miRNA expression profiling and immunhohistochemical analysis of adenoid cystic carcinomas of the breast and salivary glands

Synopsis of Ph.D. Thesis

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1. INTRODUCTION

Breast- and salivary gland-derived adenoid cystic carcinomas (bACC and sACC) show the same histomorhological appereance; however, their clinical courses differ remarkably. While bACCs present tumors with almost indolent clinical features, the long-term overall survival of sACC cases is very unfavourable, which may be due to the unique environmental endowment of their tissue of origin, or late detection of their asymptomatic development. In addition, the biological behaviour of ACCs may be influenced by molecular and genetic alterations as well. Known features of ACCs include c-kit expression (detected in a remarkable number of ACCs), as well as the presence of the MYB/NFIB fusion gene, which develops through a known translocation: t(6;9)(q22-23;p23-24). To date, most of the known molecular and genetic features of ACCs have been detected only in a few cases, and have been identified both in bACCs and sACCs, suggesting that the diverse clinical courses of bACC and sACC may be influenced by fine regulatory mechanisms of key importance. MicroRNAs (miRNAs) are short, single-stranded, tissue-specific, non-coding molecules, which play roles in a huge number mechanisms of cell phisiology. Changes in their stoichiometry may cause dramatic changes to homeostasis, and so they may induce alterations of physiologycal processes. Indeed, it is well established that miRNAs also play important roles in the pathology of malignant diseases. Through their regulatory effects on gene expression, miRNAs may cause changes in protein levels. Our research group hypothesized that the diverse clinicopathology of bACCs and sACCs may be affected by miRNAs, by fundamentally influencing the function of some genes.

2. AIMS AND OBJECTIVES

The aims of our pilot experiment were as follows:

- miRNA profiling of bACC vs. sACC cases, as well as normal breast- and salivary gland tissues
- to identify the miRNAs present in each investigated case, or those that have a special distribution within the study groups
- to determine the potential target genes of the identified miRNAs, as well as to explore related signal transductory mechanisms by the use of public databases

The aims of our expanded experiment:

In our further analysis we planned to determine the followings on an elevated number of cases:

- additional expression analysis of the previously chosen miRNAs
- semiquantitative analysis of the proteins encoded by the potential target genes of the investigated miRNAs
- to compare the results of the various study groups
- to determine the expression of estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (Her2) and Ki67 in bACC and sACC cases

3. METHODS

In our ***pilot experiment*** we investigated 2 cases of bACCs and 2 cases of sACCs, as well as 1 normal breast and 1 normal salivary gland case. Ten 5 μm thick slides were cut from formalin-fixed, paraffin-embedded (FFPE) tissue blocks of each case. If needed, macrodissection was performed, applying the Hematoxylin-Eosin (HE)-stained slides of the cases. Therafter RNA isolation was performed by Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany), which was followed by quality control (6000 Pico Chip Kit/Agilent, Palo Alto, CA, USA run on Bioanalyzer machine. On the GeneChip® miRNA Array (Affymetrix, Santa Clara, CA, USA, AF-901325) the RNA isolates hybridized with the complementary RNA-strands, attached to the surface. The miRNAs, present in the investigated tissue sample were detected by the fluorescence emmitted by the binding of the complementary strands. The fluorescent intensity was quantificated by the use of a special software (miRNA QC Tool software-t; Affymetrix, Santa Clara, CA, USA). Thereafter we selected those miRNAs, which were present in each investigated case and determined their potential target genes by IPA® (Ingenuity Pathway Analysis). Further target identification was performed in the case of miRNAs, showing special distribution between the study groups. In this case we applied the miRecords database.

