Expression of tricellulin in the human pancreas and liver as well as in primary pancreatic and hepatic tumors

PhD Thesis

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1. INTRODUCTION

Ductal adenocarcinomas of the pancreas represent 90% of the exocrine pancreatic tumors, the remaining 10% are neoplasms of cystic origin. An account of the anatomical localization and the late onset of the symptoms, the diagnosis of the disease is especially difficult. The therapeutical tools are limited, so the prognosis of the disease is very poor: after the date of the exact diagnosis, the one-year mortality is 90%, the long term survivals come from the surgically treated population. The five-year survival rate is under 7%. Functioning or non-functioning pancreatic tumors of endocrine origin can be rarely seen, only in 1-2% of all pancreatic neoplasms. While ductal adenocarcinomas show an 'adenoma-carcinoma' sequence model with certain genetic disorders in the background, the endocrine tumors have no progression model. Survival rates of endocrine pancreatic neoplasms are better than in case of exocrine tumors, the survival of the surgically treated, well-differentiated neoplasms are over 60%. Acinar cell cancers of the pancreas are 1-2% of all pancreatic cancers. The neuroendocrine origin of this type is unequivocal because of the chromogranine-, synaptophysine- and neuron-specific enolase-positivity, while the exocrine origin is confirmed by the ability of acinar production of the tumor. These are aggressive neoplasms with poor prognosis; they can metastatize to the liver at an early stage. The only efficient therapy is surgery, recurrent or inoperable cases have very poor therapeutical potential. Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver. There are many congenital and acquired factors that play important role in the etiology of this neoplasm, such as liver cirrhosis, hepatotropic viruses (HBV, HCV) and chronic hepatitis, excessive alcohol consumption, aflatoxin exposition as well as certain storage diseases. In most cases of HCC occurs in cirrhotic liver. Despite the limitation of the therapeutical possibilities, the tumor type has poor prognosis: the five-year survival rate is around 15-20%. Fibrolamellar HCC (FLHCC) is a rare variant of HCC, usually diagnosed at a younger age and in non-cirrhotic liver. The etiology of this tumor-type is unknown, the only known risk factor is focal nodular hyperplasia (FNH). Survival data are better in comparison to HCC. Given early adequate therapy, the five-years survival rate is around 30% - to which younger age and a basically healthy liver might also contribute.

The types of cholangiocarcinoma (CC) depend on the site of origin; they can be extra- and intrahepatic. The incidence of intrahepatic CC is increasing all over the world. The etiology is mostly unknown, the main risk factors are primary sclerosing cholangitis, policystic liver disease, certain parasitic diseases (e.g. sheep liver fluke), cholelithiasis and toxins. The five-

years survival rate after R0-resection is around 30%, the five-years survival rate of all diagnosed CCs is under 20%. The average survival time is between 15 and 28 months.

The tight junction (TJ) is a multiprotein membrane complex at the apical pole of epithelial and endothelial cells. The main function of this complex is the regulation of the paracellular transport mechanisms and permeability between two adjacent cells. TJs play important role in other cellular mechanisms as well, such as the formation and preservation of the polarity of these cells with regulation of diffusion at apical and basolateral cell surfaces and the receptor-mediated endocytosis at the apical membrane, as well as the exocytosis at the basolateral membrane. TJs have further function in the maintenance of the osmotic equilibrium between the cell and extracellular matrix, too. Certain TJ proteins play role in cell proliferation and regulation of selected signalling pathways, while others have elementary function in carcinogenesis, metastasis formation and tumor progression.

