Tricellulin expression and epigenetic changes in human epithelial hepatoblastomas

Doctoral (Ph.D) Theses

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1. Introduction

There have been widespread reports of an increase in childhood cancer-involving childhood liver cancer- internationally. Childhood hepatic tumors account for approximately 1 % of all pediatric solid tumors and two-thirds of these tumors are malignant. The two main malignant hepatic tumors are hepatoblastomas (HBLs) and hepatocellular carcinomas (HCCs), with HBL accounting for 90 % of malignant tumors in children younger than 5 years old. Additional rare malignant liver tumors in children are hemangioma hemangioendothelioma and mezenchymal hamarthoma. Hepatoblastoma is the most frequent malignant primary liver tumor in infancy and early childhood, with an incidency 1.6-2 patients per 1 million children per year in contrast to 0.29-0.45 patients per 1 million children per year incidency of HCC. There are 2-3 registered HB cases in Hungary yearly. HB in its early stage does not cause complaints, abdominal ultrasound examination calls attention to the disease. The best marker for the recognition and follow-up of HB is the serum AFP. The rise in serum AFP level is observable in 80-90 % of hepatoblastomas. DLK-1 protein a newly described ancillary tool in the diagnosis of HB and the expression of this protein is utilizable in the differential diagnostics of childhood malignant hepatocellular neoplasms.

HB originate from immature liver precursor cells, which come through different developmental stages, as shown by the subtypes of HB. HB subtypes have different prognostic significance. The epithelial type –being the most common type- composed of two differing epithelial components, which based on their combination, can be divided into clearly fetal and fetal/embryonal macrotrabecular and undifferentiated small cell subtypes. Few markers are available for the morphological distinction between fetal and embryonal subtypes.

Loss of cell-to-cell adhesion is one of the important mechanisms in carcinogenesis. Previously, our group observed differences in the expression of the TJ proteins CLDN-1 and CLDN-2 between the fetal and embryonal components of HB. In line with these findings, in our current study we were able to further demonstrate differences between the two epithelial HB cell types with regard to another TJ protein, TRIC. Altered TRIC expression was observed in hepatocellular carcinoma, as well as in cholangiocarcinoma

and fibrolamellar hepatocellular carcinoma and shown to be related with progression and survival.

EZH2 has enzymatic activity and functions as a histone H3 methyltransferase. Overexpression of EZH2 has also been described in many epithelial and non-epithelial tumors. EZH2 expression is elevated in invasive carcinomas and metastases, and increased expression is strongly associated with a poor clinical outcome. In HCC and CC, EZH2 overexpression is associated with aggressive and metastatic disease and poor clinical outcome. However, this mechanism has not yet been clarified in HB.

Our further aim was to explore the highly hepatocyte specific and sensitive marker Arginase-1 (Arg-1) expression in HB samples.

2. The aim of the study

Expression pattern of tight junction (TJ) proteins might be an ancillary tool in case of many human cancers as regards the differential diagnosis and prognosis, but also regarding therapy. Previously, our group demonstrated higher claudin-1 (CLDN-1) and claudin- 2 (CLDN-2) protein and mRNA expression in the welldifferentiated fetal compartment compared with the less-differentiated embryonal component of human HBs. These earlier results suggested that the CLDN pattern might indicate differentiation and also hinted at a potential association between the CLDN pattern and HB prognosis. However, there have been no data about epxression a newly described member of tight junction protein, tricellulin in human hepatoblastomas. There have been also no data about expression of EZH2 and arginase-1 proteins in human epithelial hepatoblastomas.

According to the data summarized in the introduction, the following aims were developed:

- We aimed to investigate the expression of a newly described member of the TJ family, tricellulin (TRIC), in different epithelial types of HB.
- We aimed to investigate the expression of EZH2 in different epithelial types of HB.
- We aimed to investigate the expression of arginase-1 protein in different epithelial types of HB.
- Investigating the expresssion of ß-catenin in different epithelial types of HB.
- Our aim was to analyze the overall and event free survival.
- We aimed to analyze tight junction proteins (Claudin-1 and tricellulin) and EZH2 expression in developing human liver.

