Examination of synovial sarcoma in *in vitro* miR-206 modified model system and liquid biopsy samples

PhD thesis

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1. INTRODUCTION

Synovial sarcoma (SS) as the third most common malignant soft-tissue tumor belongs to the group of tumors of uncertain differentiation. It can occur at any site of the body. The tumor usually affects young adults and teenagers and is associated with a poor prognosis. Histologically, SS is monophasic or biphasic. SS belongs to *SMARCB1*-deficient malignant soft tissue tumors.

SMARCB1 gene encodes a core subunit of the SWI/SNF chromatin-remodeling complex. *SMARCB1* is considered as a classical tumor suppressor gene. Biallelic mutation of *SMARCB1* gene was first described in malignant rhabdoid tumors (MRTs). Epithelioid sarcomas (ES) are also SMARCB1 immunonegative tumors. In our previous work we found that ESs do not harbor biallelic genetic alterations of *SMARCB1* gene. We demonstrated in this study that loss of SMARCB1 protein expression is caused by the silencing effect of micro-RNAs (miRNAs). In biphasic SS the spindle cell component is characterized by weak or negative immunostaining for SMARCB1 while the epithelial cells retain their SMARCB1 protein expression. The reason behind this can be the pathognomonic SS18-SSX fusion protein or rather epigenetic modification by miRNAs: overexpression of miR-206, miR-381 and miR-671-5p previously described in SS by our research group. MiR-206 through overexpression can act as oncomiRNA (oncomiR) and through downexpression can act as a suppressor miRNA (suppressor miR). The most potent silencer of the mRNA of *SMARCB1* was miR-206 during our previous works thus our attention turned to this miRNA.

Detection of circulating tumor cells (CTCs) and cell free nucleic acids from peripheral blood has been examined in across a wide spectrum of tumor types. While liquid biopsy is a popular diagnostic tool of detection of minimal residual disease in colon carcinoma or lung cancer few studies have investigated in sarcomas and their results were controversial.

2. OBJECTIVES

Our aim was to examine the lack of expression of *SMARCB1* tumor suppressor on mRNA and protein level using *in vitro* cell culture models. Using a newly established cell line, called SS-iASC we wanted to clarify in synovial sarcoma how the pathognomonic fusion gene (*SS18-SSX*) as genetic alteration and overexpression of miR-206 as epigenetic alteration influence the expression of *SMARCB1* in *in vitro* model systems.

Besides we also would like to analyze the presence of *SS18-SSX* chimeric gene in peripheral blood samples of SS patients who already underwent therapy.

Our main aims and hypotheses were the following:

1. We wanted to prove the expression of *SS18-SSX1* on mRNA and protein level in SS-iASC cells. We also wanted to examine the immunophenotype of the cell line.

2. After our previous *in vitro* transient miR-206 transfection experiments we planned permanent miR-206 transfection in non-tumorous and tumorous cell lines (HDFα, HT-1080 and Caco2) and two genetically modified cell line (iASC and SS-iASC). We planned to determine the expression of *SMARCB1* and other miR-206 target genes (*ACTL6A*, *CCND1*, *POLA1*, *MET*, *NOTCH3*, *G6PD*) and *SNAI1* an epithelial mesenchymal transition (EMT) marker on mRNA level.

3. We wanted to examine the expression of SMARCB1 on protein level in SS-iASC-206 cells after permanent mir-206 transfection experiments.

4. We wanted to detect the pathognomonic fusion gene (*SS18-SSX*) in the peripheral blood samples of SS patients after six months of therapy and answer the question whether the detection of the fusion transcript is a capable diagnostic tool for disease follow up.

3. METHODS

3.1. Cell cultures and permanent miRNA transfection

Two genetically engineered cell line (SS-iASC és iASC), two human tumorous (HT-1080 és Caco2) and a normal non-tumorous (HDFα) cell line was cultured. The required miR-206 was provided as OriGene human miRNA MIR206 molecule (OriGene Technologies). The cell lines were transfected with electroporation using the Neon™ Transfection System (Invitrogen by Thermo Fisher Scientific). After transfection we produced stable cell lines with antibiotic treating.

3.2. Analysis of SS-iASC cells

The newly established cell line underwent karyotyping. Expression of the *SS18-SSX1* fusion transcript was examined on mRNA level with quantitative real time PCR and nested PCR, on protein level with immunofluorescent immunocytochemical staining and Western blot analysis. In addition, immunocytochemical reactions were performed to examine the immunophenotype of the cell line.

3.3. Relative gene expression of miR-206 target genes

The effect of miR-206 overexpression on target genes (*SMARCB1*, *ACTL6A*, *CCND1*, *POLA1*, *MET*, *NOTCH3*, *G6PD*) and EMT marker *SNAI1* was examined using q-RT-PCR.

3.4. Expression of SMARCB1 on protein level

Western blot analysis, immunocytochemical staining and flow cytometry was performed in SS-iASC-206 and SS-iASC cells.

3.5. Detection of *SS18-SSX* fusion transcript in liquid biopsy samples

Blood samples were collected from 15 synovial sarcoma patient. After nucleic acid isolation nested PCR and droplet digital PCR were performed to detect the fusion transcript six month after treatment.

4. RESULTS

4.1. Introduction of SS-iASC cell line

SS-iASC preserved the same karyotypic aberrations as iASC.