TJ proteins take part in the epithelial-mesenchymal transition, too. They are built from integrant and cytoplasmatic proteins. ZO-proteins have a role not only in the structure of TJs, but in the maintenance of this cell-adhesion complex as well: it is well known that epithelial and endothelial cells are not able to form TJs in the absence of this protein family. Overexpression of occludin increases transepithelial electric resistance, which might confirm the barrier function of this protein. Earlier, the role of this protein in cell proliferation was unknown, but nowadays its presence in centrosomes has been confirmed. It can influence mitosis by a phosphorylation-assisted centrosome-separation mechanism. The first claudins (CLDN) were described in 1998, today almost 30 subtypes are known. Many of these proteins show tissue- and cell-specificity. They have two phylogenetically based subclasses: ’classic’ CLDNs and ’non-classic’ CLDNs. Their common function is the closing of intercellular gaps. CLDNs play role in the regulation of paracellular transport mechanisms as well as in lateral diffusion and cell-adhesion. It is well known that CLDNs-3 and -4 are receptors of the enterotoxin of Clostridium perfringens (CPE); after the receptor-ligand binding the depolimerization of the CLDN-molecule is observable and finally increased cell membrane-permeability can be detected. Because of this, the link between CPE-receptor and CLDNs are the focus of cancer-targeted therapeutical research.

In normal human pancreas, the ductal cells show CLDNs-1, -2, -3, -4 and -7 protein positivity, the acinar cells express CLDNs-1, -3, -4 and -7, whereas the endocrine part of the pancreas produces CLDN-3 and -7 proteins. Ductal adenocarcinomas overexpress CLDNs-1, -2, -4 and -7, whereas endocrine pancreatic cancers show only CLDNs-3 and -7 positivity. In normal liver, linear CLDN-1 and CLDN-7 membrane positivity can be detected at the apical

surface of the hepatocytes and biliary cells. Expression of CLDN-2 protein was shown to be cytoplasmatic, granular. CLDN-3 can be detected weakly in the membrane of hepatocytes, whereas it can be identified with stronger expression on the apical surface of biliary cells. CLDN-4 protein can only be expressed by biliary cells.

Actual results of barriology suggest that TJs have two subclasses: bicellular TJs between two cells and tricellular TJs between three cells. Components and function of bicellular TJs have been known for years, whereas tricellular junctions are a relatively new part of this discipline.

Tricellulin (TRIC) is a 63,6 kDa protein, built of 555 aminoacids. It has four transmembrane domains, two extracellular loops and intracellular terminals. Four isoforms are known. Although the exact function of this protein is not yet known, the maintenance of the transepithelial barrier is one of its crucial roles. After inhibition of TRIC-expression, decreased transepithelial resistance and increased paracellular permeability was detected. The fact that the diameter of the central tubule of tricellular TJs is larger than the size of many drugs can facilitate the function and role of TRIC in drug delivery in the future.

2. AIMS OF THE STUDY

There are many studies on barriology around the world, some results may have important consequences in cancer target therapy in the future. We focused on TRIC because of its known roles in cell differentiation, carcinogenesis and tumorprogression.
These investigations were done on cell lines, dominantly. TRIC protein was not tested in human liver and pancreas before. Based on all these, our aims were to answer the following questions:

2.1. Can TRIC expression be found in normal human pancreas? If yes, are there any differences in the level of expression in ductal, acinar or endocrine structures?

2.2. Can TRIC-protein be observed in pancreatic ductal adenocarcinomas?

2.3. Can any differences be detected in the expression of TRIC between normal pancreas and ductal adenocarcinomas or in adenocarciomas with altered differentiation?

2.4. Can TRIC be observed in endocrine pancreatic tumors? If yes, are there any differences in expression in the neoplasms with altered differentiation?

2.5. Can TRIC be detected in pancreatic acinar cell carcinomas?

2.6. Can TRIC-expression be found in normal human liver? If yes, are there any differences in the level of expression or in the localization between hepatocytes and cholangiocytes?

2.7. Can TRIC-protein be observed in cirrhotic, non-tumorous liver tissue?

2.8. Can TRIC-protein be observed in HCCs?

2.9. Are there any differences in the expression of TRIC in HCCs with altered differentiation?

2.10. Can TRIC-protein be detected in fibrolamellar HCCs?

2.11. Can TRIC-protein be observed in cholangiocarcinomas? If yes, are there any differences between expression in the neoplasms with altered differentiation?

