NEW DIFFERENTIATION AND DIFFERENTIAL DIAGNOSTIC MARKERS IN HUMAN HEPATOBLASTOMAS

Ph.D. Thesis Synopsis

Dr. Judit Halász

Semmelweis University,

School of Doctoral Studies of Pathology

Supervisor: Dr Zsuzsa Schaff, Professor of Pathology

Critical Examiners: Dr. Gábor Kovács, Associate Professor
Dr. Miklós Bodó, Professor of Pathology

President of the University Examination Committee:

Dr. Éva Keller, Professor of Forensic Medicine

Members of the University Examination Committee:

Dr. Margit Abonyi, Associate Professor
Dr. Károly Simon, Head of Pathological Department

Budapest
2010

1. INTRODUCTION

Hepatoblastoma is the most frequent malignant primary liver tumor in infancy and early childhood, even so it is relatively rare, since it comprises only 1 % of all childhood malignant tumors. According to the 1972-1992 study data of the Surveillance Epidemiology and End Results (SEER), childhood malignant liver tumors show a 5 % increase annually. The cause of development is not exactly clear, but it is a fact that besides genetic and environmental factors, there is a higher incidence in boys and in prematurely born, low weight children. In general, hepatoblastoma appears under the age of 3 years, but the mean age regarding diagnosis is 1 year. The prognosis of hepatoblastoma in older children and young adults is worse than in infancy and early childhood (1-3 years of age). The best marker for the recognition and follow-up of hepatocellular tumors is the serum AFP. The rise in serum AFP level is observable in 80-90 % of hepatoblastomas. Low AFP blood level (<100 ng/ml) has bad prognosis in case of hepatoblastomas. Hepatoblastomas originate from immature liver precursor cells, however, increasing evidence suggests that the tumor is derived from pluripotent stem cells of the liver. In contrast to hepatocellular carcinoma, which often develops on the ground of cirrhosis, hepatoblastoma develops in cirrhosis-free liver. Trisomy of the 2., 20. and 8. chromosomes has been described in the karyotyping of hepatoblastoma, which are assumed to be of importance in the early phase of tumorigenesis. Loss of heterozygosity (LOH) of chromosome arm 11p markers occurs commonly in hepatoblastoma identified in association with Beckwith-Wiedemann syndrome and hemihypertrophy. Patients with familial adenomatous polyposis (FAP), a syndrome of early-onset colonic polyps and adenocarcinoma, frequently develop hepatoblastomas. Germline mutations in the adenomatous polyposis coli (APC) tumor suppressor gene occur in patients with FAP, and mutations in the APC tumor suppressor gene are frequently detected in the colonic polyps and adenocarcinomas associated with FAP. A product of the APC gene is the APC protein, which has important role in cell adhesion and other signal transfer processes. The mutation of the APC gene goes with loss of function, during the course of which the cytoplasmatic degradation of the β-catenin protooncogene does not take place, it accumulates intracellularly then becomes translocated into the cell nucleus where it binds to TCF-4. During this process, with the help of transcriptional factors, certain oncogenes e.g.: c-myc, cyclin D1, TCF/LEF become activated, which enhance proliferation and dedifferentiation and inhibit apoptosis.

The mutation of β-catenin, independent of histological type, can be found in almost 50% of hepatoblastomas.

According to the **histological classification** of the tumor, epithelial (i.e. containing only epithelial elements) and mixed (i.e. containing both epithelial and mesenchymal elements) types can be distinguished. The latter can be divided into teratoid and non-teratoid subtypes. The epithelial type is composed of two differing epithelial components, which based on their combination, can be divided into clearly fetal and fetal/embryonal subtypes, respectively. Also included in this group are the macrotrabecular and undifferentiated small cell subtypes, which differ in appearance from the above. In the fetal component the growth pattern of the tumor cells is similar to the arrangement of normal liver cells: forming bundles, trabecules, whereas the embryonal component is mostly made up of clusters of elongated, ovoid shaped undifferentiated tumor cells, which show mitotic activity. The tumor cells of the embryonal component frequently form so-called pseudorosettes. The undifferentiated cells of the tumor mostly resemble blastema cells, therefore the so-called "small, round, blue cell" tumors (nephroblastoma, neuroblastoma, rhabdomyosarcoma, lymphomas) can be taken into consideration in the differential diagnostics. The two components of hepatoblastomas are frequently mixed.

According to the European SIOPEL (International Childhood Liver Tumour Strategy Group), the treatment of hepatoblastomas is based on firstly systematic chemotherapy and second on the best possible surgical removal of the tumor. In North America the Children's Cancer Study Group (CCSG), the Pediatric Oncology Group (POG) and the Children's Oncology Group (COG), in contrast to the SIOPEL group, are in favor of the surgical removal of the tumor, following which the patients are then given chemotherapeutic treatment. Despite the varying therapeutic approaches, the 5-year survival rates are similar, being above 70 %.

