 5 years), whereas those with lesions arising in the vertebral column, pelvis and shoulder girdle generally have survival rates of less than 5% at 5 years (Fang et al. 2004).

2.5.5 Oncogenic Viruses

The role of oncogenic viruses in the evolution of soft tissue sarcomas is still poorly understood, although there is strong evidence that the human herpesvirus 8 (HV8) is the causative agent of Kaposi's sarcoma (Mesri et al. 2010). In addition, there is a large body of literature supporting the role of the Epstein-Barr virus in the pathogenesis of smooth muscle tumors in patients with immunodeficiency syndrome or following therapeutic immunosuppression in the transplant setting (Deyrup et al. 2006).

2.5.6 Immunologic factors

Acquired immunodeficiency, or loss of immune surveillance, may lay a central role in the development of the relatively rare angiosarcomas that arise in the setting of chronic lymphedema (Shon et al. 2011), secondary to radical mastectomy (Stewart-Treves syndrome) (Dawlatly et al. 2011) or congenital or infectious conditions (Roy et al. 2004).

2.5.7 Genetic factors

A number of genetic diseases are associated with the development of soft tissue tumors, and the list will undoubtedly lengthen as we begin to understand the molecular underpinnings of mesenchymal neoplasia. Neurofibromatosis type 1, neurofibromatosis type 2 and familial adenomatous polyposis (FAP)/Gardner syndrome are classic examples of genetic diseases associated with soft tissue tumors (Goldblum et al. 2014)

2.5.8 Clinical Features

A definite relationship exists between soft tissue tumor type and the age of presentation (Rydholm et al. 1984). For instance, embryonal rhabdomyosarcoma is typically a tumor of infants and children, synovial sarcoma typically affects adolescents and young adults, liposarcomas and undifferentiated pleomorphic sarcomas are usually seen in middle-aged and elderly patients. It is interesting that congenital soft tissue tumors rarely behave in a malignant fashion (Kauffman et al. 1965).

Most soft tissue sarcomas are solitary. Synchronous or metachronous multiple sarcomas represent only 0.2% of all cases. Liposarcomas account for a high percentage of these cases (Grobmyer et al. 2004).

2.5.9 Diagnosis

Appropriate diagnoses are of great importance to the patient to obtain the adequate therapy. Since misdiagnoses are not uncommon, specialist centers provide valuable resources for the verification of suspected malignant mesenchymal tumors (Lehnhardt et al. 2009; Petersen et al. 2011).

For any large soft tissue tumor in which the possibility of malignancy exists, the proper initial diagnostic procedure is to obtain material through incisional biopsy or fine needle aspiration. The latter technique is being used with increased frequency in the United States, with rates of accuracy equivalent to those obtainable with frozen section (Layfield et al. 1986).

Light microscopic evaluation of hematoxylin-eosin-stained sections remains the standard technique for the initial diagnostic approach to these tumors and is sufficient in

the majority of the cases (Enjoji et al. 1984). However, there are special techniques that have been successfully applied to increase diagnostic accuracy and which sometimes are indispensable, this development applying both to adult and pediatric tumors (Iwasaki et al. 2009). These techniques include conventional special stains, electron microscopy, immunohistochemistry and molecular genetic methods (Rosai and Ackerman 2011).

Immunohistochemistry for tissue-related markers has proved to be of great value and is now extensively used to accurately classify these neoplasms: the specificity, sensitivity and applicability of this technique to routinely processed material clearly render it the method of choice in most circumstances (Heim-Hall et al. 2008). The number of available markers is very large and continues to grow (Ordóñez et al. 1998).

The systemic use of cytogenetics has shown the existence of nonrandom chromosomal alterations (mainly translocations) in association with many types of soft tissue tumor (Sandberg AA et al. 2002).

Gene fusions have been described in approximately one-third of soft tissue tumors (STT); of the 142 different fusions that have been reported, more than half are recurrent in the same histologic subtype. These gene fusions constitute pivotal driver mutations, and detailed studies of their cellular effects have provided important knowledge about pathogenetic mechanisms in STT (Mertens et al. 2016).

The findings have validated the morphologic approach to classification of soft tissue tumors, helped to refine the boundaries of some entities and offered insight into the genesis of the tumors. Furthermore, the molecular alterations (gene fusions) that result from the chromosomal translocations can now be readily demonstrated in routine paraffin-embedded tissues by reverse transcriptase polymerase chain reaction (RT-PCR) or fluorescent in situ hybridization (FISH), and such studies can be extremely helpful in the diagnosis of these tumors especially in small biopsies, tumors with unusual morphology, or tumors in unusual sites (Antonescu et al. 2006).

As an example, a break-apart FISH probe against the EWS (also known as EWSR1) gene is particularly helpful since this gene is implicated in many different soft

tissue tumor types, including Ewing sarcoma/PNET, angiomatoid fibrous histiocytoma, extraskeletal myxoid chondrosarcoma, myxoid liposarcoma, clear cell sarcoma of tendons and aponeurosis and desmoplastic small cell tumor (Chang et al. 2003).

2.5.10 Grading and Staging

Some degree of microscopic grading of soft tissue is already built into the conventional microscopic classification of these tumors. The number of grades has varied in the different systems: two (low-grade and high-grade), three (I, II and III or low-grade, intermediate-grade and high-grade) and four (I, II, III and IV) grades have been recognized (Deyrup et al. 2006).

The criteria used have included degree of cellularity, pleomorphism, mitotic activity and necrosis and have been found to be of definite prognostic value for both adult and pediatric soft tissue tumors (Coindre et al. 1988), however it is misleading to over-emphasize grading that is independent of the specific microscopic type of the sarcoma and the circumstances in which it occurs, such as the patient's age or the depth and size of the tumor (Deyrup et al. 2006).

The two grading schemes that have been most widely applied are those of the French Federation of Cancer Centers Sarcoma Groups and the National Cancer Institute (Coindre et al. 2001). The WHO meanwhile propagates the "Fédération Nationale des Centres de Lutte Contre le Cancer"; FNCLCC system (Fletcher et al. 2013)

French federation of Cancer Centers Sarcoma Group (Fédération Nationale des Centres de Lutte Contre le Cancer"; FNCLCC system) grading system

Tumor Differentiation	
Score 1	Sarcomas closely resembling normal adult mesenchymal tissue. Examples: well-differentiated liposarcomas and well-differentiated fibrosarcoma.
Score 2	Sarcomas for which the histologic typing is certain. Examples: biphasic synovial sarcoma, alveolar soft-part sarcoma, myxoid liposarcoma
Score 3	Embryonal sarcomas, undifferentiated sarcomas and sarcomas of doubtful tumor type
Mitosis count	
Score 1	0-9 mitoses per 10 high power fields
Score 2	10-19 mitoses per 10 high power fields
Score 3	More than 20 mitoses per 10 high power fields
Tumor necrosis	
Score 0	No necrosis on any examined slides
Score 1	Less than 50% tumor necrosis for all the examined tumor surface
Score 2	Tumor necrosis on more than half of the examined tumor surface

The three grade system is set-up as follows: Grade I is defined as a total of 2 or 3 when summing the scores obtained for each of the three histologic criteria; grade II represents a total of 4 or 5; grade III represents a total of 6, 7 or 8.

Two main staging systems for soft tissue sarcoma have been proposed. The one exposed by the American Joint Committee (AJC) is largely based on the TNM system, in that it uses the size of the primary tumor (T), the status of lymph nodes (N), the presence of distant metastases (M), and the tumor's histological grade (G) (Behars et al. 1992).

2.5.11 Prognosis

Prognosis of soft tissue tumors depends on a variety of parameters, many of which are interrelated.

- Tumor size: there is a definite relationship between tumor size and outcome. This is true for practically all tumor types in which this parameter has been analyzed (Rooser et al. 1988).
- Depth: Superficially located tumors (dermis and subcutaneous tissue) have a much better prognosis than deep-seated ones (intermuscular or intramuscular,

retroperitoneal) of similar microscopic type (Gerrand et al. 2003). Along similar lines, soft tissue sarcomas featuring histologic evidence of bone invasion have a poorer prognosis (Ferguson et al. 2006).