3. 3. MATERIALS AND METHODS

3.1. Patients, surgical specimens

Samples from 128 cases of surgically removed tumors were analyzed from the archive of the 2nd Department of Pathology, Semmelweis University with the permission of the Regional Ethical Committee of Semmelweis University (#172/2003): 76 pancreatic tumors, 52 liver neoplasms and 12 cirrhotic liver. Altogether 58 resecated pancreatic ductal adenocarcinomas (14 grade 1, 19 grade 2 and 25 grade 3), 15 endocrine pancreatic tumors (5 benign, 7 borderline and 3 malignant) and 3 acinar cell carcinomas were obtained. A total of 32 HCC (10 grade 1, 13 grade 2, 8 grade 3 and 1 mixed grade tumor) liver samples were examined and 20 intrahepatic cholangiocarcinomas (5 grade 1, 9 grade 2 and 6 grade 3 neoplasms) were studied. Our study also included 12 cirrhotic, non-tumorous liver samples. In the other part of our investigations we studied fibrolamellar HCCs (11 cases) and 7 cholangiocarcinomas. Five peritumoral normal pancreatic samples, five PDACs, as well as normal liver tissues and HCCs were used for immunofluorescence studies. The fresh frozen samples were stored at -80°C.

Surrounding non-tumorous pancreatic and liver samples were available in 20-20 surgically removed resection specimens.

3.2.Methods

3.2.1. Histopathology

Tissue samples were fixed immediately after removal in 10% neutral buffered (in phosphate-buffered saline, pH 7.0) formalin for 24 hours at room temperature, dehydrated in a series of ethanol and xylene, then embedded in paraffin. The 3–4 μm thick sections were routinely stained with hematoxylin and eosin (Reanal Ltd., Budapest, Hungary). In case of the diagnosis of acinar cell carcinomas, periodic-acid Schiff stain and PAS-diastase stain were used.

3.2.2. Immunofluorescence

Five peritumoral normal pancreatic samples, as well as five PDACs and 5 normal liver samples, 5 HCCs and 5 iCCCs were used for immunofluorescence studies. The frozen sections were fixed in a chilled mixture of methanol and acetone (1:1) for 5 minutes and air-dried at room temperature. Nonspecific protein binding was blocked with Protein Block Serum-Free solution (DAKO, Glostrup, Denmark). Polyclonal TRIC anti-C-terminal rabbit antibody (Invitrogen, Carlsbad, CA, USA) diluted 1:200 in Antibody diluent (Ventana,

Tucson, AZ, USA) was applied as primary antibody at 4°C overnight. Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen) diluted 1:200 in phosphate buffered saline (PBS) was used as secondary antibody at room temperature for 30 minutes in dark. Sections were covered with Vectashield mounting medium containing 4',6-diamino-2 phenylindole (DAPI) for nuclear counterstaining (Vector Labs., Burlingame, CA, USA). For TRIC and occludin, TRIC and synaptophysin as well as TRIC and collagen IV, dual immunofluorescence was carried out using primary antibodies. Frozen sections were incubated at 4°C overnight with the followings: primary antibodies of polyclonal TRIC anti-C-terminal rabbit antibody diluted 1:200, monoclonal anti-occludin mouse, monoclonal anti-collagen IV mouse and monoclonal anti-synaptophysin mouse antibodies diluted 1:100. After washing with PBS, Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen) diluted 1:200 and Alexa Fluor 488 donkey anti-mouse IgG (Invitrogen) diluted 1:200 were used as secondary antibodies.

Dual immunfluorescence was performed for TRIC-CLDN-1 and for TRIC-MDR/MRP2 in order to confirm the orientation of the TRIC protein. In case of HCCs, TRIC-CLDN-1 dual staining was carried out, whereas TRIC-CLDN-4 co-expression was detected in intrahepatic cholangiocarcinomas. Primary and secondary antibodies were the same as in case of the pancreatic dual staining.

Reactions were analyzed using fluorescent microscope (Leica DM-RXA, Wetzlar, Germany) and confocal laser scanning microscope (Bio-Rad MRC-1024 system, Bio-Rad Lab., Hercules, CA, USA). Sections were covered with Vectashield mounting medium containing DAPI for nuclear counterstaining.

3.2.3. Immunohistochemistry

Three μm thin whole sections were deparaffinized in xylene and graded alcohol. Protease 1 (Ventana) was used for 4 minutes, followed by incubation with polyclonal TRIC anti-C-terminal rabbit antibody (Invitrogen) diluted 1:50 as primary antibody at 42°C for 40 minutes. Signal amplification was perfomed by Amplification Kit (Roche, Indianapolis, USA) according to the manufacturer's guide.

