Genetic and epigenetic examination of *SMARCB1* gene in epithelioid sarcoma

PhD thesis

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1. INTRODUCTION

Loss of the classic tumuorsuppressor *SMARCB1* (*INI1*, *hSNF5*, *BAF47*) nuclear protein expression is a characteristic feature of two neoplasias; malignant rhabdoid tumour (around 100%) and epithelioid sarcoma (around 90%). However, other soft tissue tumours- such as renal medullary carcinoma, myoepithelial sarcoma, extraskeletal myxoid chondrosarcoma and epithelioid malignant peripheral nerve sheath tumour are also marked in a lesser percent by this non-functional protein. It is well known that the majority of malignant rhabdoid tumours harbour genetic alterations (biallelic deletion and/or mutation) of *SMARCB1* gene, whereas loss of function in epithelioid sarcoma (and other soft tissue tumours) appears to depend on epigenetic mechanisms.

SMARCB1 encodes a core subunit of the SWI/SNF ATP-dependent chromatin remodelling complex. Observations show that these complexes regulate gene expression, at least in part, by inducing a nucleosome conformation change that it is more approachable to the transcriptional machinery. SMARCB1 appears to play a role in the Rb-cyclin D1 pathway; however, the specific function of SMARCB1 with respect to development of different human tumours has not yet been elucidated.

Epithelioid sarcoma is an aggressive soft tissue neoplasm with unknown pathogenesis typically arising in the distal extremities of young adults. Histologically the proximal type epithelioid sarcoma frequently shows rhabdoid features therefore it can be difficult to differentiate from malignant rhabdoid tumour. Many studies reported the absence of nuclear staining of SMARCB1 in approximately 90% of epithelioid sarcomas. Recent studies revealed only 10% of epithelioid sarcoma cases with absence of SMARCB1 nuclear expression and at the same time showed biallelic mutation and/or deletion of *SMARCB1*. In cases without DNA alterations, either epigenetic changes or post-transcriptional modification could be the possible cause to prevent the expression of *SMARCB1*.

The epigenetic regulation can operate through the methylation of DNA or the different histone modifications (acetylation, methylation, phosphorylation, ubiquitination, etc.). The Polycomb repressor protein complexes involved in gene silencing and microRNAs (miRNAs) are considered as part of the epigenetic regulation. The function of DNA methylation is to establish the compact chromatin structure and to inhibit transcription. Excessively methylated specific genes including the key cell cycle regulator *CDKN2A* gene and others were identified in several sarcoma cell lines and primary tumours as well. Regarding histone methylation, the

H3K27me3 methyltransferase EZH2 (Enhancer of zeste homologue 2) is often up-regulated in different tumours such as lymphoma and other soft tissue sarcomas for example synovial sarcoma as well. The EZH2 over-expression correlates with advanced cancer stage, aggressive behaviour of the tumour and unfavourable clinical outcome.

miRNAs regulate signal transduction and cell cycle processes through the post-transcriptional inhibition of tumour suppressors- and proto-oncogenes. Changes in their expression lead to upsetting the balance of cell division, increased cell proliferation and tumour formation.

2. OBJECTIVE

The aim of our experimental work was to examine the role of genetic and epigenetic procedures in the development of epithelioid sarcoma. We would like to determine the biological mechanism responsible for the inhibition of *SMARCB1* tumoursuppressor gene expression in this type of tumour.

Key questions and assumptions summarized as follow:

1. To investigate the *SMARCB1* genetic status in order to reveal genetic aberrations (chromosome deletions, exon deletions, intraexonic alterations) responsible for the loss of gene function in epithelioid sarcoma.

2. We hypothesized that the hypermethylation of CpG islands in the *SMARCB1* gene promoter region may cause the inhibition of gene expression.

3. To investigate the possible role of EZH2 mediated histone methylation by determining the protein expression of EZH2 and its epigenetic mark H3K27me3.