- Location: Tumors of the retroperitoneum do much worse than microscopically similar lesions located in the extremities. Among the latter, local recurrence has been found to be more frequent with sarcomas of the upper extremity than those of the lower extremity (Gerrand et al. 2003).
- Microscopic type: Some soft tissue neoplasms (such as atypical lipomatous tumors) are low-grade lesions with no capacity to metastasize, whereas other neoplasms of similar cell type (such as pleomorphic liposarcoma) are highly aggressive and prone to spread distantly (Rosai and Ackerman 2011).
- Vascular invasion: This has been shown to be the strongest predictor of distant metastases in several series (Engellau et al. 2005).
- Surgical margins: Not surprisingly, adequacy of surgical margins is statistically associated with low relapse (Stojadinovic et al. 2002).
- Microscopic grade: A relationship has been found between various microscopic grading systems and outcome, which in some cases is directly related to the histotype but in others it is applied within a given histotype (Rosai and Ackerman 2011).
- Clinical stage: this determination, which incorporates several of the previously mentioned parameters, as well as the presence or absence of metastases, is the most powerful prognostic determinator (Rosai J and Ackerman LV. 2011).
- DNA ploidy: Several flow cytometric studies performed in soft tissue sarcomas of various microscopic types have shown that DNA ploidy correlates with a higher microscopic grade, a higher rate of cell proliferation and decreased survival rates (Kroese MC et al. 1990).
- Cell proliferation: Mitotic activity is incorporated in most grading schemes. Evaluation of proliferation markers such as MIB-1 and p105 has been shown to correlate with prognosis (Hasegawa et al. 2007)
- Genetic alterations: It has been shown that soft tissue tumors exhibiting mutations of TP53 or altered expression of retinoblastoma gene behave more aggressively than those lacking these changes (Kawai A et al. 1994). Claims have been made of a

relationship between the type of gene fusion in the sarcomas associated with chromosomal translocations and prognosis (as in alveolar rhabdomyosarcoma and synovial sarcoma) (Rosai and Ackerman 2011).

2.6 Synovial Sarcoma

Synovial sarcoma is a rare and aggressive soft tissue tumor that accounts for approximately 10% of soft tissue sarcomas and classically occurs in the extremities of young adults (Amary et al. 2007; Terry et al. 2007). It occurs at any age but the peak incidence is between the ages of 10 and 35 years; with a slight male predominance. The anatomic distribution is wide, but more than 60% arise in the lower limb (Bergh et al. 1999). A small but significant proportion of cases arise on the trunk, especially in the abdominal wall (Fetsch et al. 1993) in the neck (Roth et al. 1975), in the head (Shmookler et al. 1982), in the mediastinum (Suster et al. 2005) and even in the abdominal cavity (Fisher et al. 2004).

Overall 5-year survival probability is about 60-65% but falls to only around 30% at 10 years. In general, small tumor size (<5 cm), early clinical stage, early age at presentation (<10 years) and lower histologic grade (as defined by mitotic activity and necrosis) are signs of a better prognosis (Lewis et al, 2000).

Synovial sarcoma falls into two main groups, monophasic composed entirely of spindle cells and biphasic showing both epithelial and spindle cell components. The monophasic variant is more common, depending on sampling (Fletcher et al. 2002). Approximately 5-10% of cases have a poorly differentiated appearance, most often characterized by undifferentiated round cell morphology. These appear to be relatively more frequent in elderly patients with synovial sarcoma (Chan et al. 2003).

Immunohistochemically, in addition to positive staining in the obviously epithelial component, in almost all cases the spindle cell element also shows at least focal positivity for epithelial membrane antigen (EMA) and keratin; this, combined with morphologic clues, is generally the best way to distinguish monophasic lesions from malignant peripheral nerve sheath tumor (MPNST) or fibrosarcoma (Pelmus M et al.

2002). Around 30% of cases of synovial sarcoma are S-100 protein positive, similarly at least two thirds of synovial sarcomas stain positively for CD99 (Pelmus et al. 2002).

Cytogenetically, both biphasic and monophasic forms (as well as poorly-differentiated lesions) share a reproducible tumor-specific chromosome translocation, t(X;18)(p11.2;q11.2), which results in the production of one or other of two prinipal fusion genes, SYT-SSX1 and SYT-SSX2 (Sanberg et al. 2002).

2.7 BAF (Brg/Brahama-associated factors) complex and genetic mutations

Studies over the years involving drugs such as histone deacetylase and histone methyl transferase inhibitors, hydroxamic acid, sirtuins and others have suggested the important role of epigenetic modulation in cancer-indeed, nearly all cancers display epigenetic changes, and most cancer mutations, in either a direct or indirect manner, affect the epigenome (Dawson et al. 2012).

Among the most frequent mutations uncovered in human cancer sequencing efforts were mutations in genes encoding the subunits of adenosine triphosphate (ATP)-dependent chromatin remodeling complexes, most notably the mammalian SWI/SNF or BAF(Brg/Brahma-associated factors) complexes (Kadoch et al. 2013). Evidence indicating that polycomb complexes are an important primary target of mammalian SWI/SNF or BAF complexes has emerged in more recent years from the observation that mutation of the ATPase Brgl of BAF complexes leads to H3K27Me3 accumulation and repression of many genes in embryonic stem (ES) cells (Ho et al. 2011).

The advent of exome sequencing across a diverse range of human cancers has led to the realization that BAF complexes are one of the most significant tumor suppressors in humans, with a cumulative incidence of mutation of more than 20 % of human cancers sequenced to date (Kadoch et al. 2013). Many cancers (if not most) bearing BAF subunit mutations have a mutation in only one allele of the affected subunit, making them dominant tumor suppressors, rather than recessive (Kadoch et al. 2013).

The most commonly affected BAF subunit in cancer is ARID1A (BAF250A) which has been found to be mutated in a variety of tumors including endometrial, colon and rectal carcinomas (Kandoth et al. 2013). BAF 170 is mutated in gastric and colorectal cancers with microsatellite instability. The homologous subunit of BAF155 is mutated in about 10% of small cell lung cancers (Kadoch C et al. 2013).

In certain tumors, specific genes are mutated in 100% of the cancers in 100% of the cells, which provides definitive evidence that these mutations cause the development and maintenance of the tumor. An example is human synovial sarcoma which has provided ground for the discovery of a mechanism underpinning perturbation to the SS18 subnuit of BAF complexes by the t(X;18) translocation hallmark to human synovial sarcoma (Naka et al. 2010).

The t(X;18)chromosomal translocation, as illustrated in synovial sarcoma, results in the direct fusion of 78 amino acids of C-terminus of SSX to the SS18 terminus giving a fusion protein that evicts wild-type SS18 and causes displacement of BAF47. The SSX-SS18 containing complexes are then retargeted to oncogenic loci such as SOX2 and PAX6, activating these genes by displacement of PRC2 complexes and their H3K27me3 repressive marks (Kadoch and Crabtree 2013).

This oncogenic eviction of polycomb can be reversed by stoichiometrically altering the balance of SS18-SSX versus wild-type SS18 within BAF complexes thereby reversing the complex to an induced wild-type bearing normal subunit composition and hence an exciting therapeutic opportunity emerges from these findings (Kadoch and Crabtree 2013).

3. AIM OF WORK

The transcription factor SOX2 was proved to be amplified in different types of tumors including small cell lung cancer and many other forms of carcinomas, the aim of our study is to examine SOX2 expression in synovial sarcoma and compare the expression in this specific soft tissue tumor to other soft tissue tumor entitites including fibrosarcoma, rhabdomyosarcoma, liposarcoma and undifferentiated pleomorphic sarcoma.

We are also interested in testing and trying to analyze the Sox2 gene status, in positive cases, through further fluorescence in situ hybridization technique.

Our study also aims to further investigate a SOX2- H3K27me3 relationship if present.

The study attained an approval from the ethics committee of Friedrich-Schiller University of Jena, Faculty of medicine; approval number 5318-10/17.

4. Publication Original Work

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Stem cell transcription factor SOX2 in synovial sarcoma and other soft tissue tumors

Heba Zayed[a,b], Iver Petersen[a,*]

[a] Institute of Pathology, Jena University Hospital, Germany
[b] National Cancer Institute, Cairo University, Egypt

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ABSTRACT

Background: SOX2 has gained considerable interest as a pluripotency inducing gene. Co-transfection of SOX2 together with NANOG, KLF4 and c-MYC into adult fibroblasts was able to generate pluripotent stem cells. SOX2 has been reported to be expressed in synovial sarcoma, a tumor being characterized by the SS18-SSX gene fusion forming part of the SWI/SNF chromatin remodeling complex that affects histone methylation. The role of SOX2 in this tumor type as well as other soft tissue tumor entities however is still poorly characterized. We analyzed SOX2 protein expression in soft tissue tumors. Alongside we tested Histone H3 expression (H3K27me3) in SOX2 positive cases to investigate this epigenetic mark and its correlation with the SOX2 status and clinicopathological parameters.

