Role of microRNAs in sporadic pituitary tumorigenesis

Ph.D. thesis

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I. INTRODUCTION

Pituitary adenomas are among the most common intracranial tumors accounting for approximately 10-25% of all intracranial neoplasms. It is difficult to determine their prevalence because many of them are not clinically apparent and they are recognized incidentally during CT or MRI exams performed for unrelated reasons (pituitary incidentalomas). A combined analysis (including radiological and autopsy data) yielded a final prevalence rate of 16.7%.

Although pituitary adenomas are very rarely malignant (in 0.13% of cases), they may cause significant morbidity because of their mass effect or hormonal overactivity. The most common types are prolactin (PRL) producing adenomas (~50%), non-functioning adenomas (NFA) (~30%), GH producing adenomas (GH) (15-20%), corticotrop adenomas producing ACTH (5-10%) and thyrotroph adenomas producing TSH (1%). Pure gonadotropine-secreting adenomas are very rare, but it seems that the majority of NFAs are originated from gonadotrop cells.

Most pituitary adenomas are sporadic, but approximately 5% of all cases develop in a familial setting. Familial occurrence of pituitary adenomas can be observed in well-defined hereditary tumor syndromes such as multiple endocrine neoplasia type 1 (MEN1), Carney complex and familial isolated pituitary adenomas (FIPA) caused by mutations of menin, *PRKAR1A* and *AIP* genes respectively. The molecular mechanism(s) leading to pituitary tumorigenesis in sporadic cases is still largely unknown.

Although mutations in pituitary adenomas are infrequent, CpG island hypermethylation of genes involved directly or oblique in cell cycle regulation has been described (e.g.: p14, p15, p16, p18, p21, p27, pRb,

MEG3A, GADD45γ). The role of cyclin D, PTTG and HMGA2 in the pathogenesis of pituitary adenomas has been established.

MicroRNAs (miRs) are short, non-coding RNA molecules that posttranscriptionally regulate gene expression via RNA interference. Their roles have been described in development, cell proliferation, differentiation, apoptosis as well as in tumorigenesis. Only few studies, using mainly hormone producing tumors, have examined the potential role of miRs in pituitary tumorigenesis to date. These studies indicated that downregulated miR-15a and miR-16-1 may be involved in tumor growth of GH-producing adenomas and other miRs may be useful in prediction of histological subtype and the invasive behavior of these tumors.

II. OBJECTIVES

The aim of this work was to examine the functional role of miRs in pituitary tumorigenesis and to pursue the following objectives:

1. To determine the miR expression profile of NFA, GH and GH+P producing adenomas in order to identify the characteristics of miR expression profiles of tumoral and normal tissues and to determine adenoma subtype-specific miRs.
2. To examine the Wee1 kinase expression on mRNA and protein levels in pituitary tissue samples and to evaluate its regulation by miRs. To identify miR target sites on *Wee1* 3'UTR region using *in silico* bioinformatic tools and to demonstrate direct interactions between miRs and mRNAs in HeLa cell cultures.
3. To determine the miR expression profile by TaqMan Low Density Array in NFA samples and to examine the correlation between

miR expression and clinicopathological findings in order to identify miRs with potential pathogenetic functions.

4. To identify signaling pathways affected by miRs in NFAs and to perform a detailed analysis of expression levels of Smad molecules. To examine the TGBβ signaling pathway regulation through miRs.

III. PATIENTS AND METHODS

Patients. A total of 56 pituitary adenoma tissues, including 27 NFA, 14 GH-producing adenomas with and without prolactin (PRL)-secretion (GH±P) and 15 normal pituitary tissue specimens were analyzed. Adenoma tissues were removed by transsphenoidal surgery. The tumor classification was based on clinical findings, hormone levels and immunohistochemistry. Routine immunohistochemical examination included immunocytochemistry of anterior pituitary hormones for confirming the hormonal activity and staining for the MIB1 proliferation marker.