Immunofluorescent labelling and Western blotting proved the expression of *SS18-SSX1* chimeric gene product. Using both q-RT-PCR and nested PCR, the *SS18-SSX1* fusion gene mRNA product was shown to be constitutively expressed. SS-iASC cells showed focal cytoplasmic cytokeratin positivity and much stronger cytoplasmic β-catenin reaction, while no cytokeratin reaction and weak cytoplasmic β-catenin reaction was observed in iASC cells.

4.2. Effect of miR-206 on target genes after miR-206 transfection in cell cultures

To be able to examine the effect of miR-206 on *SMARCB1* and other target genes permanent miR-206 transfection was performed in SS-iASC, iASC, HT-1080, Caco2 and HDFα cell lines. After transfection and antibiotic selection HDFα-206 cells were not growing as fast as the other transfected cell lines and they died.

SMARCB1 expression was found to be significantly downregulated to 64% in SS-iASC-206 and to 83% in iASC-206. The difference most likely reflects a common effect of the chimeric SS18-SSX1 fusion protein and miR-206.

MET and *CCND1* were the only oncogenes on which miR-206 invariably acted as a suppressor, so thus these findings

collectively suggest a general suppressor function of miR-206 on *CCND1* and *MET* in these cell lines.

Target genes were mostly downregulated by miR-206 in SS-iASC-206, iASC-206 and Caco2-206 cells, while in HT-1080-206 more target gene were upregulated.

4.3. Effect of miR-206 on SMARCB1 protein expression

Western blot analysis did not detect any significant change of SMARCB1 protein expression in the transfected cell lines. SMARCB1 immunocytochemical reactions showed a focal decreased nuclear stain in a subpopulation of SS-iASC-206 cells compared with the evenly strong nuclear stain of SS-iASC cell line. Flow cytometry evaluation also demonstrated a modest decrease of SMARCB1 protein expression in SS-iASC-206 cells.

4.4. *SS18-SSX1* fusion transcript as a potential biomarker in liquid biopsy samples from synovial sarcoma patients

4.4.1. Clinical characteristics of the synovial sarcoma cohort

In our series of 15 patients with SS, the male-to-female ratio was 4:11. The median age at diagnosis was 45 years (range: 24–72). The tumor was located on the periphery in 9 (60%) cases and centrally in 6 (40%) cases. At the time of diagnosis with active SS, 10 patients (66.7%) presented with primary metastasized disease, while 5 (33.3%) displayed with localized disease. 11 (73.3%) tumors were classified

histologically as monophasic, while 4 (26.7%) were described as biphasic. One patient was only surgically treated, treatments of the remaining 14 (93.3%) patients included surgical excision and chemotherapy and radiotherapy before blood withdrawal. Recurrence occurred in 6 (40%) cases.

4.4.2. Results of nested PCR and ddPCR in the SS blood samples

Agarose gel electrophoresis after nested PCR did not detect *SS18-SSX1/2* fusion transcript in any of the patients, at the same time ddPCR detected *SS18-SSX2* fusion transcript in one patient in a small amount. This patient had primary pulmonal synovial sarcoma, underwent combined therapy and had a metastasis in the other lung. In the remaining 14 patients we could not detect the fusion transcript.

5. CONCLUSIONS

Main findings of the thesis are the following:

- We established and used SS-iASC (SS18-SSX1 carrying immortalized adipose tissue-derived mesenchymal stem cells) as an *in vitro* cell culture model for the first time which provides a suitable *in vitro* model for examining the effect of the fusion gene in synovial sarcoma.

- The SS-iASC cell line expresses *SS18-SSX1* fusion gene both on mRNA and protein level.

- The fusion gene successfully induced mesenchymal epithelial transition in the SS-iASC cell line.

- The observed gene expression patterns after permanent miR-206 transfection refer to the fact, that the action of this miRNA is largely cell context-dependent and miR-206 is a tissue- and cell-specific miRNA.

- The observed SMARCB1 mRNA and protein expressions in SS-iASC-206 cells show that oncomiR effects of miR-206 on *SMARCB1* plays an important, but not exclusive role in *SMARCB1* gene silencing in synovial sarcoma.

- Based on our findings in the synovial sarcoma cohort we can declare that release of CTCs and cell free nucleic acids is a rare occurrence after therapy and

hence in itself is not sufficient for monitoring the tumor recurrence.

6. LIST OF PUBLICATIONS

6.1. Publications related to the thesis

1. **Mihaly D**, Nagy N, Papp G, Papai Z, Sapi Z. (2018) Release of circulating tumor cells and cell-free nucleic acids is an infrequent event in synovial sarcoma: liquid biopsy analysis of 15 patients diagnosed with synovial sarcoma. Diagn Pathol, 13: 81.

IF: 2,528

2. **Mihaly D**, Papp G, Mervai Z, Reszegi A, Tatrai P, Szaloki G, Sapi J, Sapi Z. (2018) The oncomir face of microRNA-206: A permanent miR-206 transfection study. Exp Biol Med (Maywood), 243: 1014-1023.

IF: 3, 005

3. **Mihaly D**, Matula Z, Changchien YC, Papp G, Tatrai P, Sapi Z. (2017) First cloned human immortalized adipose derived mesenchymal stem-cell line with chimeric SS18-SSX1 gene (SS-iASC). Cancer Genet, 216-217: 52-60.

IF: 2,396

6.2. Publications not related to the thesis

1. Papp G, **Mihaly D**, Sapi Z. (2017) Unusual signal patterns of break-apart FISH probes usen in the diagnosis of soft tissue sarcomas. Path Oncol Res, 23: 863-871.

IF: 1,935