Methodology: In total, 60 samples of synovial sarcomas from the reference center for soft tissue tumors at the institute of pathology of the Jena University hospital were included into the study along with 343 other tissue tumors. Protein analysis was done by immunohistochemistry of tissue microarrays. All synovial sarcoma cases were confirmed by molecular testing using SS18 FISH break apart probes.

Results: SOX2 reactivity was detectable in 35 synovial sarcoma cases (58.3%) while 25 (41.7%) were negative. Only 13 cases of the other 343 soft tissue tumors, varying from nodular fasciitis to undifferentiated pleomorphic sarcoma, revealed a SOX2 expression, 12 out of these were undifferentiated high grade sarcoma. There was no obvious correlation with the clinicopathological data. H3K27me3 immunohistochemistry of the synovial sarcoma cases revealed a high statistically significant correlation between SOX2 and H3K27me3 expression ($p < 0{,}0005$, Chi square test). Similar to SOX2, there was no correlation between H3K27me3 expression and tumor grade. Six SOX2 positive synovial sarcoma cases were analyzed by FISH using a SOX2/CEN3 dual color FISH probe. None of these cases revealed an amplification of the SOX2 gene.

Conclusion: The data confirms previous studies reporting SOX2 and H3K27me3 expression in synovial sarcoma and reveals that both biomarkers are related to each other. It strengthens the notion that the tumor type is driven by epigenetic processes similar to those that are operating in pluripotent stem cells. The relevance of these parameters in the pathway pathology of synovial sarcoma, i.e. the timing and dosing of SOX2 and H3K27me3 expression initiated by the SS18-SSX driver mutation together with the interplay of these events with other signaling pathways, cellular mechanisms and additional mutations in tumor progression, will require further studies.

1. Introduction

1.1. Soft tissue tumors and synovial sarcoma

Soft tissue tumors constitute a large and heterogeneous group of neoplasms [1]. Traditionally, soft tissue sarcomas have been classified according to a histogenic concept (e.g., fibrosarcoma as a tumor arising from fibroblasts, osteosarcoma as a tumor arising from osteoblasts, and so on). However morphologic, immunohistochemical and data from experimental animals suggest that most if not all sarcomas arise from primitive pluripotent mesenchymal cells, which in the course of neoplastic transformation undergo differentiation in one or more lines [2]. Correct diagnosis is of great importance to the patient to obtain the adequate therapy. Since misdiagnoses are not uncommon, specialist centers provide valuable resources for the verification of suspected malignant mesenchymal tumors [3,4].

Synovial sarcoma is a rare and aggressive soft tissue tumor that accounts for approximately 10% of soft tissue sarcomas and classically

* Corresponding author. Present address: Institute of Pathology, SRH Wald-Klinikum Gera, Strasse des Friedens 122, D-07548 Gera, Germany.
E-mail address: iver.petersen@gmail.com (I. Petersen).

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occurs in the extremities of young adults [5,6]. It occurs at any age but the peak incidence is between the ages of 10 and 35 years; with a slight male predominance. The anatomic distribution is wide, but more than 60% arise in the lower limb [7]. A small but significant proportion of cases arise on the trunk, especially in the abdominal wall [8], in the neck [9] in the head [10], in the mediastinum [11] and even in the abdominal cavity [12]. In addition, many other localization have been reported, the largest cohort in this regard has been reported by our group [13].

Overall 5-year survival probability is about 60–65% but falls to only around 30% at 10 years. In general, small tumor size (< 5 cm), early clinical stage, early age at presentation (< 10 years) and lower histologic grade (as defined by mitotic activity and necrosis) are signs of a better prognosis [14]. Synovial sarcoma falls into two main groups, monophasic composed entirely of spindle cells and biphasic showing both epithelial and spindle cell components. The monophasic variant is more common [1]. Approximately 5–10% of cases have a poorly differentiated appearance, most often characterized by undifferentiated round cell morphology. These appear to be relatively more frequent in elderly patients with synovial sarcoma [15].

Immunohistochemically, in addition to positive staining in obvious epithelial components, in almost all cases the spindle cell element also shows at least focal positivity for epithelial membrane antigen (EMA) and keratin; this, combined with morphological clues, is generally the best way to distinguish monophasic lesions from malignant peripheral nerve sheath tumor (MPNST) or fibrosarcoma. Around 30% of cases of synovial sarcoma are S100 protein positive, similarly at least two thirds of synovial sarcomas stain positively for CD99 [16].

Cytogenetically, both biphasic and monophasic forms as well as poorly-differentiated tumors share a reproducible tumor-specific chromosome translocation, t(X;18) (p11.2; q11.2). This translocation creates an in-frame fusion and the production of one or the other of two principal fusion genes, SYT-SSX1 and SYT-SSX2 [17,18]. The abbreviation of the SYT gene was later changed to SS18. The translocation and gene fusion is demonstrable in virtually all synovial sarcoma cases but not found in any other human neoplasms. Whereas SS18, through its interactions with the SWI/SNF complex, might be expected to have a role in transcriptional activation, its fusion partner SSX associates with the polycomb repressor complex, which has opposing effects. An early observation was that SS18–SSX localizes at discrete nuclear foci within BMI1-labeled polycomb bodies [19]. More recently chromatin immunoprecipitation sequencing (ChIPSeq) results from HA-FLAG–tagged SS18–SSX, expressed in transfected C2C12 mouse myoblasts [20], correlated SS18–SSX binding with polycomb-marked nucleosomes (trimethylated histone H3K27) at a subset of genomic H3K27me3 sites.

1.2. Stem cells, induced pluripotent stem cells and SOX2

While cancer is defined by DNA mutations, differentiation and development of normal cells and tissues are governed by epigenetic modifications. In 2006, Shinya Yamanaka was the first to successfully reprogram cells using four distinct factors, thereby generating induced pluripotent stem cells (iPSCs) from terminally differentiated fibroblasts. IPSCs can be established by the over-expression of four key transcription factors, OSKM: Oct4, SOX2, Klf4 and c-Myc [21]. One of the major advantages of iPSCs is that they can be made autologously and can provide a sufficient quantity of cells by culturing, making the use of other stem cell sources unnecessary [22]. It has been demonstrated that reprogramming factor expression results in dysplasia and tumor formation in vivo, thus suggesting that OSKM has an impact on epigenetic changes that are substantially involved in the regulation of cell growth and tumorigenesis [23]. This observation is corroborated by the fact that pluripotent embryonic stem cells form teratomas upon implantation in vivo [24]. Of note, human iPSCs develop teratomas more efficiently and faster than human embryonic stem cells [25].

SRY (sex determining region Y)-box2, also known as SOX2 and being located on chromosome 3q26.33, is a transcription factor that is essential for maintaining self-renewal or pluripotency of undifferentiated embryonic stem cells and plays a critical role in maintenance of embryonic and neural stem cells [26,27]. SOX2 amplification has been found in several cancer types including glioblastoma, small-cell lung cancer (SCLC) and many forms of squamous cell carcinoma [28–30]. SOX2 has been shown to promote cellular proliferation in breast, prostate, pancreatic, cervical cancers as well as synovial sarcoma [31,32], evade apoptotic signals in prostate, gastric cancer and non-small cell lung carcinoma and promote invasion, migration and metastases in melanoma, colorectal glioma, gastric, ovarian cancer and hepatocellular carcinoma [33,34].

The aim of the present study was to evaluate the status of SOX2 in soft tissue tumors and in particular synovial sarcoma given the fact that expression has been observed in this entity [32,35].

2. Materials and methods

2.1. Tumor samples

Tissue samples of all synovial sarcoma cases between January 2013 and December 2015 were selected. Both internal patients of Friedrich-Schiller University hospital of Jena, as well as referred cases to the Institute of Pathology from other hospitals or pathology institutes were included. The Institute of Pathology of the University hospital of Jena became a national consultation and reference center after the German reunification in 1989. Not only German but also Austrian pathology institutes submit difficult cases to confirm previous diagnoses or to evaluate the suspicion of a soft tissue tumor, STT [4].