Claudins (CLDN) take part in the construction of tight junction (TJ) type cell junction structures. The molecular family was discovered in 1998, and presently 24 members are known. Each claudin molecule is made up of 4 transmembrane segments and has two extracellular loops; the N and C terminal regions are located intracytoplasmatically. Regarding their function, TJs establish intercellular connection and also close and divide into compartments the intercellular spaces. The claudins as TJ proteins primarily function as a **"gate"** (closing intercellular spaces, directing paracellular selective diffusion) and **"fence"** (maintaining the differing composition of the proteins and lipids between the apical and basolateral membrane). Certain claudin molecules (CLDN-2, -4 and -16) also function as a **selective ion channel.** The TJ-forming molecules also take part in the multistep process of

signal transfer, react to the various signals arriving from the membrane and play role in the regulation of cell growth and differentiation as well as in tumorigenesis. The lack or decreased presence, the changes in expression of claudins are accompanied by decrease in cell adhesion and increase in tumorous infiltration, i.e. by metastasis formation. In several tumors – in which the changed TJ structure presumably plays role in invasion – certain claudins have been found to show increased expression. Claudins are organ- and tissue-specific molecules. In oral epithelial carcinomas for example, increased CLDN-1 expression was shown to have relationship with enhanced invasion and aggressive histological appearance. In oesophageal epithelial tumors, higher expression of CLDN-1 was found as compared with non-tumorous epithelium. In comparison to the normal epithelium, the expression of CLDN-1 (and CLDN-7) was found to increase in cervical dysplastic alterations (CIN) and to decrease in invasive carcinomas, compared with dysplastic alterations. CLDN-2 protein showed increased expression in gastrointestinal epithelial cells and tumors deriving from them. Endothelial cells and tumors originating from these showed CLDN-5 positivity. The enhanced CLDN-4 expression in bile duct tumors can differentiate these tumors from hepatocellular carcinomas. Increase CLDN-4 expression was proved in pancreatic adenocarcinomas. Increased expression of CLDN-1 was observable in colorectal carcinomas for example, further, the nuclear localization of this protein was detected in the metastases of these tumors. Primary colorectal carcinoma cells also revealed increased expression of CLDN-1. In the lungs, high expression of CLDN-3 refers to small cell tumors, providing differentiation from atypical carcinoids, adenocarcinomas and epithelial carcinomas. In certain tumors the degree of expression refers to the prognosis. For example, CLDN-1, -3, -4 and -5 expressions are lower in diffuse type stomach tumors of bad prognosis, as compared with intestinal tumors of better prognosis. In invasive, metastatic breast tumors CLDN-1 and -7 proteins show decreased expression. CLDN-10 is a good marker of recurring hepatocellular carcinoma. No literary data can be found in respect to claudin expression in human hepatoblastomas.

Certain claudins promote the entrance of pathogens into cells, whereas others function as receptors of the pathogens. CLDN-1 (CLDN-6, CLDN-9 and occludin) molecules are regarded as having important role in the entry of the hepatitis C virus into the cell. This raises the significance of the mentioned claudin molecules in antiviral therapy. CLDN-3 and -4 are the receptors of Clostridium perfringens enterotoxin (CPE), which causes the lysis of protein-expressing cells. According to a few publications, CPE causes the lysis of tumor cells expressing the mentioned claudins (pancreatic adenocarcinoma, prostate carcinoma) – providing possibility for the introduction of targeted therapy.

Dlk-1 is a cell surface transmembrane protein, with an extracellular domain containing six EGF-like elements, a transmembrane domain and a short intracellular tail. The protein – which is member of the epidermal growth factor-like (EGF-like) so-called homeotic protein family – is coded by the DLK-1 gene. The amino acid sequence and cistein rich regions of the dlk-1 protein are very similar in their individual EGF-like repeatings to the **delta protein** (thus the naming), which is the Notch receptor ligand occurring in Drosophila. The function of the dlk-1 molecule is unknown, according to certain publications it is the negative regulator of the Notch1 signal transfer process. Dlk-1 is expressed in numerous embryonal tissues (liver, tongue, vertebrae, skeletal muscle, cartilage and pancreas), in the mature organism it is present in the glomerulosa of the cortical zone, somatotrophic cells of the hypophysis, monoaminergic neurons of the central nervous system, Leydig- and hilus cells of the testis and ovarium, the β cells of the endocrine pancreas. The dlk-1 protein is strongly expressed in the **hepatoblasts** of human and mouse embryonal livers, but is not demonstrable in the biliary epithelium and hemopoetic cell compartment. The expression of dlk-1 protein has been described in a number of human tumors: neurofibroma, myelodysplastic syndrome, glioblastoma multiforme, carcinoma of the adrenal cortex, phaeochromocytoma and adenoma of the adrenal cortex. Tight relationship between increased dlk-1 protein expression and fibrogenesis has been described in biliary atresia, according to which dlk-1 plays role in the transformation of liver stellate cells into myofibroblasts.

2. THE AIM OF THE STUDY

Recent data have shown that the prognosis of hepatoblastoma, as in the case of other childhood malignant tumors (Wilms tumor, neuroblastoma), is basically related to histological differentiation. Regarding the various histological types and/or subtypes of hepatoblastomas, studies on markers revealing the exact origin of the tumor are the subject of intensive research. The expression of certain embryonal markers in hepatoblasts is supportive of the embryonic and/or fetal progenitor cell origin of the tumor. Studies on the expression profile of claudin molecules have shown promising results in case of several organs and tumors, promoting knowledge on the exact function of the molecules as well as helping the differentiation of tumors and tumor types (subtypes) of differring prognosis.