3. Material and methods

3.1. Patients and tissue samples

In this study, 21 HB patients were enrolled with the permission of the Regional Ethical Committee of the Semmelweis University /#192/. Patients were between 3.6 months and 189.8 months of age (mean 45.9 months) at the time of diagnosis and the male/female ratio was 11/10. 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. All patients were diagnosed and treated oncologically preceding surgery, according to the SIOPEL international collaborative protocol. The treatment strategy of the SIOPEL protocol is based on pre-operative chemotherapy. Biopsy is emphasized prior to chemotherapy, although it is not mandatory.

Each of the 21 cases was classified as wholly epithelial HB, and subtyped further as pure fetal (n = 12) and embryonal/fetal (n = 9). Of the nine embryonal/fetal cases, a fetal component of <5% was observed in five cases, whereas 30–50% fetal cells were present in the other four. Both fetal and embryonal components of the latter cases were macrodissected and evaluated separately for semiquantitative immunohistochemistry (IHC). Thus, 16 fetal and nine embryonal HB samples together with 16 samples of non-tumorous surrounding liver were analysed in the study, and patterns of protein expression in the fetal and epithelial compartments were compared with each other. For the four embryonal/fetal cases showing a 30–50% fetal component, the different compartments were evaluated separately using IHC; these four cases, however, were not included in the analysis of the association of different components with overall survival and metastasis formation.

An additional 57 normal fetal liver samples were obtained from spontaneously miscarried fetuses (of between 13 and 23 weeks gestation) delivered at the First Department of Obstetrics and Gynecology, Semmelweis University, Budapest.

3.2. Histology and immunhistochemistry

The tumour and surrounding liver tissues obtained from the surgical resection specimens were processed according to routine pathology procedures for histological

examination. Briefly, tissues were fixed immediately after removal in 10% neutral buffered formalin (in PBS, pH 7.0) for 24 h at room temperature. Following dehydration in a series of ethanol and xylene, the samples were embedded in paraffin. The 3–5-lm-thick sections were stained routinely with haematoxylin and eosin (H&E) for classification of the tumours. Formalin-fixed paraffin-embedded (FFPE) sections of 3–5-lm thickness were cut and used for IHC. Reactions were carried out using a Ventana ES automatic immunostainer (Ventana Medical Systems, Inc., Tucson, AZ, USA) with the HRP multimer-based, biotin-free detection technique. Primary antibodies were used which recognised: ß-catenin (mouse monoclonal; Millipore, Billerica, MA, USA; dilution 1:200); Claudin-1 (rabbit polyclonal; Cell Marque; 1:100); EZH2 (mouse monoclonal; BD Biosciences, Mississauga, ON, Canada; 1:40); and TRIC (rabbit polyclonal; Invitrogen, Carlsbad, CA, USA; 1:50). Reagents and secondary antibodies were obtained from Ventana (iView DAB Detection Kit; Ventana).

3.3. Tissue microarray (TMA) validation

57 normal fetal liver samples were investigated by building TMA multiblocks. Two cylinders (diameter=2 cm) were removed from each paraffin block and the cores were used to create 5x10 matrix arrays, recipient blocks as described by the manufacturer (TMA Master, 3D Histec). Finally all immunohistochemical reactions were also called.

3.4. Semiquantitative analysis of immunohistochemical reactions

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 (940). The percentage of cells positive for TRIC 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. The EZH2 and b-catenin-positive nuclei were counted and expressed as percentages, and scored as follows: 0 (≤1% positivity), 1 (2–30% positivity), 2 (31–65% positivity) and 3 (66–100% positivity). Nuclear staining intensity was also evaluated as mentioned above and the scores of both area positivity and intensity were summed.

3.5. Cell culture

Our cell lines derived from American Type Culture Collection (Manassas, VA). Four cell lines were: Hep3B hepatocellularis carcioma (ATCC® HB-8064™), HepG2 hepatocellularis carcioma (ATCC® HB-8065™), HEK-293 embryonal kidney (ATCC-1573) and HUH-7 (ATCC HB- 8064) hepatocellularis carcinoma-derived cell lines.