All the specimens (n = 60), excluding some referred cases whose histopathological material was not anymore available in the institute (n = 6) and cases (n = 4) whose paraffin block was not optimal for appropriate further material retrieval, were employed in this study. The clinicopathological analysis was conducted by highly specialized soft tissue tumors expert pathologists in the referral center. Immunohistochemical analysis was performed according to standard procedures to confirm the diagnosis (EMA, Bcl2, PanCK, CK7, CD34, Ki67 and S100). Molecular confirmation was performed by FISH to confirm the t(X;18) translocation (Fig. 1).

Tissue Microarrays (TMA) of variable soft tissue tumors were also prepared in the institute and employed in this study [36]. For the construction of these, a morphologically representative region of the "donor"-paraffin blocks with soft tissue tumor was selected. From this representative region two core biopsies (diameter, 0.6 mm; height 3–4 mm) from the invading front were taken and arrayed into a new "recipient" paraffin block using a custom-built instrument [37]. After the block production was finished, 4.0-µm sections of the resulting tumor TMA block were cut for further analysis as recently described [36]. Tissue microarrays constructed included 343 soft tissue tumors (Table 2).

2.2. Immunohistochemistry

SOX2 and Histone HEK27me3 immunohistochemical staining was performed according to standard procedures using monoclonal antibodies (Anti-Human SOX2 Monoclonal Antibody, 1:100, clone SP76, Zytomed Systems; H3K27me3 antibody, mAbcam 6002, 1:200) and the recommendations of the manufacturer. SOX2 immunohistochemistry was evaluated in two cores per tumor. The average percentage was taken for statistical analysis.

Extranuclear SOX2 staining was regarded to be negative or unspecific. Staining intensity (SI) was assessed to be negative (−), weak (1+), moderate (2+) or strong staining (3+). Reactivity (R) was determined by the percentage of positive tumor cells (PP) and scored as follows: negative (0), 1–10% positive cells (1), 11–30% (2), 31–50% (3), 51–80% (4) and > 80% positive cells (5). Intense/Reactivity score

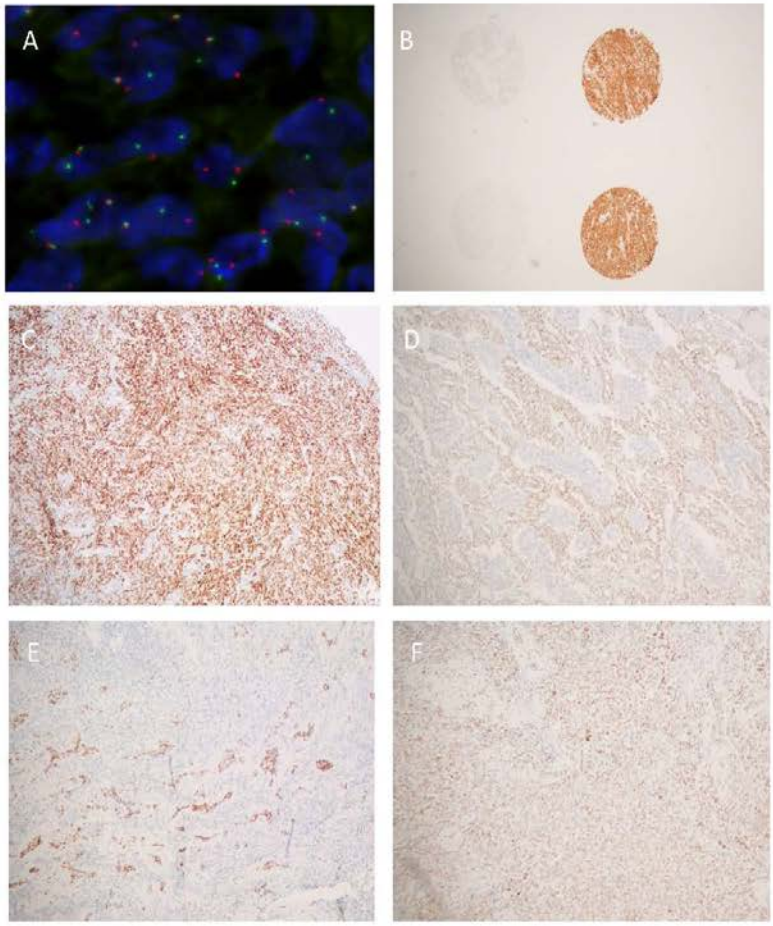

Fig. 1. SOX2 and H3K27me3 in soft tissue tumors.
A – SS18 FISH analysis of a synovial sarcoma illustrating split red and green signals in 82% of cells indicative for a t(X;18) translocation. B – Tissue microarray with one negative (left) and one positive (right) – SOX2 high grade pleomorphic sarcoma. C – Synovial sarcoma showing strong SOX2 expression (3+). D – Synovial sarcoma showing moderate SOX2 expression (2+). E – Synovial sarcoma showing weak SOX2 expression (1+). F – Histone H3K27me3 expression in a SOX2-positive synovial sarcoma.

(IRS) was calculated by multiplying PP with SI (minimum 0/maximum 15). High expression was defined as ≥8 according to the median IRS of positive stained cells.

SOX2 positive cases were further investigated for Anti-Histone H3 reactivity (H3K27me3 antibody, mAbcam 6002, 1:200). This was scored semi quantitatively as − negative; 1+ weak; 2+ moderate; 3+ strong positive.

2.3. Molecular confirmation of synovial sarcoma and SOX2-positive synovial sarcoma cases

The t(X;18) translocation hallmark of synovial sarcoma was verified by dual color fluorescence in situ hybridization (dc FISH). For this, either interphase nuclei were isolated from paraffin-embedded tumor tissue and prepared as described or tissue sections were used [38,13]. The dcFISH was performed using SPEC SS18 (18q11.2) Dual Color Break Apart Probe (Zytovision, Germany) applying the protocol provided by manufacturer. Fifty nuclei were analyzed for chromosomal rearrangements of the SYT gene region located on chromosome 18 using a laser scanning microscope LSM510 (Zeiss, Jena, Germany). DcFISH was assessed positive if at least 10% of the nuclei showed a translocation specific hybridization pattern [39].

SOX2 amplification was examined in some SOX2 positive synovial sarcoma cases, using SPEC SOX2/CEN3 Dual color Probe (Zytovision, Germany) following the protocol of the manufacturer.

2.4. Statistical methods

Data management and statistical analysis were performed using the Statistical Package for Social Sciences (SPSS) version 21. Numerical data were summarized using means and standard deviations or medians and ranges. Categorical data were summarized as percentages. Comparisons between the 2 groups with respect to normally distributed numeric variables were done using the *t*-test. For categorical variables, differences were analyzed with χ^2 (chi square) test and Fisher's exact test when appropriate. All *p*-values are two-sided. P-values < 0.05 were considered significant.

Table 1
SOX2 expression in synovial sarcoma.

	SOX2					P value
		Negative (n = 25)		Positive (n = 35)		
		No.	%	No.	%	
Age (yrs.)	Mean ± SD	46.1 ± 11.0		38.9 ± 18.5		0.066
Gender	Female	14	46.7	16	53.3	0.432
	Male	11	36.7	19	63.3	
Grade	I	0	0.0	2	100.0	0.465
	II	19	44.2	25	55.8	
	III	6	42.9	8	57.1	
Site	Extremities	13	39.4	20	60.6	0.693
	Others	12	44.4	15	55.6	
Ki67	Low Activity	16	44.4	20	55.6	0.593
	Moderate–High	9	37.5	15	62.5	
Translocation (%)	Mean ± SD	73.7 ± 21.7		77.9 ± 16.5		0.388

3. Results

3.1. Immunohistochemistry

Expression of SOX2 was investigated in 60 synovial sarcoma cases of which 2 where grade 1, 44 grade 2 and 14 grade 3. The SOX2 reactivity was positive in 35 cases (58.3%) and negative in 25 (41.7%). The SOX2 status (positive versus negative) as well as the percentage of positive cells revealed no significant correlations with clinicopathological parameters. Examples of the immunohistochemical assessment of SOX2 staining in synovial sarcoma are shown in Fig. 1. The results are summarized in Table 1. All grade 1 cases were positive (2/2), 57% of grade 2 were positive (25/44) and 57% of grade 3 as well were positive (8/14). The proliferative activity of the synovial sarcoma tumors, portrayed through Ki67 activity, indicated that 56% of the tumors exhibiting low proliferative activity were SOX2 positive (20/36) while 63% of the tumors with moderate to high proliferation revealed SOX2 positivity (15/24).