Based on the above, our aim was to study in the various epithelial components of human hepatoblastomas the presence of some types of **claudin** molecules as well as the **dlk-1** protein, which occurs physiologically in hepatoblasts. Our purpose was to find answers to the following questions:

1. **Are the studied claudin proteins expressed in human hepatoblastomas?** We wished to study the expression of CLDN-1, -2, -3, -4 and -7 proteins in human hepatoblastomas using immunohistochemical methods.

2. **Do the studied claudin proteins show differences in their expression in the various epithelial components (embryonal and fetal) of the tumor?** Our aim was to compare the expression patterns of CLDNs-1, -2, -3, -4 and -7 observed in the fetal and embryonal epithelial components, based on the results of immunohistochemical reactions and RT-PCR analyses.

3. **Could relationship be found between the histological differentiation, proliferation markers (PCNA, Ki-67) and studied claudin expressions?** We wished to study and draw comparisons between the expression of certain claudins and proliferation markers (Ki-67, PCNA) in the two epithelial components (fetal and embryonal) of different proliferative capacity of hepatoblastomas by means of immunohistochemical techniques.

4. **Can the damage to the Wnt/β-catenin signal transfer be detected in the studied hepatoblastomas?** Our aim was to study the expression of β-catenin in hepatoblastomas at protein level by means of immunohistochemistry furthermore, we wished to compare the β-catenin expression patterns observed in the embryonal and fetal components of the tumor. We also intended to perform the β-catenin gene mutation analysis of the nuclear-positive cases which indicated enhanced Wnt/β-catenin signal transfer in the two components, with the aim to verify the possible mutation(s) in the gene and to identify the nature of the mutation(s).

5. **Is the dlk-1 protein expressed in human hepatoblastoma, human hepatocellular carcinoma and other differential diagnostic tumors (ganglio/neuroblastoma, Wilms tumor, infantile hemangioendothelioma, medulloblastoma, rhabdoid tumor, AFP positiv germ cell tumors, liver MPNST?** We wished to study and compare the dlk-1 protein expression patterns of human hepatoblastomas, human hepatocellular carcinomas and the above listed tumors by immunohistochemistry and we intended to analyse their role in differential diagnostics.

3. MATERIAL AND METHODS

3.1. Samples

Retrospective studies were performed on selected formalin fixed, paraffin embedded tissues obtained from the archives of the 2nd Department of Pathology and the 1st Institute of Pathology and Experimental Cancer Research of the Semmelweis University, Budapest, with the permission of the Regional Ethical Committee of the Semmelweis University of Budapest (#172/2003).

We examined 31 human hepatoblastoma cases, 24 human hepatocellular carcinoma cases and 30 other differential diagnostic tumor cases.

14 hepatoblastoma cases were studied by immunohistochemical methods with the claudins. The mean age of hepatoblastoma patients was 3, (8 males and 6 females). The majority of the cases were epithelial type of hepatoblastoma (9) from which 2 were purely fetal, 7 were fetal/embryonal subtype and 5 mixed type of hepatoblastoma cases were also studied. Other types like macrotrabecular or small cell undifferentiated hepatoblastoma were not included. In 5 cases (fetal/embryonal) the epithelial components were macrodissected for detection of mRNA expression with Real Time RT-PCR. In 4 cases (fetal/embryonal) mutation analysis was used. In all the 14 hepatoblastoma cases AFP, HSA, CK-7, Ki-67, PCNA and β-catenin antibodies were applied.

We studied the expression of dlk-1 protein in 31 hepatoblastoma cases (17 males, 14 females; mean age 3.16 years; histology types: 21 epithelial from which 4 were fetal, 27 fetal/embryonal and 10 mixed). The presence of dlk-1 was examined with immunohistochemical methods also in 24 hepatocellular carcinomas (20 males, 4 females, mean age: 61.3 years, 13 Grade II, 11 Grade III) and in the other differential diagnostic tumor cases [AFP positive germ cell tumors (2 yolk sac, 2 teratocarcinomas, 1 seminoma, 1 immature teratoma; 6 males, mean age: 22.8 years), 5 Wilms tumors (4 males, 1 female, mean age: 5.7 years), 9 ganglioneuroblastomas (3 males, 6 females, mean age: 3.2 years), 5 medulloblastomas (3 males, 2 females, mean age: 7.6 years), 1 rhabdoid tumor (1 year old male), 2 infantile hemangioendotheliomas (2 males, mean age: 2 months), 1 mesenchymal hamartoma (6 months old female) and 1 MPNST of the liver (23 year old female)]. Detection of AFP and CK-19 immunohistochemical reactions were used also in these cases.

3.2. Methods

3.2.1. Histology and histochemical reactions

Formalin fixed and paraffin embedded tissues from tumors were stained with hematoxylin and eosin, picrosirius, Perjodid Acid Schiff (PAS), diastase-resistant PAS and Pearls' Prussian Blue.