4. We hypothesized that miRNAs as post-transcriptional regulators are responsible for silencing of *SMARCB1* gene. Bioinformatics analyses were applied for the identification of potential miRNAs targeting the 3’ UTR region of *SMARCB1*. We would like to identify the possible pathogenic miRNAs by using miRNA expression experiments. To investigate the functional effects of up-regulated miRNAs by using *in vitro* tissue culture experiments.

3. METHODS

3.1. Tissue specimens and immunohistochemistry

In this study formalin fixed, paraffin embedded (FFPE) samples of thirty-six epithelioid sarcoma and two malignant rhabdoid tumour cases were used. The SMARCB1 immunonegative epithelioid sarcoma cases were selected by using immunohistochemistry. EZH2 expression and the EZH2 mediated H3K27 trimethylation were also investigated by using immunohistochemistry.

3.2. *SMARCB1* genetic examination

For the Fluorescent *In Situ* Hybridization (FISH) BCR/ABL Dual Color Translocation Probe Set (Abbott Molecular) was used, because BCR probe covers the whole 22q11.2 chromosome region including *BCR* (22q11.23) and *SMARCB1* (22q11.23) region as well. For the analyses of *SMARCB1* sequences (9 exons), bi-directional sequencing of the purified PCR products was carried out.

3.3. Methylation-specific PCR (MSP)

After bisulfite conversion of DNA samples isolated from the epithelioid sarcoma cases harbouring the at least one intact *SMARCB1* allele MSP examination was performed by using the designed methylation specific and unmethylation specific primer sets of *SMARCB1* gene promoter region.

3.4. *In silico* miRNA target prediction

For the prediction of miRNAs targeting *SMARCB1* gene five algorithms were used: MiRBase Target v5, MirTarget2, Targetscan 4.0, RNAhybrid and Pita. From the potential miRNAs candidates, we preferred those that were identified by five, or at least four, algorithms.

3.5. *SMARCB1* gene expression

After SMARCB1 immunostaining tumour cells from seven epithelioid sarcoma cases were microdissected with PALM laser microdissection system. RNA was isolated from the dissected cells, from the whole tissue blocks and from the miRNA transfected cells also. After reverse transcription real-time quantitative PCR analysis and ΔCT method was performed for detecting the expression of *SMARCB1* mRNA level in the samples.

3.6. miRNA expression

miRNA expression analysis was carried out from the epithelioid sarcoma cases harbouring at least one intact *SMARCB1* allele by using q-RT-PCR method. The expression level of miR-1, miR-206, miR-381, miR-502, miR-548a, miR-619, miR-671-5p and miR-765 was determined.

3.7. Tissue culture and miRNA transient transfection

Two human tumour cell lines (HT-1080 human fibrosarcoma and Caco-2 human colon adenocarcinoma) and a normal non-tumour cell line (HDFα human dermal fibroblast) were cultured. The miRNAs needed for transient transfection experiments were

purchased in the form of Pre-miR™ miRNA Precursor molecules (Ambion). Cell lines were transfected by electroporation using Neon™ transfection system (Invitrogen).

3.8. SMARCB1 protein expression

The effect of *SMARCB1* silencing was examined on protein level by immunocytochemistry in the transfectant HDFα cells.

4. RESULTS

4.1. Histology and immunohistochemistry

Out of the collected 36 epithelioid sarcoma cases, 20 (56%) were distal and 16 (45%) proximal type. In 31 (86%) cases, there was no detectable expression of the SMARCB1 gene product in tumour cells with the anti-BAF47 (SMARCB1) antibody, whereas infiltrating lymphocytes and entrapped non-tumour tissue displayed immunoreactivity. Epithelioid sarcoma cases were characterized as follows: vimentin diffuse positivity, keratin (AE1/AE3) and/or EMA at least focal strong positivity, S100 negativity and SMARCB1 negativity.

4.2. *SMARCB1* genetic examination

4.2.1. Fluorescent in situ hybridization

If both *SMARCB1* allele are deleted in the tumour cells this would explain the loss of SMARCB1 nuclear protein expression. However biallelic *SMARCB1* deletion was found only in four SMARCB1 immunonegative epithelioid sarcoma cases. Monoallelic deletion of SMARCB1 was detected in 11 cases. In the remaining 16 cases chromosome abnormalities was not detected by FISH method. Polysomy was observed in five epithelioid sarcoma cases, one tumour represented trisomy and four showed tetrasomy.

