microRNA expression analysis in human hepatoblastoma

PhD thesis

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INTRODUCTION

Childhood tumors occur under 15 years of age, which is 1 % of all cancer diseases. Primary malignant liver tumors comprise 1 to 4 % of all childhood tumors, 90 % of which are hepatoblastomas (HB), this is therefore the most frequent pediatric liver malignancy. The incidence of HB is 3-4 patients per 1 million children per year; there are 3-4 registered HB cases in Hungary yearly. HB is frequently associated with genetic disorders and is characterized by high alpha-fetoprotein (AFP) sera expression. The etiology of HB however is still unknown, owing to the fact that it is a rare disease. The pathomechanism of HB is also unrevealed, unlike that of other types of cancers. HB is derived from hepatic progenitor cells, which come through different developmental stages, as shown by the HB subtypes. In contrast to hepatocellular carcinoma (HCC), which often develops on the ground of cirrhosis, steatosis or viral infection, HB develops in cirrhosis-free liver, with epigenetic, genetic origin. HB usually occurs between 6 months and 3 years of age and shows male predominance. HB in its early stage does not cause complaints, abdominal ultrasound examination calls attention to the disease. Diagnosis of HB is based on high AFP serum level, histological and radiological examinations. It is essential to diagnose and distinguish the various HB subtypes, since HCC and HB in childhood should be separable considering that HB subtypes have different prognostic significance. According to the histological classification of the tumor, HB is classified into epithelial, mixed and non-specified types, with the epithelial type being the most common, comprising 60% of all cases.

Epithelial HB can be subclassified into fetal, embryonal, macrotrabecular and cholangioblastic subtypes.

MicroRNAs (miRNA) are small (18-25 nt) noncoding, single-stranded RNAs, expressed by vertebrates, invertebrates, fungi and plants. miRNAs modulate gene expression and are involved in cell cycle, differentiation, apoptosis as well as embryonal and postnatal development. Therefore, they are essential for the normal function of cells. In general, miRNAs are negative regulators of gene expression at post-transcriptional level. Altered miRNA expression patterns have been found in several human diseases including cancer. Modified miRNA expression has been reported in liver diseases as well. Furthermore, deregulated expression of miRNAs may be related to disease-associated cell biological processes, implying that these molecules might serve as prognostic and diagnostic biomarkers for cancer diagnosis or therapy. A recent study reported altered miRNA expression in HB samples in comparison with non-tumorous liver tissues and also with HCC.

AIMS

In the present study, we focused on the characterization of the expression level of selected miRNAs in epithelial HB subtypes. Our aim was to analyze the overall and event free survival. Our goal was an *in silico* target prediction, to analyze the correlation between b-catenin and miR expression levels and to confront our HB results with HCC data based on the literature.

MATERIALS AND METHODS

Patients

In this study, 20 HB patients were enrolled with the permission of the Regional Ethical Committee of the Semmelweis University /#192/. Patients were between 3.6 months and 15.82 years of age (mean 3.69 years) at the time of diagnosis and the male/female ratio was 10/10. Surgically obtained resection samples were selected from the archives of the 1st Department of Pathology and Experimental Cancer Research as well as the 2nd Department of Pathology, Semmelweis University, between 1995 and 2011.

Tissue samples

Based on routine histological staining, epithelial HB subtypes were identified and microscopically marked for manually guided dissection. Each of the 20 cases were classified as wholly epithelial HB. Further subtyping resulted in 12 pure fetal and 8 embryonal/fetal subtypes, the latter cases differing in terms of the percentage of fetal components. In five cases, the proportion of fetal component was less than 5 %, whereas for the remaining three embryonal/fetal cases this was between 30 and 50 %. Thus, a total of 15 fetal and 8 embryonal HB samples, along with 15 non-tumorous surrounding liver (SL) samples were available for the study.

Determination of miR expression levels

After total RNA isolation reverse transcription and quantitative real time PCR were performed.

miRNA target prediction *in silico*

The targets of miRNAs, which showed significant differences in survival analysis were mapped by miRanda algorithm. Signaling pathway analysis was performed by WebGestalt system with Benhamini-Hochberg method. Statistical significance was set at p value of <0.01 and the minimum number of genes within the KEGG pathway for each category was set to 5.

Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded (FFPE) sections of 3–5 μm thickness were cut and used for IHC. Reactions were carried out manually using Novolink Polymer Detection System Kit (Leica Biosystems, Wetzlar, Germany) and ZNF207 antibody (Santa Cruz, Dallas, Texas, USA). For semiquantitative analysis of IHC reactions, 10 randomly selected areas of each HB section were chosen and 100 cells per field were counted using a high-power field objective (×40). The percentage of cells positive for ZNF207 was scored as follows: 0 (≤1% positivity), 1 (2–30% positivity), 2 (31–65% positivity) and 3 (66–100% positivity). In addition, the intensity of each reaction was scored as weak (1), moderate (2) or strong (3). The scores for percentage of positivity and intensity were summed (IHC score) for statistical analysis.

Statistical analysis

The relative expression data were analyzed by means of nonparametric statistical tests (Mann–Whitney U, Kruskal–Wallis ANOVA and median test, Spearman's Rank-Order correlation). Overall survival (OS) and event

free survival (EFS) were determined using the Kaplan–Meier method, comparisons between survival functions for different strata were assessed with log-rank statistics. All statistical analyses were carried out using STATISTICA software version 9.1 (StatSoft Inc., Tulsa, OK). Statistical significance was set at p value of <0.05.