3.2.2. Immunohistochemistry

3.2.2.1. Immunohistochemical reactions with CLDN-1, -2, -3, -4 and -7 antibodies

Paraffin embedded, 3-4 μm thick sections were used for immunohistochemistry. After endogen peroxidase blocking and antigen retrieval the following primary antibodies were used: CLDNs-1 (CAT.: 187362), -3 (CAT.: 341700) and -7 (CAT.: 349100) were polyclonals, CLDN-2 (CAT.: 187363) and -4 (CAT.: 187341) antibodies were monoclonals (Zymed, San Francisco, CA, USA).

The biotinylated secondary antibody and avidin-streptavidin-enzyme conjugate were used according to the protocol of automated Ventana system. Reagents and secondary antibodies were produces of Ventana (Tuscon, AZ, USA). Diaminobenzidine (DAB: Ventana, Tuscon, AZ, USA) was used for visualisation, and counterstained with hematoxylin.

For negative controls, the appropriate antibody was omitted and either the antibody diluent alone or isotype matched IgG serum was used. Positive controls recommended by the manufacturer (Zymed Inc, San Francisco, CA, USA) were used to confirm correct immunohistochemical staining for CLDNs, that is normal skin epithelium for CLDN-1, normal colon mucosa for CLDNs-2, -3, -4 and normal ductal cells in breast for CLDN-7.

Reactions were scored positive where linear membrane staining was seen, except for CLDN-2, where cytoplasmic granular reaction was detected as well.

3.2.2.2. ***Immunohistochemical reactions with d***lk, AFP, β-catenin, HSA, CK-7, CK-19, Ki-67 and PCNA antibodies

Dlk-1 (goat polyclonal, R&D Systems - AF1144, Minneapolis, MN, USA)**, AFP** (rabbit polyclonal, DAKOCytomation – A0008, Glostrup, Denmark**), CK-19** (mouse

monoclonal, Biogenex – MU246-UC, San Remon, CA, USA) **and β-catenin** (mouse monoclonal, BD Transduction - 610154, San Diego, CA, USA) antibodies were applied manually. **HSA** (mouse monoclonal, Novocastra - 760-4350, New Castle, UK), **CK-7** (mouse monoclonal, DAKOCytomation - M7018, Glostrup, Denmark), **Ki-67** (mouse monoclonal, DAKOCytomation - M7240, Glostrup, Denmark) and **PCNA** (rabbit polyclonal, DAKOCytomation - M0879, Glostrup, Denmark) antibody reactions were carried out with automated Ventana system (Ventana Medical Systems Inc, Tucson, AZ, USA). Ventana iVIEW kit DAB detection Kit was used for visualisation.

In case of **AFP, CK-19** and **dlk-1** manual immunohistochemical reactions Vectastain ABC Elite Kit (Vector Laboratories, Burlingname, CA, USA) and for **β-catenin** Dako Cytomation EnVision+ System Labelled Polymer-HRP Anti-Mouse (Dako North America, Inc. USA) detection system were applied. 3,3'-diaminobenzidin (DAB) (CELL MARQUE, USA) was used as chromogen. For negative controls, the appropriate antibody was omitted and either the antibody diluent alone or isotype matched IgG serum was used. Human liver and biliary cells for **HSA** and **CK-7,** transitional cells for **CK-19,** lymph node for **β-catenin, Ki-67** and **PCNA** were used as positive controls to confirm correct immunohistochemical staining. Rat liver tissue with oval cells (2-2-acetylaminofluorene/partial hepatectomy protocol) for **AFP** and **dlk-1** was used as positive control (Am J Pathol 164, 1347-1359, 2004).

3.2.3. Evaluation of immunohistochemical reactions

For semiquantitative analysis with **CLDN**, **HSA**, and **CK-7** ten randomly selected areas of each HB were analyzed using high-power field objective (X400) with 100 cells counted in each field. Slides with divergent scores were discussed and reevaluated. For these slides, the percentage of positive tumor cells was scored as follows: 0 (0-5% positivity), 1 (6%-20% positivity), 2 (21%-40% positivity), 3 (41%-60% positivity), 4 (61%-80% positivity), 5 (81%-100% positivity). The score zero was evaluated as negative immunohistochemical reaction. For PCNA, Ki-67 and β-catenin slides the positive nuclei (labeling indices) were counted and expressed in percentage.

The intensity (-/+++), percentage and pattern (cytoplasmatic/membranous/canalicular) of the **dlk-1** staining were evaluated by semiquantitative statistical analysis.

3.3. Molecular biological methods

3.3.1. Real-time (RT-) PCR

Total RNA from formalin fixed paraffin embedded tumor tissues was isolated with High Pure RNA Paraffin Kit (Roche, Indianapolis, Indiana, USA). Samples were microdissected to select the areas containing purely exocrine or endocrine pancreatic tumors. Proteinase K digestion time was 16 hours for each sample. All purifications were performed in accordance with the manufacturer's protocol. Total RNA (500 ng) was reverse transcribed for 50 minutes at 42°C in 30 µl with MulV reverse transcriptase (Applied Biosystems, Foster City, CA, USA) in the presence of RNAse inhibitor (Applied Biosystems) using Random Hexamers (Applied Biosystems).