Measurement of mRNA and miR expression. The tumor specimens were immediately frozen in liquid nitrogen after adenoma removal and stored at -80 ^{o}C until total RNA isolation was performed. mRNA and miR expressions were determined by quantitative real-time PCR using TaqMan assays. For defining miR expression profile TaqMan Low Density Array (TLDA) was applied and expression level of 678 different miRs was measured.

Target prediction and vector construction. A total of 6 in silico algorithms, DIANA-mirPath, TargetScan, PicTar, MiRBase (later MicroCosm), microRNA.org, and RNA22 were used for target prediction.

Wee1 3'UTR (ENSG00000166483) was amplified by PCR from genomic human DNA, then the amplified sequence was cloned (5'→3') into pGL3 Control vector at 3'end of the firefly luciferase gene using XBaI restriction site (pWee+). The reversely inserted (3'→5') *Wee1* 3'UTR was used as negative control vector (pWee-). The target sites of miR-128a and miR-516a-3p were mutated by site-directed mutagenesis using mutant primers, and the mutant 3'UTRs were also cloned (5'→3') into pGL3 Control vector. Sequences of pWee+, pWee- and mutant pWee vectors were verified by direct DNA sequencing

Transient transfection, cell culture experiments. Transient transfections of HeLa cells were executed using 100 nM pre-miR precursor molecules with Lipofectamine 2000. The effect of miR-20a, miR-93, mir-128a, miR-155 and miR-516a-3p was tested on wild type (pWee+), negative control (pWee-) and mutant 3'UTR compared to Control miR. Luciferase assay was performed twenty-four hours later using Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's protocol. Each experiment was carried out in six independent replicates and repeated four times. Firefly luciferase activity was adjusted for transfection efficiency by normalizing to Renilla luciferase activity of each sample.

The cell growth and proliferation were determined after transient transfection of pre-miR precursors using AlamarBlue assay at two time points (48 h and 72 h after transfections) according to the manufacturer's protocol.

Detection of Wee1 protein by immunhistochemistry and Western blot. Formalin-fixed, paraffin-embedded tissues from 21 surgically removed human pituitary tissues (9 NFA samples, 7 GH±P producing and 5 normal tissues) were used for immunohistochemical analysis with anti-Wee1 (ab37597, Abcam) antibody. The specificity of anti-Wee1 antibody was

tested by immunoblotting on snap-frozen, human pituitary samples after homogenization and protein isolation. Wee1 protein detection was also performed on HeLa cell lysates after pre-miR transient transfection using anti-Wee1 (#4936; Cell Signaling) antibody. Protein bands were quantified with QuantityOne 4.6.7 (BioRad) software, normalized to β-Actin.

Pathway analysis and statistics. For pathway analysis the most significantly overexpressed miRs were loaded into DIANA-mirPath tool. Using TargetScan v5. an enrichment analysis of multiple miR target genes were performed by a Pearson's Chi-squared test comparing each set of miR targets to all known KEGG pathways. KEGG (Kyoto Encyclopedia of Genes and Genomes, Kanehisa and Goto, 2000) is a database resource that provides knowledge about several genomes as well as their relationships to biological systems. It has been utilized as a systematic knowledge base for molecular and network biology.

Statistical analysis was performed using Integromics RealTime StatMiner 3.0 (TIBCO Software Inc., Palo Alto, USA) and Statistica 7.0 softwares (StatSoft Inc., Tulsa, USA). Shapiro-Wilks normality test was used for controlling data distribution. For comparing groups one-way ANOVA with Fisher post-hoc test, T-test, Mann-Whitney U Test were applied depending on data distribution and the number of groups. Pearson's or Spearman rank test was used for correlations. Hierarchical cluster analysis was performed by "Complete linkage clustering" method using Euclidean distance for similarity measuring. A value of $p<0.05$ was considered to be significant.

IV. RESULTS

IV.1. MicroRNA expression in pituitary adenoma subtypes

The expression of 678 different miRs was measured in pooled normal pituitary, GH-producing adenomas, GHP-producing adenomas and NFA samples. Without preamplification 250 miRs were detected in pituitary tissues, of which 174 were expressed in all four pools, 9 miRs were detected only in normal pool, and 7 miRs in all three adenoma pools but not in the normal tissue.