In our ***expanded experiment*** we performed further miRNA expression assays and protein expression analysis on an elevated number of cases (16 sACCs, 14 bACCs, 9 normal breast and 11 normal salivary gland tissues). By miRNA expression assays we determined the quantity of the previously selected miRNAs, for which FFPE tissue blocks were used. As the first step RNA isolation was performed (Life Technologies total RNA isolation kit; AM1975) which was followed by quantification (NanoDrop 1000 Spectrophotometer; Thermo Fisher Scientific Inc., Waltham, MA, USA). For validation, qPCR was performed by

applying miRNA specific primers and TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies; katalógusszám: 4440047) run on LightCycler 480 Instrument II (Roche Applied Science) machine. We then performed protein expression analysis of those proteins identified as potential targets of the selected miRNAs. Additionally, we analyzed proteins commonly assessed in clinical diagnostic routines to asscribe specific clinicopathological features of breast cancer. We determined the expression levels of cyclin D1 and Bcl-2 proteins in each investigated study group (bACCs, sACCs, normal breast and salivary gland tissues). The expression of ER, PgR, Her2 and Ki67 was determined in the tumorous cases. For statistical evaluation, ANOVA (ANalysis Of VAriance) and Tukey-tests were performed by using STATISTICA (v 9.1, StatSoft Inc., Tulsa, OK) and GraphPad PRISM. (v 5.001, GraphPad Software Inc, La Jolla, CA) programs.

Differences were considered as statistically significant if the p value was under 0,05. Our experiments were performed as included in the No.101/2012 ethical approval of the Regional and Institutional Committee of Science and Research Ethics (TUKEB) of the Semmelweis University.

4. RESULTS

Of the investidgated 847 human miRNAs, we identified in our ***pilot experiment*** 57 miRNAs, which was present in each investigated study case. Eight miRNAs (miR-17*, miR-125a-3p, miR-134, miR-181a-2*, miR-206, miR-379, miR-382 és miR-1275) were only present in sACC cases, while miR-1234 was only absent in these tumors and was detected in every other tissue sample. Another 572 miRNAs were not detected in any of the investigated tissue samples. To elucidate miRNA functions, we performed IPA® pathway analysis to predict the interactions of the 57 miRNAs, present in each study case and to determine potential interactions with their target genes. According to these results, in bACC cases, the following genes might be affected: TP53, DGCR8, LAMTOR3, AKT and PRIM1. In the sACC cases, another set of genes was identified by this analysis: PTEN, PIK3CA, ESR1, IGFR1 and FOXO1. To identify further target genes, we focused on those miRNAs that displayed a specific distrubution within the investigated study groups. MiRNAs showing decreased expression in bACCs compared to normal breast tissues, while being increased in sACCs compared to normal salivary gland tissues (let-7b; let-7c; miR-17; miR-20a; miR-24; miR-195; miR-768-3p), were classified as members of „subgroup A". MiRNAs that were overexpressed in bACCs, but whose levels were decreased in sACCs compared to their normal counterparts (let-7e; miR-23b; miR-27b; miR-193b; miR-320a; miR-320c; miR-768-5p; miR-1280; miR-1826), were labelled as members of „subgroup B". Additional potential miRNA-target gene interactions were searched by the use of miRecords, considering exclusively the previously validated interactions. In this analysis we identified hundreds of target genes, which contained genes regulating cell cycle (CCND1, CDK4, CDK6, CDC25A), or apoptosis (BCL2, BCL2L11); transcription factors E2F1, E2F2, E2F3) and some further genes with more or less

known functions in malignancies. In this manner potential roles for VEGFA, HMGA2, NOTCH1 and MYC emerged.