Real-time PCR was performed with 2 µl cDNA template in a total volume of 25 µl, using the ABI Prism 7000 sequence detection system (Applied Biosystems). Each PCR was conducted in 25 µl volume of SYBR Green Supermix (Bio-Rad, Hercules, CA, USA, 1708851). Real-time PCR was performed in duplicates in 96-well plates, for 2 minutes at 95°C for initial denaturing, then 40 cycles at 95°C for 20 seconds, at 63°C for 30 seconds and at 72°C for 1 minute.

3.3.2. β-catenin mutational analysis

Four cases of those selected for real-time RT-PCR quantification were chosen for b-catenin mutational analysis. Two to eight 5-lm-thick sections of fetal and embryonal components were dissected from each tissue block. DNA was extracted using PCR Template Kit (Roche). Primers—forward primer 5'-AGCTGATTTGATGGAGTTG-3' (815-833), reverse primer 5'-ACCAGCTACTTGTTCTTGAG-3' (1027-1046), GI: 6682315) were used to amplify a 232-base pair (bp) DNA fragment of exon 3 of the b-catenin gene for point mutation analysis in the region described to contain activating mutations. DNA sequencing was performed using an ABI 373 Automated Sequencer (Applied Biosystems), with the ABI Prism Dye Terminator Kit (Applied Biosystems). Each mutation was verified in both sense and antisense direction.

3.4. Statistical analysis

For the statistical analysis of immunohistochemical scores with **claudin, β-catenin**, proliferation markers (**Ki-67 és PCNA**), **CK-7** and **HSA** antibodies, the Mann-Whitney U test was used to compare the expression of proteins in the different epithelial cell types of HBs. Statistical analysis with **dlk-1** antibody was performed by Fisher's exact test using contingency tables.

In the Real Time-RT-PCR metodology data analysis and statistical evaluation were performed by REST (Relative Expression Software Tool, www.wzw.tum.de/gene-quantification) and Pairwise Fixed Reallocation Randomization Test. For relative quantification the average of β-actin as reference gene was used. Probability values of less than 0.05 ($P<0.05$) were accepted as being significant.

4. RESULTS

4.1. Study of claudin expression in the tumor-free, surrounding liver using immunohistochemical techniques. The hepatocytes gave mild linear membranous reaction mostly only on the luminar poles in case of CLDN-1, whereas weak granular positivity could be detected along the luminal poles in the cytoplasm in case of CLDN-2. **CLDN-3, CLDN-4 and CLDN-7** proteins were not expressed in the hepatocytes, whereas expression of the studied claudins was observable in the bile ducts.

4.2. Study of claudin expression in hepatoblastoma using immunohistochemical technique. From the studied claudins, only CLDN-1 and -2 proteins showed strong expression in the epithelial (fetal, embryonal) components of the tumor. In case of CLDN-1 and CLDN-2 reactions, clear differences in expression were observable between the fetal and embryonal components of hepatoblastoma: CLDN-1 and CLDN-2 showed significantly higher expression in the fetal as compared with the embryonal component of hepatoblastoma. Except for sporadical, weak positivity, the embryonal component was found to be negative.

4.3. Study of proliferation markers (Ki-67, PCNA) and other markers (HSA, CK-7) in human hepatoblastoma by immunohistochemistry. The Ki-67 and PCNA proteins showed significantly higher nuclear expression in the embryonal component as compared with the fetal component. HSA showed strong expression in the fetal component, but was found negative in the embryonal component. CK-7 reaction was negative.

4.4. Study of CLDN-1, -2, -3, -4 and -7 mRNA expressions in the fetal and embryonal components of hepatoblastoma by Real Time RT-PCR. Study of the mRNA expression in the epithelial, i.e. fetal and embryonal components of hepatoblastoma showed results that correlated with the results of immunohistochemical reaction analyses. mRNA expressions of CLDN-1 (23-fold, $P = 0.001$) and CLDN-2 (8.5-fold, $P = 0.001$) were significantly higher in the fetal than in the embryonal component. CLDN-3, -4 and -7 mRNA expressions (3.5-, 2- and 2.2 folds, respectively) were higher in the fetal component, the differences, however, were not significant.

4.5. Study of β-catenin expression in human hepatoblastoma by immunohistochemistry. From the 14 hepatoblastoma cases studied, 13 cases showed β-catenin nuclear staining. The β-catenin expression in the fetal and embryonal components did not show significant differences.

4.6. Study of β-catenin gene mutation using molecular biological technique (mutation analysis). The nucleotide sequence of PCR amplicons in the β-catenin gene originating from the macrodissected samples (4 fetal/embryonal) showed missense mutation in one case each of the fetal and embryonal component samples. Both mutations were found in the 37. codon of the 3. exon, where the **TCT – TGT** switch resulted a **serin – cistein** exchange.

4.7. Study of dlk-1 protein expression in human hepatoblastoma by means of immunohistochemistry. Every dlk-1 immunohistochemical reaction carried out in the studied hepatoblastoma cases gave positive result. In the majority of the tumorous cases the staining was found to be focal, whereas every surrounding tumor-free livers tissue specimen gave negative result. Positive reaction was detected only in the epithelial (fetal, embryonal) components of the tumor, the mesenchymal elements (mixed hepatoblastoma) were negative. Dlk-1 protein expression did not show significant differences between the two epithelial components.