Using T-test 14 miRs (hsa-miR-509-3-5p, hsa-miR-508-5p, hsa-miR-452, hsa-miR-330-5p, hsa-miR-200a*, hsa-miR-503, hsa-miR-424, hsa-miR-449a, hsa-miR-199-5p, hsa-miR-654-5p, hsa-miR-20b, hsa-miR-431, hsa-miR-485-5p, hsa-miR-370) were identified whose expression were significantly different between adenomas and normal tissues.

When compared to the normal pituitary tissue pool, overexpression of miRs in NFA pool and underexpression of miRs in hormone secreting adenoma pools (GH, GHP) were observed. In addition, 20 adenoma subtype-specific miRs were detected: 5 miRs (hsa-miR-1, hsa-miR-760, hsa-miR-196b, hsa-miR-188-5p, hsa-miR-146b-3p) were expressed only in the GH-producing adenoma pool, 3 miRs (hsa-miR-205, hsa-miR-132*, hsa-miR-523) only in the GHP-producing adenoma pool, and 12 (hsa-miR-217, hsa-miR-96, hsa-miR-216a, hsa-miR-202, hsa-miR-215, hsa-miR-501, hsa-miR-502, hsa-miR-18a, hsa-miR-338, hsa-miR-450a, hsa-miR-10b, hsa-miR-329) only in the NFA pool.

When examining the difference between GH-producing and GHP-producing adenomas, 17 miRs were detected only in GH-producing adenomas and 21 miRs were expressed in GHP-producing adenomas but not in GH-producing adenomas. Despite these differences in GH-producing

and GHP-producing adenomas, a significant correlation (R=0,93; p<0,01) was found in miR expression levels between the two groups, allowing us to combine these samples into one pool and to use these combined data as one data set (GH±P). There were 109 miRs showing different expression in hormone producing adenomas and NFA.

IV.2. miRs regulated Wee1 kinase expression in pituitary adenomas

For overexpressed miRs, target predictions were performed using miRBase, TargetScan, PicTar algorithms and Wee1 kinase appeared as a potentially important target.

IV.2.1.Difference in mRNA and protein expression of the Wee1 kinase

Using immunostaining the Wee1 protein was found to be downregulated compared to normal tissue.in GH-producing adenomas and NFA samples but not in ACTH-producing adenomas, In addition, the phospho-Wee1 also showed similar protein expression pattern. The ratios of normal/phospho protein were the same in normal pituitary and adenoma tissues, suggesting that the difference was not related to the protein activity. Western blot experiments revealed similar results, but statistical comparison was not performed due to the small number of samples. Despite this difference at protein level, we failed to document differences at mRNA level, suggesting a possible existence of posttranscriptional regulation of the Wee1 kinase expression.

IV.2.2. Identification of miRs targeting the Wee1 3'UTR and their expression in pituitary tissues

Using 3 target prediction softwares, 91 miRs potentially targeting the *Wee1* 3'UTR were identified, of which 11 miRs were predicted by all three

algorithms. MiR-155, miR-20a, miR-93, miR-128a and miR-516a-3p were selected for further validation. We found that all five miRs were overexpressed in NFA samples, while miR-155 and miR-93 were overexpressed in NFA and GH producing adenomas as well.

IV.2.3.C Effect of predicted miRs on Wee1 3'UTR and identification of miR binding sites in Wee1 3'UTR.

HeLa cells were transfected with wild type (pWee+) or negative control *Wee1* 3'UTR (pWee-) and 100 nM Control or specific pre-miR precursor. These experimets showed that pre-miR-128a, pre-miR-516a-3p and pre-miR-155 significantly inhibited the luciferase assay activity by 0.58±0.06, 0.75±0.07, and 0.48±0.11, respectively (mean±SD; $p<0.01$), as compared to the control pre-miR precursor molecule, but they failed to exert a significant effect on pWee1-. MiR-20a and miR-93 precursors had no significant effect on luciferase activity on pWee+. To explore whether the effects of miR-128a, and miR-516a-3p are additive, we co-transfected HeLa cells with the combination of the two pre-miR molecules and evaluated the luciferase assay activities. The combination of pre-miR-128a and pre-miR-516a-3p significantly decreased luciferase activity compared to the control pre-miR precursor.