3.6. Immunofluorescent staining

In order to be able to predict exact protein localization of TRIC and EZH2 immunofluorescence examinations were performed with primary antibodies. Samples were fixed in liquid nitrogen. Frozen sections were incubated with antibodies against tricellulin (TRIC) and EZH2, and fluorescence Alexa Fluor 488 (FITC) and/or Alexa 568 labelled secondary antibodies. We examined and photographed the frozen and labelled sections with a Leica DMRXA fluorescent microscope. Then, we fixed the images with the help of Leica CW4000 FISH visualisation and documentation Software.

3.7. Statistical analysis

Overall scores of immunoreactions related to the three histology groups, namely fetal and embryonal/fetal subtypes of HB and non-tumorous surrounding liver, were compared using the non-parametric Kruskal–Wallis ANOVA test. Association between the presence of distant metastasis and EZH2 expression was investigated with Fisher's exact test. EFS and OS were determined using the Kaplan–Meier method, in which event HB cases were divided into four groups based on whether TRIC and EZH2 IHC scores were lower or higher than the median TRIC or EZH2 values. Comparisons between survival functions for different strata were assessed with log-rank statistics. All the analyses were performed using STATISTICA software version 9.0 (StatSoft Inc., Tulsa, OK, USA). Statistical significance was set at $p < 0.05$.

4. RESULTS

4.1. Arginase-1, ß-catenin, EZH2 and tricellulin expression in non-tumorous surrounding liver. Arginase-1 showed 100% nuclear/cytoplasmic strong positivity in hepatocytes. Biliary structures (including arteries and veins) showed no staining with arginase-1 anitbody.

All the samples of non-tumorous surrounding liver proved to be negative for nuclear/ cytoplasmic **ß-catenin** staining, but the hepatocytes showed a membranous reaction.

None of the samples of non-tumorous surrounding liver showed staining with **EZH2** antibody.

Tricellulin immunoreaction in the surrounding liver was seen as small dots or lines along the membranes of hepatocytes and bile ducts, of varying intensity.

4.2. Arginase-1, ß-catenin, EZH2 and tricellulin expression in different epithelial types of HB by immunohistochemistry. All cases of hepatoblastomas showed immunoreactivity for **Arg-1** with strong, diffuse positivity. No significant differences were detected between fetal and embryonal subtypes. These data demonstrated that Arg-1 is a useful marker for HBs.

Cytoplasmic and/or nuclear staining of **ß-catenin** was detected in 20 (95%) of the 21 HB cases. Nuclear staining could be detected in 17 (81%) of the 21 cases. In contrast, all the samples of non-tumorous surrounding liver proved to be negative for nuclear/cytoplasmic ßcatenin staining, but the hepatocytes showed a membranous reaction. The nuclear ß-catenin IHC score (cumulative score of percentage and intensity) was increased significantly in the embryonal component when compared with the fetal component and surrounding liver. In comparison to the surrounding liver, fetal tumour cells also displayed significantly higher nuclear ß-catenin expression. Conversely, cytoplasmic ß-catenin expression showed no significant differences in the two epithelial tumour compartments. Nevertheless, diffuse cytoplasmic staining was considerably higher in both fetal and embryonal cells when compared with the surrounding hepatocytes

Nuclear **EZH2** reaction was detected in both embryonal and fetal cells (in 100% of HB cases), ranging from moderate to very intensive/strong reaction. In contrast, none of the samples of non-tumorous surrounding liver showed staining with EZH2 antibody.

The nuclear IHC score was significantly higher in the embryonal component when compared with the fetal component whereas both embryonal and fetal compartments displayed a significantly elevated IHC score in comparison to the surrounding liver samples.