Reactions were carried out using Ventana Benchmark XT automated immunohistochemical staining system (Ventana) with HRP multimer based, biotin-free detection technique according to the protocol provided by the manufacturers. Secondary antibodies and reagents were obtained from Ventana and reactions were visualized by UltraView™ Universal DAB Detection Kit (Ventana). Sections from mouse duodenum, previously determined to express TRIC, were used as positive controls in case of the pancreas, whereas human duodenum was

used for the liver. For negative control, primary antibodies were substituted with Antibody diluent (Ventana).

3.2.4. Evaluation of TRIC immunohistochemistry

TRIC immunohistochemical reactions were evaluated by digital morphometrical analysis. All slides were digitalized by scanning with Mirax Pannoramic MIDI and Mirax Pannoramic SCAN digital slide scanners (3D Histech Ltd., Budapest, Hungary). Fifteen non-overlapping fields of view of each slide of interest were selected from pancreatic samples at 40x virtual objective magnification, whereas from liver samples at 60x virtual objective magnification. The extension of immunostained areas was measured using Leica QWin V3 image analysis and processing software (Leica Microsystems Imagine Solutions Ltd., Cambridge, UK). We defined the number of pixels of the positive areas, which were compared with the number of pixels of the whole fields of view, then the software defined the percent ratio of the immunpositive areas (area %). We counted the average values of the 15 raw data of each sample, then calculated the new, final value as 'Area %'.

3.2.5. Statistical analysis

Independent groups showing asymmetrical sample distribution were compared using Mann-Whitney U test complemented by post hoc method. Overall survival analyses were performed using the Kaplan-Meyer method. Overall survival intervals were determined as time period from initial diagnosis to time of death. Comparisons between survival functions for different strata were assessed by means of log-rank statistics in case of pancreatic samples and with Spearman-rank correlation tests in case of liver samples. Differences were considered significant when $p < 0.05$. All statistical analyses were done using Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA) software program.

3.2.6. Western blot

Proteins were extracted from fresh-frozen, -80°C stored samples of 5 PDACs, 5 peritumoral normal pancreas and 6 specimens each of HCCs, iCCs and normal livers. A total of 50 mgs each of the samples were homogenized, followed by centrifugation at 13200 rpm at 4°C for 5 minutes. Isolation was performed by using lysis buffer [20mM TRIS pH 7.5, 150 mM NaCl, 2mM EDTA, 1% TritonX-100 containing protease-inhibitor complex (Sigma Aldrich Co., St. Louis, MO, USA)]. Measurements of protein concentrations were performed

using Bradford-analysis. Equal quantities (30 µgs) of protein samples were loaded in each lane, run on 10% SDS-polyacrylamide electrophoresis at 200V for 35 minutes, then transferred to nitrocellulose membranes at 100V, 4°C for 75 min. For aspecific protein-blocking, fat-free milk (5%, PBS) was used for 30 minutes. Blots were incubated with polyclonal TRIC anti-C-terminal rabbit antibody (1:500, Invitrogen) and anti-GAPDH antibody (1:5000, AbDSerotec, Kidlington, UK) at 4°C overnight. After washing in 0.1% TRIS, the secondary antibodies as anti-mouse GAPDH (1:2000, AbDSerotec) and HRP conjugated anti-rabbit antibody (1:2000, DAKO) were applied at room temperature for 120 minutes. Following three series of washings in TRIS-Buffered Saline Tween-20 (TBST), signals were visualized by chemiluninescent detection (BioRad, Hercules, CA, USA), then the reactions were evaluated by Kodak gel documentation system (Kodak, Rochester, NY, USA).

3.2.7. RT-PCR

RNA-isolation

Five formalin-fixed, paraffin-embedded normal pancreatic tissue samples, 10 samples of grade 1, 11 samples of grade 2 and 9 of grade 3 PDACs were selected for real time RT-PCR analysis. Total RNA was isolated from five 5-10 µm macrodissected sections (necrosis, bleeding and connective tissue excluded) using RNeasy FFPE Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's guide. Absorption levels of RNA extracts were obtained using NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Reverse Transcription of RNA

cDNA samples were prepared from 1 µg total RNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA, USA) as specified by the manufacturer.