4.8. Study of dlk-1 protein expression in hepatocellular carcinoma and other tumors by immunohistochemistry. Almost all hepatocellular carcinomas (except one atypical case) and other differential diagnostics-related tumors (with the exception of mature ganglion cells in ganglioneuroblastoma) showed dlk-1 negativity.

5. CONCLUSIONS/NEW FINDINGS

5.1. We were the first to describe the expression of claudins in human hepatoblastomas. In the epithelial components of hepatoblastoma CLDN-1 and CLDN-2 proteins showed strong expression, CLDNs-3, -4 and -7 did not show appreciable positivity.

5.2. Among epithelial components of hepatoblastoma CLDN-1 and CLDN-2 proteins showed significantly higher expression in the fetal component as compared with the embryonal component. The embryonal component was negative, showing only sporadical positivity. The results of Real-Time RT-PCR analyses correlated with the immunohistochemical results. These findings refer to the fact that CLDN-1 and CLDN-2 proteins can be regarded as differential markers in hepatoblastomas.

5.3. In hepatoblastomas higher tissue differentiation is accompanied by strong CLDN-1 and CLDN-2 expression and low proliferative ability. Our studies proved an inverse correlation between the expression of CLDNs-1 and -2 signalling higher tissue differentation and cell proliferation.

5.4. Almost all hepatoblastoma cases (13 out of 14) demonstrated β-catenin nuclear accumulation, the distribution of which did not correlate with histological subtype. From the selected cases, one case each of the fetal and embryonal components proved the occurrence of point mutation in the β-catenin gene (3. exon). These findings refer to the fact that damage to the Wnt/β-catenin signal transfer plays a role in the pathogenesis of hepatoblastomas independently of histological subtype.

5.5. All our hepatoblastoma cases expressed dlk-1 protein. The distribution of the expression did not correlate with histological subtype. All but one (23 of 24) hepatocellular carcinomas and every other tumors studied by us relevant differential diagnostically proved negative for dlk-1. Based on the above, we have proven that the dlk-1 protein is suitable for distinguishing hepatoblastoma primarily from hepatocellular carcinoma, the expression of this protein is therefore utilizable in the differential diagnostics of childhood malignant hepatocellular neoplasms.

6. LIST OF PUBLICATIONS

Total Impact Factor (IF): 18,378

Publications related to the Dissertation (IF: 4,98):

1. **Halász J**, Holczbauer A, Páska C, Kovács M, Benyó G, Verebély T, Schaff Z, Kiss A. (2006) Claudin-1 and claudin-2 differentiate fetal and embryonal components in human hepatoblastoma. Hum Pathol, 37: 555-61. **IF: 2,810**

2. Dezso K, **Halász J***, Bisgaard HC, Paku S, Turányi E, Schaff Z, Nagy P. (2008) Delta-like protein (DLK) is a novel immunohistochemical marker for human hepatoblastomas. Virchows Arch, 452: 443-8. **IF: 2,082**

3. Jakab Cs, **Halász J**, Kiss A, Szász A. M, Schaff Zs, Rusvai M, Kulka J. (2008) Külső pozitív kontrollok alkalmazása claudin-expressziós immunhisztokémiai vizsgálatokban. Magyar Állatorvosok Lapja, 130: 433-438. **IF: 0,088**

*****equal contributor and first author

Publications not related to the Dissertation:

1. Jakab C, **Halász J**, Kiss A, Schaff Z, Rusvai M, Gálfi P, Abonyi TZ, Kulka J. (2009) Claudin-5 protein is a new differential marker for histopathological differential diagnosis of canine hemangiosarcoma. Histol Histopathol, 24: 801-13. **IF: 2,194**

2. Aigelsreiter A, Janig E, Sostaric J, Pichler M, Unterthor D, **Halasz J**, Lackner C, Zatloukal K, Denk H. (2009) Clusterin expression in cholestasis, hepatocellular carcinoma and liver fibrosis. Histopathology, 54: 561-70. **IF: 4,131 (2008)**

3. Jakab Csaba, **Halász Judit**, Kiss András, Schaff Zsuzsa, Pekár Magdolna, Keszthelyi Rita, Meczker Ágnes, Kulka Janina. (2007) Szöveti multiblokk (tissue micro-array-TMA) technika az állatorvosi onkopatológiai vizsgálatokban. Magyar Állatorvosok Lapja, 129: 310-315.
IF: 0,104

4. Jakab Cs, Dudás Gy Z, Horváth É, Tóth A, **Halász J**. (2008) A májbiopsziás vizsgálatok jelentősége a kisállatpraxisban. Magyar Állatorvosok Lapja, 130: 39-47. **IF: 0,088**

5. Jakab Cs, **Halász J**, Kiss A, Schaff Zs, Pekár M, Szabára Á, Kulka J. (2008) Claudin-5 fehérje expressziójának vizsgálata kutyák emlőmirigyeinek és emlőcarcinomáinak nyirokér endothelsejtjein immunhisztokémiai módszerrel. Magyar Állatorvosok Lapja, 130: 296-303. **IF: 0,088**