In addition, 343 soft tissue tumors, varying from nodular fasciitis to undifferentiated pleomorphic sarcoma, were analyzed in this study and revealed minimal SOX2 expression. Only 13 cases (3.8%) were positive, 12 (92.3%) out of these were undifferentiated high grade sarcoma (Table 2). The twelve patients of undifferentiated high grade pleomorphic sarcoma ranged in age from 29 to 79 years with female predominance (females = 8, males = 4). Analyzing the clinical data, there were no obvious correlations with clinicopathological data: 6 cases were from the lower extremity, 4 from the trunk, 1 form the head and neck region (occipital) and 1 other tumor was located in the skin. Still, it is interesting that all SOX2 positive tumors were high grade sarcomas.

The positive and negative synovial sarcoma cases were further subjected to Anti-Histone H3K27me3 immunohistochemistry. The expression data was related to the SOX2 reactivity as illustrated in Table 3. The statistical analysis revealed a high statistically significant correlation between SOX2 and H3K27me3 expression ($p < 0.0005$, Chi square test). Out of 35 SOX2 positive synovial sarcoma cases, 25 (71, 4%) were H3K27me3 positive and 10 (28, 6%) were negative. Concurrently, out of 25 SOX2 negative cases, only 3 (12%) were H3K27me3 positive and 22 (88%) were negative. Similar to SOX2, there was no correlation between H3K27me3 expression and tumor grade in synovial sarcoma. An example of the immunohistochemical assessment of Histone (H3K27me3) in a SOX2-positive synovial sarcoma is shown in Fig. 1.

Table 2
SOX2 expression in other soft tissue tumor types (non-synovial sarcoma).

Diagnose	SOX2			
	Negative		Positive	
	Number	%	Number	%
Adult fibrosarcoma	1	0.3	0	0.0
Alveolar rhabdomyosarcoma	1	0.3	0	0.0
Alveolar soft part sarcoma	3	0.9	0	0.0
Dedifferentiated liposarcoma	3	0.9	0	0.0
Endometrial stromal sarcoma, low grade	2	0.6	0	0.0
Epithelioid sarcoma	2	0.6	0	0.0
Extraskeletal myxoid chondrosarcoma	1	0.3	0	0.0
Inflammatory myofibroblastic tumour	1	0.3	1	7.7
Ischaemic fasciitis	3	0.9	0	0.0
Low grade fibromyxoid sarcoma	10	3.0	0	0.0
Low grade myofibroblastic sarcoma	32	9.7	0	0.0
MPNST	1	0.3	0	0.0
Myositis ossificans	4	1.2	0	0.0
Myxoinflammatory fibroblastic sarcoma	3	0.9	0	0.0
Nodular fasciitis	66	20.0	0	0.0
Proliferative fasciitis	9	2.7	0	0.0
Undifferentiated high grade pleomorphic sarcoma	179	54.2	11	84.6
Undifferentiated pleomorphic sarcoma with giant cells	7	2.1	1	7.7
Undifferentiated pleomorphic sarcoma with prominent inflammation	2	0.6	0	0.0
Non-synovial sarcoma soft tissue tumors	330 (96.2%)		13 (3.8%)	

3.2. FISH analysis of synovial sarcoma cases

Fluorescence in situ hybridization (FISH) detection of t(X;18) translocation of synovial sarcoma was carried out in all the 60 cases. All samples showed a split apart signal using a SS18/SYT dual color probe (Fig. 1). The percentage of positive cells varied from 44% to 96%. Of the immunohistochemically SOX2 positive synovial sarcoma cases, six were randomly selected and analyzed by FISH using a SOX2/CEN3 dual color FISH probe. None of these cases, revealed an amplification of the SOX2 gene.

Table 3
Histone H3K27me3 expression in correlation with SOX2 reactivity in synovial sarcomas.

			SOX2		Total
			Negative	Positive	
H3K27me3	Negative	Count	22	10	32
		% of Total	36.7%	16.7%	53.3%
	Positive	Count	3	25	28
		% of Total	5.0%	41.7%	46.7%
Total		Count	25	35	60
		% of Total	41.7%	58.3%	100.0%

4. Discussion

4.1. Role of SOX2 in synovial sarcoma and other cancer types

Synovial sarcoma is classified as a soft tissue tumor of uncertain cellular origin [1]. It may be derived from primitive mesenchymal cells that can undergo differentiation and has been characterized as a stem cell malignancy [40,41]. In the last decades, a major change has taken place in cancer biology in emphasizing the importance of cancer stem cells and their relationship to embryonic stem (ES) cells whose biology is governed by stem cell transcription factors like SOX2. Embryonic stem cells proliferate without apparent limit, they can readily be propagated clonally and are not subject to contact inhibition or anchorage dependence. These are typical features of transformed cells and, indeed, ES cells are tumorigenic. Thus ES cells can be considered as conditional tumor cells [42]. The accumulated understanding of the mechanisms underlying pluripotency in ES cells led to attempts to revert somatic cells into a pluripotent state using the Oct3/4, SOX2, Klf4 and c-Myc reprogramming factors. SOX2 is an essential transcription factor, which not only has a role during neurogenesis and embryonic foregut development, but also allows reprogramming of adult cells to pluripotent stem cells [43]. Evidence has recently been accumulating to support the hypothesis that solid tumors contain a small subpopulation of cells called cancer stem-like cells, which exhibit self-renewing capacities and are responsible for tumor maintenance and metastases [44]. SOX2 has been suggested as a marker for cancer stem cells in various tumor types [45,30].

The objective in our study was (1) to examine SOX2 expression in synovial sarcoma, (2) to compare the expression in synovial sarcoma to that in other soft tissue tumors and (3) to analyze mechanisms with impact on SOX2 expression like SOX2 gene status and H3K27me3 expression in synovial sarcoma. The study was carried out on 60 cases of synovial sarcoma and 343 cases of different other soft tissue tumors that were referred to the Jena consultation and reference center of soft tissue tumors. A wide panel of biomarkers was used to analyze and confirm the diagnosis of all included soft tissue tumors. Standard, full-sized tissue sections were used to construct tissue microarrays (TMAs) for analyzing SOX2 and other biomarkers.

About 60% of all synovial sarcoma cases were positive to SOX2. All grade 1 cases were positive (2/2) while less than 60% of grade 2 and grade 3 cases revealed SOX2 expression. Similarly, there was only a minor difference of SOX2 positivity in the low proliferative group (56%) versus moderately to highly proliferating tumors (63%). Together with the fact that the majority of undifferentiated sarcomas were SOX2 negative, this suggests that SOX2 does not seem to be essential for tumor progression, but may play an essential role in tumor initiation.

The relationship between SOX2 expression and tumor behavior is highly controversial. It is intriguing that some studies reported better tumor behavior with SOX2 expression. Züllig and colleagues were able to study the process of early lymphatic metastases in squamous cell carcinoma (SCC) of the oral cavity and demonstrated a significant association between high cancer cell-expressed SOX2 protein and significant lower risk of lymph node metastases [46]. According to them, this result is consistent with findings in lung SCC, reporting high SOX2 protein expression levels and SOX2 amplification to be correlated with better overall survival [47]. This is consistent with earlier data from the group of Perner on lung SCC, they reported that SOX2 amplification and overexpression was associated with better outcome [48]. In contrast, Neumann et al. correlated elevated SOX2 expression with lymph-node metastasis and distant spread of right-sided colon cancer in a matched pair collection of 57 carcinomas with distant spread and 57 cases without metastasis. Overall high SOX2 expression was reported in 21.1% of cases. Surprisingly, absence of SOX2 was associated with advanced T-category, T3/T4 [49]. In our study, SOX2 reactivity did also not correlate with higher tumor grades.

SOX2 amplification is quite characteristic for SCC pathogenesis and this can be correlated with the fact that lung SCC similar to SCC from other organs typically has a 3q overrepresentation/amplification. In contrast, adenocarcinomas of the lung harbor much less SOX2 amplifications [28]. It should be noted that alterations of chromosome 3, in particular 3p deletions being frequently associated with 3q gains, are considered early events in head and neck as well as lung carcinogenesis [50,51]. Interestingly, it has been shown that SOX2 and PIK3CA, both located at 3q26-q28 and generally coamplified in lung SCC, cooperate in the transition of lung dysplasia into cancer [52].