For our ***expanded experiments***, we selected 19 miRNAs, based on the results of our preliminary experiments and analyzed their expression in normal breast- and salivary gland-derived tissues, such as bACCs and sACCs: let-7b; let-7c; miR-17; miR-20a; miR-24; miR-195; miR-768-3p, let-7e; miR-23b; miR-27b; miR-193b; miR-320a; miR-320c; miR-768-5p; miR-1280; miR-1826, miR-17*; miR-379; miR-125a-3p; miR-382; miR-134; miR-1275; miR-206; RNU43 RNU48. We found the expression of miR-17 and miR-20a elevated in bACCs compared to normal breast tissues ($p_{miR\text{-}17\_bN\_vs\_bACC}$=0,017 and $p_{miR\text{-}20a\_bN\_vs\_bACC}$=0,024). In the expression of these miRNAs no significant difference was detected between sACCs and their controls, neither among the control groups. Comparing salivary gland-derived tissues, we identified two miRNAs, whose expression was significantly lower in sACC cases compared to controls: let-7b and miR-193b ($p_{let\text{-}7b\_sN\_vs\_sACC}$=0,032 and $p_{miR\text{-}193b\_sN\_vs\_sACC}$=0,023). In the expression of these miRNAs no significant difference was detected between tumorous and normal breast tissues, neither between the control groups. In comparing normal breast and salivary gland tissues, miR-23b and miR-27b was found elevated in salivary glands ($p_{miR\text{-}23b\_bN\_vs\_sN}$=0.007 and $p_{miR\text{-}27b\_bN\_vs\_sN}$=0.024) with no significant difference between other study groups. Between the two tumorous groups (bACCs and sACCs) we found no significant difference in the expression levels of any investigated miRNAs.

In the course of our validatory experiments, by the use of the miRWalk database, we identified CCND1, BCL2 and MYC genes as regulated targets of all miRNAs, showing significant difference between normal and tumorous tissues (miR-17; miR-20a; let-7b and miR-193b). The database miRTarBase listed only CCND1 as a common target of each of these miRNAs. The expression of cyclin D1, Bcl-2 and c-myc proteins was planned to be determined by

immunohistochemical analysis. However, we could not optimize the immunohistochemical reactions for c-myc protein despite the multiple changes in the applied protocol, therefore we could not obtain any results for this protein.

Comparing breast-derived tissues, we found ***cyclin D1*** overexpressed in tumours compared to normal controls ($p_{cyclinD1\_bN\_bACC}<0.0001$). Similarly, the expression of cyclin D1 was elevated in sACC cases, compared to normal salivary glands ($p_{cyclinD1\_sN\_sACC}<0.0001$).

In analyzing the expression of ***Bcl-2***, we obtained similar results: in breast-derives tumours, Bcl-2 was overexpressed compared to normal ($p_{cBcl-2\_bN\_bACC}=0.005$); salivary gland-derived tumours also had significantly higher levels of Bcl-2 than their controls ($p_{Bcl-2\_sN\_sACC}=0.042$).

Comparing the tumorous tissues (bACCs and sACCs), no significant difference was detected in the expression of cyclin D1, nor in the case of Bcl-2 ($p_{cyclinD1\_bACC\_sACC}=0,113$ and $p_{Bcl-2\_bACC\_sACC}=0,110$). Likewise, no significant difference was detected in the expression of these proteins by comparing the control groups ($p_{cyclinD1\_bN\_sN}=0,126$ and $p_{Bcl-2\_bN\_sN}=0,068$).

The results of the immunohistochemical analysis of ***ER, PgR, Her2 and Ki67*** were similar to the published literature data. We detected weak ER and PgR positivity in 1 bACC and 1 sACC case. Neither the bACC, nor the sACC samples expressed Her2 protein. We detected low proliferation levels both in bACCs and sACCs, with the exception of two sACC cases.