6. Jakab C, **Halász J**, Kiss A, Schaff Z, Szász AM, Rusvai M, Tóth ZA, Kulka J. (2008) Evaluation of microvessel density (MVD) in canine mammary tumours by quantitative immunohistochemistry of the claudin-5 molecule. Acta Vet Hung, 56(4): 495-510.
IF: 0,624

7. Jakab C, **Halász J**, Szász AM, Kiss A, Schaff Z, Rusvai M, Gálfi P, Kulka J. (2008) Expression of claudin-1, -2, -3, -4, -5 and -7 proteins in benign and malignant canine mammary gland epithelial tumours. J Comp Pathol, 139: 238-45. **IF: 1,398**

8. Jakab C, **Halász J**, Szász AM, Batmunkh E, Kiss A, Schaff Z, Rusvai M, Gálfi P, Kulka J. (2008) Expression and localisation of claudin-1, -2, -3, -4, -5, -7 and -10 proteins in the normal canine mammary gland. Acta Vet Hung, 56(3): 341-52.
IF: 0,624

9. Fuszek P, Horvath HC, Speer G, Papp J, Haller P, Fischer S, **Halasz J**, Jaray B, Szekely E, Schaff Z, Papp A, Bursics A, Harsanyi L, Lukovich P, Kupcsulik P, Hitre E, Lakatos PL. (2006) Location and age at onset of colorectal cancer in Hungarian patients between 1993 and 2004. The high number of advanced cases supports the need for a colorectal cancer screening program in Hungary. Anticancer Res, 26: 527-31. **IF: 1,479**

10. Fuszek P, Horváth HC, Speer G, Papp J, Haller P, **Halász J**, Járay B, Székely E, Schaff Z, Papp A, Bursics A, Harsányi L, Lukovich P, Kupcsulik P, Hitre E, Lakatos PL. (2006) A colorectalis rákok lokalizációjának változása Magyarországon 1993 és 2004 között. Orv Hetil, 147(16): 741-6.

11. Sobel G, **Halász J**, Bogdányi K, Szabó I, Borka K, Molnár P, Schaff Z, Paulin F, Bánhidy F. (2006) Prenatal diagnosis of a giant congenital primary cerebral hemangiopericytoma. Pathol Oncol Res, 12(1): 46-9. **IF: 1,241**

12. Csak T, Folhoffer A, Horvath A, **Halász J**, Diczházi C, Schaff Z, Szalay F. (2006) Holmes-Adie syndrome, autoimmune hepatitis and celiac disease: a case report. World J Gastroenterol, 12(9): 1485-7.

13. Lakatos PL, Gyori G, **Halasz J**, Fuszek P, Papp J, Jaray B, Lukovich P, Lakatos L. (2005) Mucocele of the appendix: an unusual cause of lower abdominal pain in a patient with ulcerative colitis. A case report and review of literature. World J Gastroenterol, 11(3): 457-9.

14. Vereckei A, Vándor L, **Halász J**, Karádi I, Lengyel M. (2004) Infective endocarditis resulting in rupture of sinus of Valsalva with a rupture site communicating with both the right atrium and right ventricle. J Am Soc Echocardiogr, 17: 995-7. **IF: 1,427**

15. **Halász J**, Kovács M, Illyés G, Máttyus I, Szolnoki J, Szabó A, Verebély T, Schaff Z. (2004) Malignant rhabdoid tumour in the liver. Orv Hetil, 145(27): 1439-43.

16. Lakatos PL, **Halász J**, Keresztes K, Fuszek P, Jäckel M, Poros A, Morvay K, Lakatos L. (2004) A familiaris adenomatosus polyposisról egy eset kapcsán. Orv Hetil, 145(15): 813-7.

17. Horvath A, Folhoffer A, Lakatos PL, **Halasz J**, Illyes G, Schaff Z, Hantos MB, Tekes K, Szalay F. (2004) Rising plasma nociceptin level during development of HCC: a case report. World J Gastroenterol, 10(1): 152-4.

18. Lakatos PL, Lakatos L, Fuszek P, Lukovich P, Kupcsulik P, **Halász J,** Schaff Zs, Papp J. A nyelőcső és a gastrooesophagealis junctio daganatainak gyakorisága és szövettani megoszlása 1993-2003 között. (2005) Orv Hetil, 146 (9): 411-6.

19. Csák T, Folhoffer A, Horváth A, Osztovits J, **Halász J,** Diczházi Cs, Schaff Zs, Szalay F. (2006) Autoimmun hepatitis, coeliakia és Holmes-Adie-szindróma együttes előfordulása. Magy Belorv Arch, 59: 55-58.

Quotable abstracts related to the Dissertation:

1. **Judit Halasz**, A. Kiss, A. Holczbauer, Cs. Paska, M. Kovács, G. Benyo, K. Galantai, T. Verebely and Zs. Schaff. Expression of claudins in human hepatoblastoma. Z Gastroenterol 2005; 43.