Primer design

Gene-specific primers were designed by AlleleID 6.01 primer design software (Premier Biosoft International, CA, USA) for real time RT-PCR. Isoform specificity and primer sizes were checked by BioEdit biological sequence alignment editor software (Tom Hall Ibis Therapeutics, Carlsbad, CA, USA). Primer specificity was checked by BiSearch software (Hungarian Academy of Sciences, Institute of Enzymology, Budapest, Hungary). Primer specific amplification degree (58°C) was optimized by gradient PCR.

PCR and Real-Time PCR

Real-time RT-PCR analysis was performed using SYBR Green technology on ABI Prism 7000 Sequence Detection System (Applied Biosystems), according to manufacturer's instructions. Abelson murine leukemia viral oncogene homologue 1 (ABL-ab) was used as internal control gene. Primer specific amplification was controlled by 2% agarose gel electrophoresis as well as melting temperature analysis. The final 20 µl reaction mixture contained Power SYBR Green PCR Master Mix (Applied Biosystems), 10 pM of forward and reverse primers and 100 ng cDNA as template. Amplification conditions were as follows: incubation at 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds, at 60°C for 60 seconds and at 72°C for 15 seconds, with subsequent melting analysis: heating to 95°C for 20 seconds, cooling to 45°C for 10 seconds, then reheating to 95°C. Primer specific amplification was checked by 2% agarose gel electrophoresis and melting analysis.

4. RESULTS

4.1. „Can TRIC expression be found in normal human pancreas? If yes, are there any differences in the level of expression in ductal, acinar or endocrine structures?”

The presence of TRIC was seen in acinar cells, ductuli and bigger ducts by immunohistochemistry. The protein was detectable with a dot-like pattern at tricellular points, close to the apical poles of ductal and acinar cells, whereas linear membrane positivity was identified at bicellular junctions. TRIC and occludin co-localization was detected at the apical membranes of acinar and ductal cells with double immunostaining.

Endocrine parts of the pancreas did not express TRIC protein. For the confirmation of our results, Western-blot analysis was carried out.

We did not notice any differences in the level of expression of TRIC depending on the anatomical structure of the pancreas.

4.2. „Can TRIC-protein be observed in pancreatic ductal adenocarcinomas?”

Spotty expression of TRIC was noticed in grade 1, grade 2 and grade 3 ductal adenocarcinomas at the luminal pole of the tumor cells.

4.3. „Can any differences be detected in the expression of TRIC between normal pancreas and ductal adenocarcinomas or in adenocarciomas with altered differentiation?”

Compared to normal pancreas, well differentiated ductal adenocarcinomas showed significantly lower linear TRIC-positivity. TRIC expression decreased in parallel to the dedifferentiation of the tumors. TRIC production was decreased significantly in grade 2 carcinomas as compared with normal pancreas and grade 1 adenocarcinomas. This result was confirmed by digital morphometric analysis. Grade 3 ductal adenocarcinomas expressed the protein at a significantly lower level as compared with normal pancreatic tissue, as well as with grade 1 and grade 2 tumors. We found the same results by digital morphometric analysis. Decrease of TRIC protein in parallel to the level of dedifferentation was confirmed by Western blot, respectively.

Log-rank analysis was carried out for the survival data, which showed significant relation between the grade of ductal adenocarcinomas and the survival of patients. Significant

correlation between the expression of TRIC and the grade of the tumors suggested indirect relationship between TRIC-expression and survival. In conclusion, well differentiated adenocarcinomas with high TRIC-expression showed better survival data as compared with undifferentiated tumors with low TRIC-expression.

Relative expression of TRIC-mRNA by quantitative real time RT-PCR was significantly higher in normal panncreatic tissue compared with grade 1, grade 2 and grade 3 adennocarcinomas. Significant difference of TRIC-mRNA between the adenocarcinoma groups was not identified, although in the grade 3 group it was higher than in the grade 1 and grade ??? groups, as well as in grade 2 tumors compared to grade 1 neoplasms.