5. CONCLUSIONS

In this work we performed the first parallel and comparitive miRNA-profliling of breast- and salivary gland-derived adenoid cystic carcinoma cases (bACCs and sACCs). In our preliminary experiment we identified miRNAs (present in each of our investigated cases: bACCs, sACCs and in normal breast and salivary gland controls and miRNAs) which showed special distribution between the study groups. By using IPA® and miRecords databases, **CCND1**, CDK4, CDK6, CDC25A, **BCL2**, BCL2L11, E2F1, E2F2, E2F3, VEGFA, HMGA2, NOTCH1 and **MYC** genes emerged among the potential target genes of the identified miRNAs. According to our validatory experiments we found miR-17 and miR-20a overexpressed in bACCs compared to normal breast tissues. Additionally, the expression of let-7b and miR-193 was downexpressed in sACCs compared to controls. The expression of miR-23b and miR-27b was higher in normal salivary gland tissues than in normal breast tissues. The statistical analysis of miRNA expression showed no statistical difference between the two tumours. CCND1, BCL2 and MYC were identified as common target genes of miR-17, miR-20a, let-7b and miR-193b. Through immunohistochemical analysis of cyclin D1 and Bcl-2 we detected overexpression of these proteins in the tumorous tissues both in breast- and salivary gland-derived tissues. While we found no significant difference in the expression of these proteins between bACC and sACC cases: their expression was elevated in both tumors compared to their adherent controls, and between normal controls. The results of the analysis of estrogen receptor (ER), progesterone receptor (PgR) and human epidermal growth factor receptor (Her2) expression was similar to the literature data. Our investigated tumorous samples showed mostly low Ki67 expression.

In conclusion, our study found differences of miRNA expression in ACCs in both organs compared to normal tissue contols, however the miRNA expression

of ACCs of the two organs did not differ significatly – at least for the miRNAs analyzed in this study.

Our results may contribute to the more exact understanding of the hidden epigenetic features of the diverse clinicopathological characteristics of bACC and sACC cases.

6. NEW RESULTS

1. I performed parallel miRNA-expression profiling of breast- and salivary gland-derived adenoid cystic carcinomas (bACCs and sACCs) for the first time.
2. In our preliminary experiment we identified for the first time miRNAs (57), which were expressed in each of our investigated cases: bACCs, sACCs and in normal breast- and salivary gland-derived tissues, showing specific distribution between the different study groups.
3. Simirarly, in the preliminary phase of our investigations, we detected, that some of the miRNAs were only present in sACC samples (miR-17*, miR-125a-3p, miR-134, miR-181a-2*, miR-206, miR-379, miR-382 and miR-1275), while one single miRNA was detected in each study group exept sACCs (miR-1234).
4. We performed detailed target prediction (IPA® and publicly available databases, containing miRNA-target gene interactions). In this analysis, the possible roles of the following miRNAs arose: *BIM*, *BMPR2*, ***BCL2***, ***CCND1***, *CDC25A*, *CDK6*, *IL8*, *JAK1*, *MAP3K12*, *MEF2D*, ***MYC***, *RUNX1*, *VEGFA*, *HMGA2*, *NOTCH1* and *PLAU*.
5. According to the results of our validatory experiments, we reported, for the first time, the overexpression of two miRNAs (miR-17 and miR-20a) in bACC cases, and the underexpression of let-7b and miR-193b in sACC cases, compared to their normal controls. The expression of miR-23b and miR-27b differed between normal breast- an salivary gand-derived tissues. We found no significant difference in the miRNA expression of bACCs and sACCs.
6. Common regulated targets of miR-17, miR-20a, let-7b and miR-193b were identified by miRWalk and miRTarBase databases.

7. Of the proteins encoded by these target genes we had the opportunity to determine the expression of cyclin D1 and Bcl-2 by immunohistochemical analysis: we found the overexpression of both proteins in tumors compared to control tissues.
8. By determing the estrogen receptor (ER), progesterone receptor (PgR) and Her2 expression of our bACC cases, each of those proved to belong to the group of triple-negative breast cancers. Simirarly, each of our sACCs expressed neither of these proteins.
9. In a few cases, we determined the Ki-67 expression of the tumors. Except of a few cases, both bACCs and sACCs showed low proliferation rates. The highest proliferation was detected in two poorly differentiated breast-derived adenoid cystic carcinoma tissue.

PUBLICATIONS RELATED TO THE DISSERTATION

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QUOTABLE ABSTRACTS RELATED TO THE DISSERTATION

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