In SCLC, SOX2 amplifications were reported in 27% of cases being correlated with SOX2 expression. Inhibition of SOX2 protein expression by transfection of short hairpin RNA in SCLC cell lines with SOX expression resulted in reduced cell proliferation. It was hypothesized that SOX2 may represent a putative lineage-survival oncogene in SCLC. In addition, it was mentioned that induction of SOX2 in lung epithelial cells increased the number of neural progenitor cells [29].

Generally, SOX2 protein expression is widespread and was proven in the majority of primary SCC as well as breast cancer [53], testicular germ cell tumors [54], gastric [55] and pancreatic adenocarcinoma [56].

4.2. SOX2 regulation and its interplay with SSX-SS18, H3K27me3 and signaling pathways

In squamous lung and esophageal cancers, aberrant SOX2 expression was linked to the genomic amplification of its chromosomal location on chromosome 3q26.33. Chromosome 3q copy number gains are a common event in breast cancers and have been implicated as an independent predictor of poor prognosis in node-negative breast cancers [57]. Therefore some of the positive SOX2 synovial sarcoma cases were further subjected to a molecular study via FISH to detect amplification on the genetic level, but all were negative. None of the positive cases revealed an amplification, thus gene copy number alterations do not seem to play a role in SOX2 upregulation in this tumor entity.

This seems to be similar in some carcinoma subtypes. Claudia Lengerke and colleagues analyzed lymph node metastases of breast cancer to explore whether aberrant SOX2 expression is a result of gene amplification as reported in other carcinomas. Surprisingly, with the exception of one case of low level amplification in a score 3 primary tumor, the majority of analyzed samples did not show SOX2 gene amplifications, suggesting that at least in breast carcinomas expressing SOX2, the aberrant gene expression is mostly driven by other mechanisms [58].

Which other mechanisms have influence on SOX2 expression? A highly relevant study with respect to the interplay between the SS18-SSX fusion, histone modification and SOX2 expression was published in 2013 [32]. Kadoch and Crabtree reported that the SS18-SSX fusion protein of synovial sarcoma leads to alterations in the human SWI/SNF chromatin remodeling complex. Similar to SS18, the fusion protein incorporates into the SWI/SNF complex resulting in the exclusion of tumor suppressor gene BAF47 (also known as INI1 and SMARCB1), another component of the complex, resulting in its inactivation. The SWI/SNF complex antagonizes the activity of the Polycomb repressive complex 2 (PRC2) being responsible for trimethylation of lysine 27 of histone H3 (HeK27me3) by the methyltransferase EZH2 forming an essential component of the PRC2 complex and being considered a repressive mark for gene transcription [32,59]. This was actually the reason why we analyzed global H3K27me3 expression in our study.

Interestingly, we found that SOX2 expression was correlated with global H3K27me3 expression in synovial sarcoma. In contrast, Kadoch and Crabtree reported that the altered SWI/SNF complex in synovial sarcoma binds to SOX2 gene locus resulting in SOX2 activation by a local decrease in H3K27me3 [32]. This situation may be similar to seminoma in which a repressive H3K27me3 mark at the SOX2 locus is responsible for SOX2 repression while there seems to be H3K27me3

expression at least in a subset of neoplasms of this entity [60,61].

As mentioned, methylation of Histone H3 at the lysine 27 residue is mediated by the histone methyltransferase EZH2. Enhancer of zeste homologue 2 (EZH2) showed high expression in cells possessing embryonic gene expression signature, while its amount declines with tissue maturation and differentiation [62]. Abnormal overexpression of EZH2 has been reported in a wide variety of tumor types including carcinomas, lymphomas, cutaneous melanoma, and soft tissue sarcomas [63].

Studies have revealed a complicated interaction between SOX2 and the WNT signaling pathway. It was reported that SOX2 antagonized WNT signaling to inhibit the differentiation of adult stem cells and osteoblast lineage, it enhanced tumorigenesis and self-renewal property of osteosarcomas by promoting the transcription of negative regulators of WNT signaling [64]. Other pathways apart from Wnt/β-catenin signaling that have been associated with SOX2 are Hippo/YAP, Survivin/MAP4K4, EGFR/FOXO6, PI3K/Akt, Hedgehog and JAK/STAT [30,65]. It seems that SOX2 itself, like other SOX genes, does not possess sufficient affinity for DNA binding and that for transcription activity the recruitment of other protein partners like Nanog, OCT4 and Sall4 is required [30].

Kimura and colleagues recently explored specific markers and discovered that synovial sarcoma cell lines possessed heterogeneity by way of containing a sphere-forming subpopulation highly expressing Nanog, Oct4 and SOX2. By expression microarray analysis, CXCR4 was identified to be highly expressed in the sphere subpopulation and correlated with stem-cell associated markers [35]. According to their study, stem-cell associated markers including SOX2 and SS18/SSX were highly expressed in the sphere/forming population of synovial sarcoma, hence SS18/SSX and its sphere-specific binding proteins might regulate tumor-initiating cells via epigenetic and/or transcriptional deregulation.

4.3. Tumor progression of synovial sarcoma

High expression of EZH2 is generally associated with advanced stages of tumor progression, aggressive tumor behavior, and dismal clinical outcome [66]. In synovial sarcoma, endogenous EZH2 expression correlated with H3K27me3 at PcG target genes. It has been reported that high expression of EZH2 and H3K27me3 helps to distinguish poorly differentiated synovial sarcoma from monophasic and biphasic subtypes and is associated with unfavorable clinical outcome [41,67]. Our study does not provide such evidence, but it is important to mention the limitation of our tumor collective lacking data on metastatic spread and survival. Generally, the impact of H3K27me27 expression on cancer prognosis seems to be complex as there are also many studies reporting a better outcome in tumors with high expression, e.g. in colorectal and breast cancer [68,69].

Regarding synovial sarcoma, metastasis and tumor progression seem to be driven not only by epigenetic modifications like histone methylation but also additional chromosomal changes apart from the defining t(X;18)(p11.2;q11.2) translocation and the activation of other genes and pathways distinct from the SS18-SSX gene fusion event [6,70]. The CINSARC signature of 67 genes correlating with chromosomal instability and prognosis in undifferentiated sarcoma was also highly significant in stratifying synovial sarcoma with respect to metastatic outcome [71]. Specific microRNAs being detectable in the blood have been correlated with clinical outcome. And apart from EZH2, IGFBP7, specific matrix metalloproteases, Secernin-1, NY-ESO-1, the CXCR4 pathway as well as PI3K/AKT/mTOR and RAS/MAPK signaling have been associated with metastatic risk [70]. Furthermore, it is important to note that synovial sarcoma is characterized by a recurrent pattern of DNA methylation that can be used to separate and diagnose this entity against 50 other soft tissue tumor types using genome-wide methylation analysis and bioinformatical classification algorithms. The methylome analysis provides in addition a global gene copy profile which may help to establish prognostic subgroups of this entity [72,73].

In summary, our study confirms the importance of the SS18-SSX gene fusion and its downstream targets SOX2 and H3K27me3 in synovial sarcoma. The entity provides a paradigm of a tumor that is primarily driven by alterations in the epigenome. The strength of the SS18-SSX alteration lies in the fact that the fusion gene has influence on two major players in epigenetic regulation. On one hand it leads to the inactivation of tumor suppressive functions of the SWI/SNF nucleosome remodeling complex and on the other hand it changes Histone chromatin marks that leads to the activation of the cancer stem cell transcription factor SOX2 and other genes that are normally suppressed by the polycomb repressive complexes 1 and 2 [6,41,74]. The understanding of the pathway pathology of synovial sarcoma has advanced substantially in recent years and it is foreseeable that this will help in establishing effective therapy of this potentially lethal disease.

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5. DISCUSSION

Soft tissue sarcomas are rare and represent less than 1% of all cancer types. In recent years between 3500 and 3750 new cases were registered in Germany each year. They can roughly be subdivided into two groups: entities with single, well-characterized driver mutations and sarcomas with a complex genotype (Petersen 2017).

Synovial sarcoma is classified as a soft tissue tumor of uncertain cellular origin (Fletcher et al. 2013). It gets its name from its microscopic similarity and proximity to the synovium, but in reality the development of tumor cells is not necessarily of synovial origin. While it is a soft tissue tumor typically found in the arms or legs and usually close to tendon sheaths and joint capsules, it can also occur in other locations, such as the heart, brain, and prostate. Synovial Sarcoma accounts for 5%–10% of all STS (Spurrell et al. 2005) and 10%–20% of STS in adolescents and young adults (Nielsen et al. 2015).