Membranous **TRIC** expression was detected in 19 (90%) of the 21 HB samples and in all the 16 surrounding liver samples. Extensive intense TRIC expression was seen in the fetal compartments of HBs along the membranes of tumour cells. In contrast, immunoreaction was less intense and the percentage of positive areas was lower in the embryonal areas of tumours with spotty accumulations, especially at the apical pole of the pseudorosettes formed by the embryonal tumour cells. Immunoreaction in the surrounding liver was seen as small dots or lines along the membranes of hepatocytes and bile ducts, of varying intensity. Intriguingly, TRIC nuclear immunostaining was observed in 5 (24%) of the 21 HB samples (four fetal and one embryonal), although only a small percentage (2–3%) of tumour cells per field displayed nuclear immunostaining. In these cells, membranous TRIC expression could not usually be observed; however, in one of the five cases there were a few cells with both nuclear and membranous TRIC expression. The IHC scores for TRIC expression in fetal tumour cells proved to be increased significantly in comparison to embryonal cells and surrounding non-tumorous liver cells.

4.3. EZH2 and tricellulin expression in hepatoblastoma lung metastasis. In one cases of distant hepatoblastoma metastasis expression pattern of EZH2 and tricellulin were the same as in the primary tumor.

4.4. Tricellulin immunofluorescent staining in hepatoblastoma samples. In five cases, which were all fetal subtype, tricellulin staining could be detected along the membranes of hepatocytes and bile ducts, of varying intensity.

4.5. Association of EZH2 expression with nuclear ß-catenin expression. Expression of EZH2 was observed in 100% (21 of 21) cases, whereas nuclear ß-catenin expression was detected in only 81% (17 of 21) of the HB cases. Both intensity and area positivity scores were elevated in the embryonal component in both EZH2 and nuclear ß-catenin reactions: poorly differentiated embryonal cells showed significantly increased

EZH2 and nuclear ß-catenin IHC scores when compared with the well- differentiated fetal subtype. Furthermore, neither EZH2 nor nuclear ß-catenin staining could be detected in the surrounding non-tumorous liver samples.

4.6. Survival analysis. No significant differences were detected between fetal (n = 12) and embryonal/fetal cases (n = 5) in OS or EFS. Mean OS was 97.4 months (range 20–209), while mean EFS was 76.3 months (range 11–209). Hepatoblastoma cases were classified further into TRIC-low, TRIC-high, EZH2-low and EZH2-high groups, according to their position above or below the median of cumulative IHC score values of TRIC and EZH2. The TRIC-low group was associated with a signifcantly shorter OS (mean 75.1 months) than the TRIC-high group (mean OS 122.4). No mortality was observed in the TRIC-high group and only one event occurred in the EZH2-low group. In the EZH2-high group mean OS was 87.1 and mean EFS 64.0 months. In the EZH2-low group, mean OS was 98.9 months and mean EFS 94 months. Comparison of the EZH2-low and -high groups revealed no significant differences in EFS or OS. However, the group showing high EZH2 expression was associated with the presence of distant metastases. There was no difference in EFS and OS in case of ß-catenin and arginase-1.

4.7. Tricellulin expression in cell-lines. HUH-7 and HEK-293 cell lines expressed only cytoplasmic TRIC expression, normal membranous staining could not been detected. Hep3b hepatocellular carcinoma-derived cell line showed only cytoplasmic TRIC staining. On the other hand, in HepG2 cell line expressed nearly 100% strong, intranuclear TRIC staining.

4.8. Developing normal human fetal liver immunohistochemistry. **Claudin-1** (CLDN-1) expression was investigating in developing liver. Claudin-1 showed strong expression mainly along the hepatocyte membrane and was strongly expressed in the biliary epithelium from week 14 to week 23. This finding indicates that CLDN-1 also has a major role in liver development.

We concluded that **TRIC** is expressed in the developing human liver from week 14 to week 23. The expression of TRIC is restricted to the hepatocytes and biliary epithelium. These results indicate that TRIC has a major role in the normal development of the liver.

EZH2 expression was also investigated in the developing liver of 57 cases of spontaneous abortion. We concluded that EZH2 is strongly expressed in the developing human liver from week 14 to week 23, proving that EZH2 is involved in and necessary for fetal hematopoiesis. EZH2 was found to be negative in the liver of 1 year old babies and in adults.

4.9. Developing normal human fetal liver immunofluorescent study. 10 cases were investigating with EZH2 and TRIC antibody. All cases expressed strong nuclear EZH2 and membranous TRIC staining pattern.