4.4. „Can TRIC be observed in endocrine pancreatic tumors? If yes, are there any differences in expression in the neoplasms with altered differentiation?”

Neuroendocrine tumors were all TRIC-negative independent of the stage of the differentiation.

4.5. „Can TRIC be detected in pancreatic acinar cell carcinomas?”

Spotty immunreaction was detected at the apical pole of the abortive, gland-like tumor cells.

4.6. „Can TRIC-expression be found in normal human liver? If yes, are there any differences in the level of expression or in the localization between hepatocytes and cholangiocytes?”

Spotty aggregation of the protein was seen at tricellular junctions by immunofluorescence, whereas linear membrane positivity was detected at bicellular junctions. Normal hepatocytes expressed TRIC heterogenously at the apical surfaces of hepatocytes and cholangiocytes, too. TRIC-CLDN-1 and TRIC-MRP2 co-localization were seen by double immunofluorescence, confirming the exact localization of TRIC in normal hepato- and cholangiocytes. TRIC-CLDN-1 co-localization was detected in HCCs, whereas TRIC-CLDN-4 double staining in iCCs. Linear immunostaining was identified between two tumor cells, and spotty TRIC-expression was seen at tricellular junctions in fibrolamellar HCCs. Our

immunnohistochemical results were similar to the outcome of the immunofluorescent microscopy. These results were confirmed by Western blotting.

4.7. „Can TRIC-protein be observed in cirrhotic, non-tumorous liver tissue?”

Linear and spotty immunopositivity was detetcted in cirrhotic liver samples. Both small and large bile ducts showed spotty positivity. We could not identify any correlation between the level of expression and the severity of cirrhosis.

4.8. „Can TRIC-protein be observed in HCCs?”

Results of TRIC immunohistochemistry in HCCs were also found to be heterogeneous. A number of HCCs were TRIC negative, whereas others showed overexpression of the protein. Normal biliary cell-like TRIC expression was identified in the pseudoglandular structures of well- and mildly differentiated tumors, close to the apical surfaces. In the trabecular cells of the nesty-structured tumor mass linear immunopositivity was detectable. Certain poorly differentiated tumors epxressed TRIC very intensively. In six HCC samples nuclear positivity could be observed in 1-2%. Further investigations are needed for the precise explanation of this phenomenon.

4.9. „Are there any differences in expression of TRIC in the HCCs with altered differentiation?”

We did not detect any significant differences in TRIC-expression between normal liver tissue, cirrhotic liver and primary liver carcinomas. In almost half of the HCCs, twofold TRIC-expression was measured as compared with the normal liver. Correlation between tumor differentiation, tumor size or age of patients was not found in HCCs and CCs. Kaplan-Meier analysis was used to calculate the survival data of liver cancers. We could not identify any correlation between grade, size or age and the overall survival, however, survival time was signifantly lower in the high TRIC-producing HCC-group ($p < 0.05$). The risk ratio was significantly higher in the ’TRIC-high’-group by univariant Cox-regression analysis (3.12; CI: 1.35-7.2; $p<0.01$). Similar to pancreatic ductal adenocarcinomas, survival of the ’TRIC-high’-group was significantly higher compared to the ’TRIC-low’-group ($p < 0.05$). Decreasing TRIC expression by dedifferentation of cholangiocellular tumors resulted in significant differences in survival, too ($p < 0.05$).

60kDa product was observed in all HCCs by Western blot. Another product was seen at 80kDa in two-thirds of HCCs. TRIC expression was observable in 30% of well differentiated CCs. A larger product was detectable at 80 kDa and a smaller one at 50kDa. Conventional HCCs showed the highest TRIC-mRNA expression among liver carcinomas, whereas fibrolamellar HCCs produced less TRIC-mRNA. Significant differences were not detected.

4.10. „Can TRIC-protein be detected in fibrolamellar HCCs?"