Although its cellular origin is unclear, Synovial sarcoma (SS) is generally divided into two main histological subtypes: monophasic and biphasic. Monophasic SS is characterized by the presence of spindle cells and the absence or near-absence of glandular epithelial cells, whereas biphasic SS has equal presence of both spindle cells and glandular epithelial cells (Spurrell et al. 2005). In addition, monophasic SS displays fibrous and sarcomatous cells that are relatively uniform and small and form sheets. In contrast, biphasic SS presents with an epithelial appearance. Another characteristic of SS is the unique chromosomal translocation (t X;18), which results in fusion of the *SYT* gene to the *SSX1*, *SSX2*, or, on rare occasions, the *SSX4* gene (Nielsen et al. 2015).

Recently, major changes took place in the understanding of cancer biology, emphasizing the importance of cancer stem cells, and supporting the suggested theory that tumors arise from these cells rather than terminally differentiated cells.

Embryonic cells, whose biology is governed by stem cell transcription factors like SOX2, and cancer stem cells, are thought to be closely related. The Embryonic stem cells proliferate without apparent limit; they can be propagated clonally and are

not subject to contact inhibition or anchorage dependence. These are typical features of transformed or tumor cells and this is why embryonic stem cells can be considered as conditional tumor cells (Smith 2001). Evidence has recently been accumulating to support the hypothesis that solid tumors contain a small population of cancer-stem like cells, which exhibit self-renewing capacities and are responsible for tumor maintenance and metastases (Visvader and Lindeman 2008).

The aim of our study was to:

(1) Examine SOX2 expression in synovial sarcoma.

Transcription factor SOX2 was proved to be amplified in different types of tumors including small cell lung cancer and many forms of squamous cell carcinoma (Karachaliou et al. 2013)

(2) Compare the expression of SOX2 in synovial sarcoma to other soft tissue tumors.

To fulfill this aim, we analzyed tissue microarrays that were prepared in the institute of Pathology, Jena University and included a wide variety of soft tissue tumors ranging from Fibrosarcoma, Rhabdomyosarcoma, dedifferentiated Liposarcoma, undifferentiated pleomorphic sarcoma and many more.

(3) Analyze mechanisms that may affect SOX2 expression.

This included an analysis to understand the SOX2 gene status utilizing fluorescence in situ hybridization (FISH) technique and studying the H3K27me3 in synovial sarcoma.

Role of SOX2 in synovial sarcoma and other cancer types

The study was carried out on 60 cases of synovial sarcoma and 343 cases of the different other soft tissue tumors that were referred to the Jena consultation and reference center of soft tissue tumors. All the tumors were intensively histologically studied via at least two soft tissue tumors experts and were further subjected to a wide

panel of biomarkers to direct and confirm the histological diagnosis. All the synovial sarcoma cases were subjected to FISH to analyze the characteristic t(x;18) translocation.

The other 343 cases of various soft tissue tumor entities were employed in the production of the tissue microarrays.

Out of the above mentioned 60 synovial sarcomas, 35 (60%) were SOX2 positive and 25 (40%) were negative. The expression was variable but worth noticing was the fact that all the grade 1 tumors were positive while less than 60% of grade 2 and grade 3 revealed a positive staining. Similarly, there was a minor difference of SOX2 positivity in the low proliferative group (56%) versus moderately to highly proliferating tumors (63%). This suggests that SOX2 does not seem to be essential for tumor progression, but may play a role in the tumor initiation itself (Zayed & Petersen 2018).

This is also supported by the fact that the majority of the undifferentiated sarcomas were SOX2 negative in first place, again hinting that the transcription factor Sox2 is probably not essential in the tumor progression phase.

This was also stated by Abd El-Maqsoud et al. (2014) who identified Sox2 expression in ductal carcinoma in situ (DCIS) cases with a higher expression rate than seen in invasive carcinomas, suggesting a role of Sox2 in the initial stages of breast carcinogenesis. In this study, Sox2 expression was significantly associated with comedo type, negative hormone receptor status, and the triple-negative phenotype. However a positive association of Sox2 expression with high-grade DCIS was not reached.

The relationship between SOX2 expression and tumor behavior is highly controversial. The fact that some studies reported even better tumor behavior with SOX2 expression is to be highlighted. For example, in a study conducted by Züllig and colleagues, they managed to investigate and analyze the process of early lymphatic metastasisis in squamous cell carcinoma of the oral cavity and demonstrated a significant association between cancer cells that strongly expressed SOX2 and a lower risk of lymph node metastases. Lu, (2010) and colleagues also reported that patients with squamous tumors with expression of SOX2 mRNA above the median enjoyed a better prognosis than those with lower levels of expression.

This is also consistent with Wilbertz and colleagues who reported in squamous cell lung carcinoma that their data clearly demonstrate an association between elevated SOX2 expression and indicators of better patient outcome, most importantly prolonged overall survival. Furthermore, increased levels of SOX2 amplification indicate a better histological differentiation grade and a trend to improved patient survival.

Consistently, Bass et al.'s (2009) findings, indicated that patients with lung squamous cell carcinomas harboring an embryonic stem cell-like gene expression signature, including SOX2 expression, exhibited significant better survival than patients with tumors lacking this signature.

On the contrary, Neumann et al. demonstrated that increased expression of either SOX2 or nuclear β-catenin was associated with distant metastasis in right-sided colon cancer. Additionally, SOX2 was also associated with lymph-node metastases. According to them, this data underlined the importance of stemness-associated markers for the identification of colon cancer with a high risk for distant spread.

According to Russo et al., SOX2 overexpression upregulated pluripotency and epithelial-mesenchymal transition (EMT) transcription factors, along with growth, angiogenic and lymphangiogenic factors, and promoted prostate cancer cell invasiveness.

In our study, SOX2 reactivity did also not correlate with higher tumor grades. There was also no detectable relation with tumor proliferative activity which was assessed by Ki67 immunostaining.

SOX2 amplification

Sox2 amplification is characteristic for squamous cell carcinoma (SCC) pathogenesis and this can be correlated with the fact that lung SCC, similar to SCC from other organs, typically has a 3q overrepresentation/amplification. In contrast, adenocarcinomas of the lung harbor much less SOX2 amplifications (Karachaliou 2013). It should also be noted that alterations of chromosome 3, in particular 3p deletions being frequently associated with 3q gains, are considered early events in head and neck as well as lung carcinogenesis (Bockmühl et al. 1996 ; Petersen et al. 1997).

Interestingly, it has been shown that SOX2 and PIK3CA, both located at 3q26-q28 and generally coamplified in lung SCC; cooperate in the transition of lung dysplasia into cancer (Kim et al. 2016).

It was also reported that SOX2 regulates cell cycle-related genes positively or negatively. CDKN1A, which induces G1 arrest, is repressed by SOX2 in lung carcinoma cells, pancreatic cancer cells (Chen et al., 2012) and gastric cancer cells (Otsubo et al. 2008). CDKN1B, which also induces G1 arrest, is repressed by SOX2 in pancreatic cancer cells and gastric cancer cells. CCND1, which accelerates the cell cycle, is activated by SOX2 in gastric cancer cells and MCF7 breast cancer cells (Chen et al. 2008) SOX2 represses cell cycle inhibitors and activates cell cycle accelerators; however, the pattern of gene regulation is not universal in different cancer cell types.

In squamous cell lung carcinoma, SOX2 amplifications were reported in 27% of cases being correlated with SOX2 expression. Inhibition of SOX2 protein expression by transfection of short hairpin RNA in SCLC cell lines with SOX2 expression resulted in reduced cell proliferation. In addition, it was mentioned that induction of SOX2 in lung epithelial cells increased the number of neural progenitor cells (Rudin et al. 2012).

According to a study carried out by Gut et al. (2018) based on 55 squamous cell carcinomas of the vulva, SOX2 amplification was found in 20.8%; 27.3% of vulvar carcinomas showed SOX2 protein overexpression. SOX2 amplification was correlated with SOX2 overexpression in their data set ($P<0.01$). Amplification of the SOX2 locus was associated with high tumor grade ($P<0.05$) and human papillomavirus (HPV) positivity ($P<0.01$). SOX2-amplified tumors showed more frequently a basaloid phenotype than nonamplified carcinomas. SOX2 protein overexpression was also correlated with basaloid phenotype and positive HPV status of vulvar carcinomas.