4.2.2. Analysis of SMARCB1 sequence

Epithelioid sarcoma cases without biallelic deletion of 22q11.2 chromosome region were screened for *SMARCB1* mutation analysis. Nine exons of *SMARCB1* gene were PCR amplified and direct sequenced for revealing the possible somatic mutations in altogether 27 epithelioid sarcoma cases. Among those cases that showed monoallelic deletion two of them revealed mutation of the retained allele. One of them displayed a 10 base pair long duplication (ATCCTAGCC) in the exon 5, the other showed a single base pair point mutation (GGG>AGG) in the exon 7. Altogether we found six cases -including the four cases showing biallelic deletion with FISH- harbouring *SMARCB1* deletion/mutation.

4.2.3. Validation of FISH result on SMARCB1

In order to rule out the possibility that FISH probes were missing small *SMARCB1* deletions (for example total exon deletions), and also the possibility of contaminating stroma, we used laser capture microdissection to select only tumour cells, followed by PCR amplification of the nine exons of *SMARCB1*. All three epithelioid sarcomas harbouring monoallelic *SMARCB1* deletion and the two cases with wild type *SMARCB1* gene resulted in PCR products with all the primers of *SMARCB1* nine exons showing that there was no biallelic deletion in our selected cases.

4.3. Examination of *SMARCB1* mRNA expression

As a result of investigation of the *SMARCB1* gene expression, low level of *SMARCB1* mRNA was found in RNA samples (16 cases) extracted from tissue blocks. We suspected that the stromal or other normal cells (eg, endothelium, lymphocyte, fibroblast, etc) might cause contamination and it results in the appearance of the *SMARCB1* mRNA in a variable expression levels among tumour samples depending on the ratio of normal and tumour tissue. Therefore, we decided to carry out laser capture microdissection to collect only tumour cells, which showed no expression of *SMARCB1* gene; correspondingly, mRNA could not be detected. For the positive control, we also collected individual stromal and/or normal cells from the tumour-free area, which revealed increased levels of *SMARCB1* transcript.

4.4. *SMARCB1* epigenetic examination

Biallelic genetic alteration of *SMARCB1* was not found in altogether 25 SMARCB1 immunonegative epithelioid sarcoma cases. Therefore, among the epigenetic regulation processes first the *SMARCB1* promoter methylation and then the EZH2 protein expression and the EZH2 mediated H3K27 trimethylation were examined.

4.4.1. SMARCB1 promoter methylation

Twenty-five bisulfite converted epithelioid sarcoma DNA was examined by methylation-specific PCR (MSP) and there were no methylated cytosins in the promoter of *SMARCB1* gene. MSP analysis revealed that there were no methylated alleles in the promoter, so *SMARCB1* tumoursuppressor displayed unmethylated status in epithelioid sarcoma.

4.4.2. EZH2 mediated H3K27 trimethylation

Using EZH2 immunohistochemistry we determined that EZH2 overexpression is not a general feature of epithelioid sarcoma. Twenty-five epithelioid sarcomas (69%) were negative for EZH2, the remaining eleven cases (31%; nine proximal and two distal types) were EZH2 positive. The H3K27me3 antibody which recognizes only the tri-methylated lysine 27 on histone 3 revealed also negative immunohistochemical reaction in all the examined epithelioid sarcoma tumour cells.

4.4.3. Identification of miRNAs targeting SMARCB1 mRNA

4.4.3.1. In silico target prediction

The selected five algorithms revealed 80 potential miRNAs targeting the 3'-untranslated region of *SMARCB1*. Matching results of all five algorithms revealed only one miRNA, namely, miR-206. Seven miRNAs (miR-1, miR-381, miR-502, miR-548a, miR-619, miR-671-5p, and miR-765) were found as matching results in at least four algorithms. Based on this result, we selected the aforementioned eight miRNAs for further validation.