Similar to conventional HCCs, linear membrane positivity was seen at the apical pole of tumor cells. Significantly higher TRIC-expression was detected in normal liver compared to iCCs, HCCs and first studies of fibrolamellar HCCs. iCCs showed significantly lower TRIC expression than conventional HCCs. Later we repeated our investigations on further fibrolamellar HCCs, but did not find significant differences between normal and tumorous TRIC-expressions. This can probably be explained by the higher case number-resulting in more accurate statistical analysis.

4.11. „ Can TRIC-protein be observed in cholangiocarcinomas? If yes, are there any differences between expression in the neoplasms with altered differentiation?"

Parallel to the dedifferentiation of iCCs, significant decrease of TRIC was observed, similar to pancreatic ductal adenocarcinomas. Grade 3 iCCs showed weak immunopositivity, whereas strong, spotty TRIC-positivity was detected in the glandular parts of the well differentiated tumors. We found very weak expression in no-glandular-like types of HCCs. Our results with iCCs were partly different: we found significantly less TRIC-expression when compared with HCCs in this group.

In our study, HCCs and CCs could be classified as 'TRIC-high' and 'TRIC-low' groups by digital morphometric analysis. The stage of tumor-differentiation, the size and age of patients are independent factors by Spearman rank correlation.

5. CONCLUSIONS

1. TRIC protein was detected in all of the acinar and ductal cells of the exocrine pancreas. We could not identify any expressional or localizational differences in TRIC between ducts and acini. TRIC was not expressed in the endocrine part of the pancreas.

2. TRIC was detected in pancreatic ductal adenocarcinomas at protein- and mRNA-levels.

3. Expression of TRIC protein was significantly decreased with the dedifferentiation of pancreatic adenocarcinomas when compared with each other and the normal pancreatic tissue. Molecular analyses revealed the mRNA-expression of TRIC to be significantly higher in the normal pancreas as compared with adenocarcinomas showing any differentiation.

4. TRIC protein was not expressed in endocrine pancreatic tumors, at either protein-, or mRNA-level.

5. Intense, granular TRIC-production was seen at the apical pole of the tumor cells of acinar cell carcinomas of the pancreas.

6. Heterogeneous TRIC-expression was seen in normal liver, the protein was detected at the apical surface of the hepatocytes, as well as at the cholangiocytes. TRIC showed co-localization with CLDN-1 and MRP2, confirming the apical localization of the TRIC protein.

7. We detected TRIC protein in macro- and micronodular cirrhotic liver samples, too. The expression pattern was similar to the pattern of the normal liver. Granular and linear immunopositivity were seen intranodularly. Differences in the level of expression between the normal liver and cirrhotis samples were not observable. We did not detect any correlation between the level of expression and the severity of the cirrhosis.

8. TRIC protein was identified in HCCs with altered differentation. The level of the expression was heterogeneous.

9. Expressions of grade 1 and 2 neoplasms were similar to the expression of the normal biliary epithelium. In grade 3 cases, only linear membrane positivity was detectable. Nuclear positivity was detected in six case of HCCs, 1-2% of the tumor cells. Further investigations are needed for the exact interpretation of this phenomenon. Correlation was not seen between

the differentiation and the size of the tumors or the age of the patients. Survival time was significatly shorter in HCCs with higher TRIC-expression.

10. The prescence of TRIC protein was verified in fibrolamellar HCCs in perimembranous localization. Expression of the protein was significantly higher in normal liver than in fibrolamellar HCCs.

11. TRIC protein was detected in CCs apically, showing both linear and granular pattern.

12. Significant decrease in TRIC-expression was seen in intrahepatic CCs during the dedifferentation of the tumors. Survival rates of CCs - similarly to pancreatic ductal adenocarcinomas - were significantly better in the TRIC-overexpressing group, controversial to HCCs.

6. LIST OF OWN PUBLICATIONS

Publications related to the dissertation:

Korompay A, Borka K, Lotz G, Somorácz A, Törzsök P, Erdélyi-Belle B, Kenessey I, Baranyai Z, Zsoldos F, Kupcsulik P, Bodoky G, Schaff Z, Kiss A. Tricellulin expression in normal and neoplastic human pancreas. *HISTOPATHOLOGY.* 60:(6B) pp. 76-86. (2012) **IF: 2.857**

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