For the identification of binding sites of miRs, site directed mutagenesis was performed for each predicted site by mutant primers. Mutant plasmids were transfected and luciferase activity was measured. The miR-128a precursor decreased the luciferase activity to 52%, the one-site mutated plasmid reduced the activity to 67% while the double mutant plasmid had no effect (activity was decreased only to 95%). The miR-516a-3p produced an 63% decrease compared to wild type 3'UTR. The results revealed that the Wee1 3'UTR contains two binding sites for miR-128

(between nucleotides 28 and 34, and between nucleotides 252 and 258), and one binding site for miR-516-3p (between nucleotides 40 and 46).

IV.2.4. Functional consequences of miRs targeting the Wee1 3'UTR in HeLa cells

The influence of these 3 miRs on Wee1 protein and cell proliferation was examined in HeLa cells after transfection with pre-miR precursor molecules. Wee1 protein levels significantly decreased after transfection with pre-miR precursors and the most remarkable decrease was observed with a combination of the 3 pre-miR precursors. Decrease in HeLa cell proliferation was observed after transfection of all pre-miR precursors alone or in combination with the exception of mir-128a pre-miR alone at 48 and 72 hours after transfection.

IV.3. miR expression in hormonally inactive pituitary adenomas

IV.3.1. miR expression profile in NFA

In addition to TLDA experiments, expression profile of miRs was determined in individual samples obtained from 8 NFA and 4 normal pituitary glands were determined using Low Density Array supplemented with preamplification. Using preamplification 194 of the 678 miRs were undetectable in pituitary tissue and miRs not expressed at least in the half of the samples were also excluded from further studies. 5 miRs (miR-198, miR-299-5p, miR-497*, miR-548c-3p, miR-622) were expressed only in normal tissue, and 3 miRs (miR-124*, miR-515-5p, miR-872) only in NFA samples. Of the 478 expressed miRs 92 overexpressed and 70 were underexpressed with an at least two fold change in NFA samples compared to normal tissues. Cluster analysis separated two groups among NFA samples, but there was no difference between these two NFA groups in

immunohistochemical, laboratory or clinical findings. Although each of the 8 NFA specimens originated from macroadenomas, expression of 6 miRs (miR-450b-5p, miR-424, miR-503, miR-542-3p, miR-629 és miR-214) showed significant negative correlation with tumor size. Among them 4 miRs (miR-450b-5p, miR-424, miR-503, miR-542-3p) mapped to the same chromosomal region (Xq26.3).

IV.3.2. Pathway analysis

When significantly overexpressed miRs were loaded into the DIANA-mirPath tool, several possible pathways have been revealed. Among the 63 pathways identified, MAPK, Akt/mTOR, Wnt and Notch signaling have been already known as being altered in pituitary adenomas. As the most significant finding, the TGFβ signaling pathway showed 205 miR-mRNA interactions involving 51 genes and 39 miRs. Of the 39 miRs, 19 had significant influence on the TGFβ pathway.

IV.4. Role of the TGFβ signaling pathway in the pathogenesis of nonfunctioning pituitary adenomas

Smad 3, a key transcription factor in the TGFβ signaling transduces signal from receptor to nucleus. In our experiments we found that Smad3, Smad6 and Smad9 were significantly downregulated in NFA compared to normal pituitary tissues. Correlation analysis revealed that expression of 74 miRs showed a significantly negative correlation with Smad3 expression. Of the 74 miRs, 30 miRs were expressed differently in normal and adenoma tissues, and 20 differently expressed miRs (19 overexpressed and one underexpressed) potentially targeted the *Smad3* 3'UTR.