4.4.3.2. Expression of miRNAs targeting *SMARCB1*

The miR-206 (5.7-fold), miR-381 (8.17-fold), miR-671-5p (6.55-fold) and miR-765 (10.12-fold) were found to be significantly (*p<0.001*) over-expressed in epithelioid sarcoma samples when compared with the three control groups (rhabdoid tumour, SMARCB1immunopositive epithelioid sarcoma and epithelioid sarcoma with biallelic-deleted *SMARCB1*) and normal fat tissue. The qRT-PCR analysis of miR-1 and miR-502 showed similar or down-regulated expression in epithelioid sarcoma samples compared with control groups and calibrator. At the same time, miR-548a and miR-619 were not detectable in the aforementioned tissues.

4.4.4. Regulation of SMARCB1 level by miRNAs in transfected cell cultures

4.4.4.1. Examination of the *SMARCB1* gene expression

To test the functional silencing effect of the four over-expressed miRNAs on *SMARCB1* expression, we transfected HT-1080, Caco-2 and HDFα cells with specific miRNA molecules, then measured *SMARCB1* mRNA levels by q-RT-PCR. In HT-1080 cells after 24 hr of transfection with miR-206, miR-381, and miR-671-5p significantly (*p<0.001*) decreased *SMARCB1* gene expression to 34.27%, 74.23%, and 72.45%, whereas after 48 hr of transfection only miR-206 and miR-671-5p inhibited significantly (*p<0.001*) *SMARCB1* gene expression to 54.15% and 74.74%, compared with the negative control miRNA molecule. The miR-381 failed to exert a significant effect on *SMARCB1* after 48 hr of transfection. The miR-381 and miR-671-5p significantly (*p<0.001* and *p<0.01*) suppressed *SMARCB1* expression to 43.53%, 79% and 72.45% in Caco-2 cells and to 31.2%, 56.7% and 56.8% in HDFα cells, respectively, compared with negative control miRNA. Surprisingly, miR-765 had no functional effect on *SMARCB1*, although this miRNA showed the highest expression level in epithelioid sarcoma samples. The miR-206 proved to be the most effective gene silencer molecule *in vitro.*

Combinations of miR-206/381 and miR-206/671-5p significantly (*p<0.001*) down-regulated *SMARCB1* expression to 44.59% and 35.47%, but the silencing with miR-206 alone proved to be more effective in the HDFα cells.

Thus, the synergistic silencing effect of the miRNAs was not proved in our experimental system.

4.4.4.2. SMARCB1 protein expression

The influence of the three selected miRNAs, miR-206, miR-381 and miR-671-5p (causing the most effective gene silencing) on SMARCB1 protein was examined in transfected HDFα cells by using immunocytochemistry 24 and 48 hrs after transfection. SMARCB1 intranuclear negativity was observed after transfection of miR-206 alone as well as miR-206/381 and miR-206/671-5p combinations after 48 hr of transfection.

5. CONCLUSIONS

The main findings of my dissertation summarized as follow:

- The majority of epithelioid sarcomas (81%) do not harbour biallelic genetic alterations of *SMARCB1* tumour suppressor gene. Moreover, neither the gene promoter hypermethylation nor the most common histone methylation mechanism EZH2 mediated H3K27 trimethylation and the up-regulation of *EZH2* gene was responsible for the inhibition of *SMARCB1* transcription.

- In SMARCB1 immunonegative epithelioid sarcoma tumour cells there was no detectable *SMARCB1* mRNA expression therefore post-translational modifications are not responsible for the loss of protein expression.

- The role of RNA interference was confirmed as the cause of *SMARCB1* inactivation in epithelioid sarcoma. Significant over-expression was found relating four miRNAs: miR-206, miR-381, miR-671-5p and miR-765.

- The miR-206, miR-381, miR-671-5p and their combinations are functionally active for the silencing of *SMARCB1* gene.