RESULTS

Macroscopy and histology of HB

By microscopy, the tumors appeared as well-defined solid lesions with typical histopathology. Fetal components consisted of small, uniform cells resembling fetal hepatocytes with eosinophilic cytoplasms and round to oval basophilic nuclei. The cells were occasionally arranged in trabecules. In contrast, the cells of embryonal components were less differentiated and showed large nuclei with scant cytoplasm. The cells often formed pseudorosettes. The surrounding liver samples revealed no significant alterations, the structural architecture was preserved and occasionally mild non-specific inflammation and fibrosis were seen near the tumor tissue.

miRNA expression analysis

Comparing HB samples with SL samples, lower levels of miR-17-5p, miR-122, miR-195, miR-210, and miR-214, but a higher level of miR-221 expression was observable. Expressions of miR-17-5p, miR-195, miR-210, and miR-214 were lower, whereas the level of mir-221 was higher in the

fetal subtype upon comparison with the embryonal subtype. In the embryonal component only the expression of miR-122 was lower as compared with SL samples. A statistically significant difference was revealed between the fetal and embryonal components in that expression of miR-18a was higher in the embryonal than in the fetal component.

Survival analysis

Overall survival (OS) showed considerable differences in relation to low miR-21, high miR-21 ($p<0.02$), low miR-222, high miR-222 ($p<0.03$) and low miR-224, high miR-224 ($p<0.01$) expressions. High miR-222, miR-224, and low miR-21 expressions were found to be correlated with shorter OS (Fig. 4) with an average survival time of 92.50, 57.63, and 55.56 months, respectively for these miRNAs. The average survival times in relation to low miR-222, miR-224 and high miR-21 expressions were 101.78, 132.78, and 144.50 months, respectively. In respect to HB, regardless of subtype, miRNA expression did not correlate with EFS.

***In silico* target identification**

Functional annotation and pathway analysis was performed using the WebGestalt system. Of the 203 genes (the common target genes of miR-21, miR-222 and miR-224), 198 IDs were unambiguously mapped to 198 unique Entrez Gene IDs and these were used in the KEGG enrichment analysis. Altogether four pathways passed the criteria ($p<0.01$ and the minimum number of genes within the KEGG pathway for each category was set to 5). The MAPK signaling pathway was chosen as tumor biology

of HB associated with this pathway. Of the 203 genes, 149 were present on the Affymetrix arrays. For these, JetSet was used to identify the best available Affymetrix probe sets for each gene. Students t-test was performed for comparison of each gene between normal and tumorous tissues. The analysis identified the ZNF207 gene.

Immunohistochemistry

Nuclear immunostaining was observed in HB and non-tumorous samples. According to the IHC scores, ZNF207 expression proved to be significantly increased in the embryonal tumor cells as compared with surrounding non-tumorous liver cells ($p<0.008$) and in the comparison between HB tumorous and non-tumorous samples (<0.03). Comparison of the ZNF207-low and -high groups revealed significant differences in EFS ($p<0.04$), showing that high ZNF207 expression was associated with better EFS.

Correlation between beta-catenin and miR expression levels

Inverse correlation was detected between nuclear beta-catenin expression and miR-17-5p ($p<0.03$), miR-122 ($p<0.003$), miR-195 ($p<0.0009$), miR-210 ($p<0.0042$) and miR-214 ($p<0.008$) expression levels. Inverse correlation was also observed between cytoplasmic beta-catenin expression and miR-17-5p ($p<0.006$), miR-122 ($p<0.001$), miR-195 ($p<0.01$), miR-210 ($p<0.003$) and miR-214 ($p<0.03$) expressions,whereas positive correlation was observed between miR-221 ($p<0.04$) and cytoplasmic beta-catenin expressions.

CONCLUSIONS

In conclusion, we were the first to demonstrate that higher expression of miR-18a was found in the embryonal than in the fetal HB component furthermore, lower expressions of miR-17-5p, miR-122, miR-195, miR-210, and miR-214 and higher expression of miR-221 were found in HB samples as compared with SL samples. In addition, we established that high miR-21 and low miR-222 and miR-224 levels were associated with increased OS of HB patients. In *in silico* target identification revealed that a less known protein, ZNF207 showed significant difference between embryonal HB and SL tissue, but did not show significant difference in relation to OS. Nuclear and cytoplasmic beta-catenin immunostaining displayed inverse correlation with miR-17-5p, miR-122, miR-195 and miR-210 expression levels, suggesting a beta-catenin characteristic miRNA pattern. These results indicate that miRNA profiling has the potential for use as a diagnostic and predictive tool in HB.

PUBLICATIONS

Total Impact Factor (IF): 6,179

Publications related to the Dissertation (IF:5,533):

1. **Gyugos, M.,** G. Lendvai, I. Kenessey, K. Schlachter, J. Halasz, P. Nagy, M. Garami, Z. Jakab, Z. Schaff and A. Kiss. (2014) microRNA expression might predict prognosis of epithelial hepatoblastoma. Virchows Arch. 2014 Apr; 464(4): 419-427. Doi:10.1007/s00428-014-1549-y.
   IF: 2,676
2. K.Schlachter, **Gyugos, M.,** J. Halász, G. Lendvai, K. Baghy, M. Garami, B. Gyöngyösi, Z. Schaff, A. Kiss (2014) High Tricellulin expression is associated with better survival in human hepatoblastoma. Histopathology**.**2014 Apr 16. doi: 10.1111/his.12436.
   IF: 2,857

Publications not related to the Dissertation (IF: 0,646):

1. A. Dencs, A. Farkas, **M. Gyugos**, A. Kurcz, E. Puskas, B. Treso, E. Rusvai, E. Barcsay and M. Takacs. (2011) Phylogenetic analysis of a nosocomial transmission of hepatitis B virus at a paediatric haematology ward. Acta Microbiol Immunol Hung, 58: 23-29.
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