V. CONCLUSIONS

V.1. In my study determination of whole genome miR expression profile in pituitary tissues led to the identification of miRs which may have a potential role in pathogenesis to pituitary adenomas. The results indicated that increased miR expression is characteristic for NFA while GH- and GHP-producing adenoma exhibit decreased miR expression as compared to normal pituitary tissues. It is possible that several signaling pathway or different levels of one pathway may be involved in pituitary tumorigenesis. The heterogeneity of pituitary adenomas described by previous mRNA and miR expression experiments has been also confirmed with my miR profile analysis in these pituitary tissues. Additionally, I have identified 9 miRs which were exclusively present in normal pituitary tissues.

The miR expression profiles of GH- and GHP-producing adenomas showed strong correlation, presumably reflecting their similar biochemical and clinical behaviors observed in patients with these tumors.

The results of these experiments may serve as a start-up for further investigations. Based on bioinformatical analysis of several signaling pathways, my following studies have been focused on the exploration of alterations of cell cycle and TGFβ signaling in pituitary adenomas.

V.2. In my studies I confirmed the pathogenetic role of the Wee1 kinase in NFA and GH±P-producing pituitary adenomas. Wee1 regulates the G2/M transition by inactivation of the Cdk1-Cyclin B complex. In previous reports Wee1 was found to be downregulated in colon cancer tissues and colon cancer cell lines suggesting its tumor suppressor function. Wee1 was also underexpressed in non small-cell lung cancer cells. A

multivariate analysis suggested that both cyclin B1 and Wee1 expression were significant prognostic factors in non small-cell lung cancer and that the loss of Wee1 expression may exert a potential role in promoting tumor progression.

Impairment of Wee1 function may lead to the loss of the G2/M checkpoint which in turn may allow the accumulation of DNA damage. The fact that Wee1 inhibitors combined with DNA damaging drugs may cause mitotic catastrophe in cancer cells may support this presumption. It is assumed that in pituitary adenomas the G1/S checkpoint, the main regulator of cell cycle (restriction point) remains intact, but an impairment of the G2/M may lead to the development of benign adenomas.

In my studies I showed that Wee1 protein expression is regulated by miRs (miR-128a, miR-516a-3p and miR-155), and I identified and experimentally validated their binding sites in *Wee1* 3'UTR. These data suggest that the three miRs, together with miR-195 reportedly targeting Wee1 in pituitary adenomas, may play a role in the pathomechanism pituitary adenomas.

V.3. The expression profile of miRs in NFA indicated the presence of potentially important miRs in these tissues. It was found that 162 miRs showed different expression pattern in NFA tissues, and several of them reportedly had an oncogenic potential (e.g.: miR-93, miR-20a, miR-26a, miR-191, miR-140). Expression of miR-140 was significantly elevated in colon cancer stem-like cells which exhibit slow proliferating rate supporting the assumption that this miR may have a particular role in the development of slowly growing pituitary adenomas.

V.4. Using pathway analysis TGFβ signaling was proved to have pathogenetic role in NFA. I showed that this signaling pathway is repressed in NFA through downregulation of Smad3 expression. The studies identified 20 miRs targeting Smad3, including miR-140 which has been already validated by others in earlierv studies. Based on these results it seems that the TGFβ signaling is affected not only in prolactinomas reported in other studies but also in NFA, and that this signaling pathway may be influenced posttrancriptionally by miRs. It may be concluded that through downregulation of the Smad3, inhibition of the TGFβ pathway results in a shift to alternative, non-Smad downstream signaling e.g. Ras-MAPK, p38, c-Jun N-terminal (JNK) MAP kinase, PI3K-Akt pathways. Thus, our pathway analysis based on miR expression alteration in NFA may support the impact of previously-described pathogenic pathways including PI3K-Akt-TOR, MAPK, Wnt, and Notch.

In summary my work demonstrates that miRs having posttranscriptional impact with known oncogenic and tumorsuppressor roles in other neoplasms may have a pathogenetic role in pituitary adenomas as well.

LIST OF PUBLICATIONS

Publications directly related to the PhD thesis

Original articles:

Butz H., Likó I., Boyle B., Lendvai N., Igaz P., Czirják S., Korbonits M., Rácz K., Patócs A Methods of microRNA research and it's application according to pituitary adenomas (in Hungarian)
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