- The discovered epigenetic regulation is a new mechanism in the carcinogenesis of epithelioid sarcoma: *SMARCB1* tumour suppressor gene is regulated by onco-miRNAs (miR-206, miR-381 and miR-671-5p), which probably play important roles in the pathogenesis of this tumour.

- Based on our results, it is possible to distinguish malignant rhabdoid tumour from epithelioid sarcoma (mainly the proximal type variant).

6. PUBLICATION RECORDS

6.1. Publications related to the theme

1. **Papp G**, Krausz T, Stricker TP, Szendrői M, Sápi Z. SMARCB1 expression in epithelioid sarcoma is regulated by miR-206, miR-381, and miR-671-5p on Both mRNA and protein levels. Genes Chromosomes Cancer. 2014, 53(2):168-76.
   IF.: 3,546

2. **Papp G**, Changchien YC, Péterfia B, Pecsenka L, Krausz T, Stricker TP, Khoor A, Donner L, Sápi Z: SMARCB1 protein and mRNA loss is not caused by promoter and histone hypermethylation in epithelioid sarcoma, Mod Pathol 2013, 26(3):393-403.
   IF.: 5,253

6.2. Publications not related to the theme

1. Changchien YC, Tátrai P, **Papp G**, Sápi J, Fónyad L, Szendrői M, Pápai Z, Sápi Z: Poorly differentiated synovial sarcoma is associated with high expression of enhancer of zeste homologue 2 (EZH2), J Transl Med 2012, 10:216
   IF.: 3,459

2. Changchien YC, Katalin U, Fillinger J, Fónyad L, **Papp G**, Salamon F, Sápi Z: A challenging case of metastatic intra-abdominal synovial sarcoma with unusual immunophenotype and its differential diagnosis, Case Report Pathol 2012, 2012:786083

3. Changchien YC, Haltrich I, Micsik T, Kiss E, Fónyad L, **Papp G**, Sápi Z: Gonadoblastoma: Case report of two young patients with isochromosome 12p found in the dysgerminoma overgrowth component in one case, Pathol Res Pract 2012, 208:628-632
   IF.: 1,213

4. Balogh Z, Szemlaky Z, Szendrői M, Antal I, Pápai Z, Fónyad L, **Papp G**, Changchien YC, Sápi Z: Correlation between DNA ploidy, metaphase high-resolution comparative genomic hybridization results and clinical outcome of synovial sarcoma, Diagn Pathol 2011, 6:107 **IF.: 1,638**

6.3. Oral and poster presentations

1. **Papp G**, Changchien YC, Péterfia B, Sápi Z. A *SMARCB1/INI1* gén genetikai és epigenetikai vizsgálata epithelioid sarcomában. (Oral presentation), PhD Scientific Days 2012, Budapest, 2012. április 12-13.

2. Changchien YC, Katalin U, Fillinger J, Fónyad L, **Papp G**, Salamon F, Sápi Z. A Challenging Case of Metastatic Intra-abdominal Synovial Sarcoma with Unusual Immunophenotype and Its Differential Diagnosis. (Poster presentation), Technology Transfer in Diagnostic Pathology 7th Central European Regional Meeting, Siófok, 2012. május 15.

3. Changchien YC, Haltrich I, Micsik T, Kiss E, Fónyad L, **Papp G**, Sápi Z. Gonadoblastoma: 2 case reports from young patients with gonadal dysgenesis and the molecular approach. (Poster presentation), 2nd Pannonia Congress of Pathology, Siófok, 2012, május 18.

4. **Papp G**, Changchien YC, Péterfia B, Sápi Z. A *SMARCB1* gén genetikai és epigenetikai vizsgálata epithelioid sarcomában. (Oral presentation) - *I. prize*, Fiatal patológusok találkozója (FIPAT 2012), Zamárdi, 2012. szeptember 21-22.

5. **Papp G**, Sápi Z. *SMARCB1* mRNA expression is regulated by microRNAs in epithelioid sarcoma. (Oral presentation), 25th European Congress of Pathology (ECP 2013). Lisbon, Portugal, 2013. augusztus 31-szeptember 4.