SOX2 regulation and its interplay with SSX-SS18, H3K27me3 and signaling pathways

In squamous lung and esophageal cancers, aberrant SOX2 expression was linked to the genomic amplification of its chromosomal location on chromosome 3q26.33. Chromosome 3q copy number gains are a common event in breast cancers and have

been implicated as an independent predictor of poor prognosis in node-negative breast cancers (Janssen et al. 2003)

Therefore some of the positive SOX2 synovial sarcoma cases were further subjected to a molecular study via FISH to detect amplification on the genetic level, but all were negative. None of the positive cases revealed an amplification, thus gene copy number alterations do not seem to play a role in SOX2 upregulation in this tumor entity (Zayed and Petersen 2018).

Our results were consistent with Lengerke and colleagues (2011) who analyzed lymph node metastases of breast cancer to explore whether aberrant SOX2 expression is a result of gene amplification as reported in other carcinomas. With the exception of one case of low level amplification in a score 3 primary tumor, the majority of analyzed samples did not show SOX2 gene amplifications, suggesting that at least in breast carcinomas expressing SOX2, the aberrant gene expression is mostly driven by other mechanisms.

Different studies were conducted in attempt to understand the SOX2 overexpression mechanism and associated genetic alterations. In 2013, the interplay between SS18-SSX fusion, histone modification and SOX2 expression was highlighted by Kadoch and Crabtree (2013). They reported that the SS18-SSX fusion protein of synovial sarcoma leads to alterations in the human SWI/SNF chromatin remodeling complex. Similar to SS18, the fusion protein incorporates into the SWI/SNF complex resulting in the exclusion of the tumor suppressor gene BAF47 (also known as INI1 and SMARCB1), another component of the complex, resulting in its inactivation. The SWI/SNF complex antagonizes the activity of the Polycomb repressive complex 2 (PRC2) being responsible for trimethylation of lysine 27 of histone H3 (HeK27me3) by the methyltransferase EZH2 forming an essential component of the PRC2 complex and being considered a repressive mark for gene transcription (Kadoch et al. 2016). This was actually the reason why we considered analyzing global HeK27me3 expression in our synovial sarcoma cases.

The statistical analysis revealed a high statistically significant correlation between SOX2 and H3K27me3 expression ($p<0.0005$, Chi square test). Out of 35

SOX2 positive synovial sarcoma cases, 25 (71, 4%) were HeK27me3 positive and 10 (28, 6%) were negative (Zayed and Petersen 2018). Kadoch and Crabtree also reported that the altered SWI/SNF complex in synovial sarcoma binds to SOX2 gene locus resulting in SOX2 activation by a local decrease in H3K27me3. This situation may be similar to seminoma in which a repressive H3K27me3 mark at the SOX2 locus is responsible for SOX2 repression while there seems to be H3K27me3 expression at least in a subset of neoplasms of this entity (Kushwaha et al. 2016; Kristensen et al. 2012).

Surface and colleagues (2010) reported that methylation of Histone H3 at the lysine 27 residue is mediated by the histone methyltransferase EZH2. Enhancer of zeste homologue 2 (EZH2) showed high expression in cells possessing embryonic gene expression signature, while its amount declines with tissue maturation and differentiation. Abnormal overexpression of EZH2 has been reported in a wide variety of tumor types including carcinomas, lymphomas, cutaneous melanoma, and soft tissue sarcomas (Chang and Hung 2012).

Kimura and colleagues (2016) also explored specific markers and discovered that synovial sarcoma cell lines possessed heterogeneity by way of containing a sphere-forming subpopulation highly expressing Nanog, Oct4 and SOX2. By expression microarray analysis, CXCR4 was identified to be highly expressed in the sphere subpopulation and correlated with stem-cell associated markers. According to their study, stem-cell associated markers including SOX2 and SS18/SSX were highly expressed in the sphere/forming population of synovial sarcoma, hence SS18/SSX and its sphere-specific binding proteins might regulate tumor-initiating cells via epigenetic and/or transcriptional deregulation.

Generally, the impact of H3K27me27 expression on cancer prognosis seems to be complex as there are also many studies reporting a better outcome in tumors with high expression, e.g. in colorectal and breast cancer (Bae et al. 2014; Benard et al. 2014).

Tumor progression of synovial sarcoma

In synovial sarcoma, endogenous EZH2 expression correlated with H3K27me3 at PcG target genes. It has been reported that high expression of EZH2 and H3K27me3 helps to distinguish poorly differentiated synovial sarcoma from monophasic and biphasic subtypes and is associated with unfavorable clinical outcome (Changchien et al. 2012). However our study does not provide such evidence, but it is important to mention the limitation of our tumor collective lacking data on metastatic spread and survival.

Metastasis and tumor progression in synovial sarcoma seem to be driven not only by epigenetic modifications like histone methylation but also additional chromosomal changes apart from the defining t(X;18)(p11.2;q11.2) translocation and the activation of other genes and pathways distinct from the SS18-SSX gene fusion event (de Necochea-Campion et al. 2017). The CINSARC signature of 67 genes correlating with chromosomal instability and prognosis in undifferentiated sarcoma was also highly significant in stratifying synovial sarcoma with respect to metastatic outcome (Lagarde et al. 2013).

Future sarcoma diagnostics

Recent studies assure the inevitable importance of understanding tumors' specific genetic mutations and utilizing this aspect in diagnostic measures as well as future elaborated gene based therapeutic regimes. For example in our study we clearly demonstrated the importance of the SS18-SSX gene fusion and its downstream targets SOX2 and H3K27me3 in synovial sarcoma. The entity provides a paradigm of a tumor that is primarily driven by alterations in the epigenome. The strength of the SS18-SSX alteration lies in the fact that the fusion gene has influence on two major players in epigenetic regulation. This leads to the inactivation of tumor suppressive functions of the SWI/SNF nucleosome remodeling complex and changes Histone chromatin marks that leads to the activation of the cancer stem cell transcription factor SOX2 as well as other genes that are normally suppressed by the polycomb repressive complexes 1 and 2 (Banito et al. 2018).

In a study conducted by Koelsche and colleagues (2018) they stated that undifferentiated solid tumors with small blue round cell histology and expression of CD99 mostly resemble Ewing sarcoma; however, this group of smal round cell tumors may also include other tumors such as mesenchymal chondrosarcoma, synovial sarcoma, or small cell osteosarcoma. They assured that definitive classification usually requires detection of entity-specific mutations. Hence they generated genome-wide DNA-methylation profiles of 30 small blue round cell tumors not otherwise specified: 14 (47%) assigned to Ewing sarcoma, 6 (20%) to small blue round cell tumors with CIC alteration, 4 (13%) to small blue round cell tumors with BCOR alteration, which is a methylation group composed of small blue round cell tumors with BCOR–CCNB3 fusion and clear cell sarcoma of the kidney with BCOR internal tandem duplication, two (7%) to synovial sarcomas, two (7%) to malignant rhabdoid tumors, and one (3%) to mesenchymal chondrosarcomas.They also assured that genetic analyses validated the predicted sarcoma subtypes in most cases.

It is important to note that synovial sarcoma is characterized by a recurrent pattern of DNA methylation that can be used to separate and diagnose this entity against 50 other soft tissue tumor types using genome-wide methylation analysis and bioinformatical classification algorithms. The methylome analysis provides in addition a global gene copy profile which may help to establish prognostic subgroups of this entity (Petersen 2017; Köelsche et al. 2015).

6. CONCLUSION

Our study proved a significant SOX2-expression in about 60% of the 60 synovial sarcoma cases investigated, whereas other soft tissue tumor entities showed very sporadic insignificant expression in only 13 out of 343 variable tumors.

Alongside our findings support other studies reporting SOX2 and H3K27me3 expression in synovial sarcoma which reveals that both biomarkers are related to each other.

This fact strengthens the notion that the tumor type is driven by epigenetic processes similar to those that are operating in pluripotent stem cells. The relevance of these parameters in the pathway pathology of synovial sarcoma, i.e. the timing and dosing of SOX2 and H3K27me3 expression initiated by the SS18-SSX driver mutation together with the interplay of these events with other signaling pathways, cellular mechanisms and additional mutations in tumor progression, will require further studies.

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8. Appendix

Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der FriedrichSchiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Professor Dr. Iver Petersen,

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe

und dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Ort, Datum Unterschrift des Verfassers

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