5. Conclusions and new findings

1. This is the first report describing the expression of TRIC in HB.
2. Well-differentiated fetal epithelial type HB cells sexpressed TRIC with significantly higher intensity and area positivity than the less-differentiated embryonal epithelial cells. Based on these data TRIC might have role in the differentiation, progression. aggressiveness and prediction of survival of patients treated with HB.
3. Our current study proved that high TRIC expression is associated with a considerably more favourable overall survival of HB patients as compared with low TRIC expression.
4. The observation of nuclear TRIC positivity in HB samples and cell lines is of interest, as localization of TJ protein would not usually be expected to this site in the cell.
5. The expression of EZH2 was significantly higher in the embryonal component when compared with the fetal component. Accordingly, epigenetic regulation might have a major role in HB progression. EZH2 might be an ancillary marker available for the morphological distinction between fetal and embryonal subtypes. However, the group showing high EZH2 expression was associated with the presence of distant metastases, suggesting that EZH2 might have role in HB aggressiveness in case of treated patients.
6. In our current study, 95% of the tumour samples showed abnormal (nuclear/cytoplasmic) ß-catenin staining. The nuclear ß-catenin IHC score (cumulative score of percentage and intensity) was increased significantly in the embryonal component when compared with the fetal component and surrounding liver.
7. In addition, coexpression of EZH2 and nuclear ß-catenin was observed in 81% of HB cases and the poorly differentiated embryonal type of HB exhibited significantly stronger staining of both proteins. These data are in accordance with the literature; however, the role of EZH2 in Wnt signalling needs further exploration in HB.

8. Our further aim was to explore the highly hepatocyte specific and sensitive marker Arginase-1 (Arg-1) expression in HB samples. All cases of hepatoblastomas showed immunoreactivity for Arg-1 with strong, diffuse positivity. No significant differences were detected between fetal and embryonal subtypes. These data demonstrated that Arg-1 is a useful marker for HBs.
9. We concluded that TRIC is expressed in the developing human liver from week 14 to week 23. The expression of TRIC is restricted to the hepatocytes and biliary epithelium. These results indicate that TRIC has a major role in the normal development of the liver. Similarly to TRIC, Claudin-1 (CLDN-1) expression was investigating in developing liver. Claudin-1 showed strong expression mainly along the hepatocyte membrane and was strongly expressed in the biliary epithelium from week 14 to week 23. This finding indicates that CLDN-1 also has a major role in liver development.
10. We concluded that EZH2 is strongly expressed in the developing human liver from week 14 to week 23, proving that EZH2 is involved in and necessary for fetal hematopoiesis. EZH2 was found to be negative in the liver of 1 year old babies and in adults.

6. List of publications

Total Impact Factor (IF): 7,711

Publications related to the Dissertation (IF: 5,151):

Schlachter K , Gyugos M , Halasz J , Lendvai G , Baghy K , Garami M , Gyongyosi B , Schaff Z , Kiss A

High Tricellulin Expression Is Associated With Better Survival In Human Hepatoblastoma.

HISTOPATHOLOGY 65:(5) pp. 631-641. (2014)

IF: 3.301

Hajosi-Kalcakosz S, Dezso K , Bugyik E , Bodor C , Paku S , Pavai Z , Halasz J , Schlachter K , Schaff Z , Nagy P

Enhancer of zeste homologue 2 (EZH2) is a reliable immunohistochemical marker to differentiate malignant and benign hepatic tumors.

DIAGNOSTIC PATHOLOGY 7: Paper 86. (2012)

IF: 1.850

Publications not related to Dissertation (IF: 2.560)

Gyugos M , Lendvai G , Kenessey I , Schlachter K , Halász J , Nagy P , Garami M , Jakab Z , Schaff Z , Kiss A

microRNA expression might predict prognosis of epithelial hepatoblastoma

VIRCHOWS ARCHIV-AN INTERNATIONAL JOURNAL OF PATHOLOGY 464:(4) pp. 419-427. (2014)

IF: 2.560