2. Dezső K, **Halász J**, Paku S, Bisgaard HC, Schaff Zs, Nagy P. DLK a potential new immunohistochemical marker for hepatoblastoma: 43rd Annual Meeting of the European Association for the Study of the Liver, Milan, Italy, April 23-27, 2008 Journal of Hepatology 48**:**(S2) *p. S153.* (2008)

3. **Halász J**, Holczbauer Á, Kiss A, Páska Cs, Kovács M, Benyó G, Galántai I, Schaff Zs. Expression of claudins in human hepatoblastoma. 20th European Congress of Pathology, Paris, France, September 3-8, 2005 Virchows Arch 447: 2005 (2005)

4. **Halász J**, Horváth A, Folhoffer A, Szalay F, Nagy P, Schaff Zs. Hepatocellular carcinoma in biliary cirrhosis with stem cell markers XXVI International Congress of the International Academy of Pathology, Montreal, Canada, 16-21 September, 2006 Modern Pathol 19 S3: 122-123 (2006)

Quotable abstract not related to the Dissertation:

1. Horváth A, Hantos M, Tekes K, **Halász J**, Illyés Gy , Schaff Zs, Folhoffer A, Lakatos PL, Szalay F. Hepatocellular carcinoma in a patient with primary biliary cirrhosis. Is nociceptin a marker for HCC? FALK Symposium, Freiburg, Germany, 2004. Z Gastroenterol 2003;41:439.

2. A Horvath, Folhoffer, T Csak, PL Lakatos, MB Hantos, K Tekes, A Szijarto, P Kupcsulik, A. Kiss, **J Halasz**, Z Schaff, F Szalay. High nociceptin content in human hepatocellular carcinoma tissue. DDW, New Orleans, USA, 2004. Gastroenterol 2004, 40 Suppl 1: A250.

3. Aigelsreiter, A; Janig, E, **Halasz, J**, Lackner, C, Sostaric, J, Pichler, M, Stumptner, C, Unterthor, D, Kufferath, I, Zatloukal K, Denk, H. (2007): Clusterin expression in cholestasis and liver fibrosis Virchows Archive 2007; 451(2):132-132.-21th European Congress of Pathology; SEPT 8-13; Istanbul, TURKEY.

4. Aigelsreiter, A; **Halasz J**, Lackner C, Sostaric J, Reihs R, Stark K, Kufferath I, Pichler M, Stumptner C, Zatloukal K, Denk H (2007): Hepatocellular carcinomas with Mallory bodies and/or Intracellular hyaline bodies: Correlation with steatohepatitic features Virchows Archive 2007; 451(2):370-370.-21th European Congress of Pathology; SEPT 8-13, 2007; Istanbul, TURKEY.

5. Győri G, Fuszek P, Járay B, **Halász J**, Schaff Zs, Lukovich P, Abonyi M, Lakatos PL. Mucocele of the appendix: An unusual cause of lower abdominal pain in a patient with ulcerative colitis. A case report. 46th Annual Meeting of the Hungarian Society of Gastroenterology, Balatonaliga, June 1-5, 2004 Z. Gastroenterol 42: 414, 2004 (2004).

Non-quotable abstracts not related to the Dissertation:

1. Horváth A, Hantos M, Tekes K, **Halász J**, Illyés Gy , Schaff Zs, Folhoffer A, Lakatos PL, Szalay F. Hepatocellular carcinoma in a patient with primary biliary cirrhosis. Is nociceptin a marker for HCC? 45th Annual Meeting of the Gastroenterology Society, Balatonaliga, Hungary, 2003.

2. **Halász J**, Horváth A, Folhoffer A, Lakatos PL, Illyés Gy, Hantos BM, Tekes K, Nagy P, Szalay F, Schaff Zs. Primary biliaris cirrhosis in hepatocellular carcinoma. 63th Congress of Pathology, Balatonszéplak, Hungary, 2004.

3. **Judit Halász**, Margit Kovács, György Illyés, István Máttyus, Judit Szolnoki, Antal Szabó, Tibor Verebély, Zsuzsa Schaff. Malignant rhabdoid tumor in the liver. FALK Symposium, Freiburg, Germany, 2004.

4. **Judit Halasz**, A. Kiss, A. Holczbauer, Cs. Paska, M. Kovács, G. Benyo, K. Galantai, T. Verebely and Zs. Schaff. Expression of claudins in human hepatoblastoma. FALK Symposium, Berlin, Germany, 2005.

5. P. Valyi, K. Danyi, **J. Halasz**, Z. Schaff, A. Fazekas, K. Bogi. Expression of the tight junction associated claudins in normal human gingiva. Joint meeting of the Continental European Division and Scandinavian Divison of the IADR, Amsterdam, Netherland, 2005.

6. P. Valyi, K. Danyi, **J. Halasz**, Z. Schaff, A. Fazekas, K. Bogi. Expression of occludin and ZO-1 in healthy stratified gingival epithelium. Joint meeting of the Continental European Division and Scandinavian Divison of the IADR, Amsterdam, Netherland, 2005.

7. **Halasz J**., Kiss A., Holczbauer A., Paska Cs., Kovacs M., Benyo G., Verebely T., Schaff Zs. Claudin expression in human hepatoblastoma. 64th Congress of Pathology, Pécs, Hungary, 2005.

8. **Halasz J**., Verebely T., Kovacs M. VIP secreted ganglioneuroblastoma which caused WDHA-syndrome. 64th Congress of Pathology, Pécs, Hungary, 2005.