FINAL REPORT ON OTKA RESEARCH PROJECT # 1 0 1 4 3 5

"Novel prognostic and diagnostic markers in primary and metastatic liver tumors"

As the first line, fine needle aspiration biopsy, surgical biopsy surgical resection specimen samples of the study were selected and evaluated based on the pathology inclusion criteria of the research focus. First, surgical resection specimens of 50 hepatocellular carcinomas (HCC) and 120 colorectal carcinomas (CRC), the surrounding nontumorous colon mucosa (CRCS), and the liver metastases of the identical patients (CRCM), then nontumorous surrounding liver tissue samples (SL) were selected. A special group of HCCs was also identified where diagnoses were gained by fine needle aspiration cyology (FNAB), (40 cases). This group of HCC patients represent two groups of patients: those who received Sorafenib treatment and those who did not. Sorafenib treated patients were further categorized in well reacting (treated for more than 9 months) and poorly reacting patient groups (treated for less than 9 months). The clinicopathological parameters (laboratory parameters, grading & staging, recurrence free survival, progression free survival and overall survival) were followed throughout the investigations and completed by the time of the publications. Studying the groups of CRC, CRCS, CRCM and SL samples of the identical patients provides a uniques opportunity to study tumor progression. It took the first year to complete the tissue microarray (TMA) and identify the exact tumor/nontumorous tissue ratio in the almost 1 000 tissue cylinders taken for the TMAs. In order to obtain isolated site specific RNA for mRNA and microRNA expression analysis macrodissection of the tissue blocks of the TMA cylinder specific area was carried out. Hepatoblastoma samples were similarly collected and categorized. Following RNA and DNA isolation and completion of the TMAs the specific mRNA, microRNA and TMA based immunohistochemical investigations were performed. RNA isolation was completed with RNeasy FFPE kit (Quiagen) and microRNA (miR) specific transcription was carried out: miR-17-5p, miR-18a, miR-21, miR-34a, miR-122, miR-195, miR-210, miR-214, miR-221, miR-222, miR-223 and miR-224 expressions were analized (ABI).

The changed expression of claudin family members - which transmebrane proteins are the major components of the tight junction – might characterize carcinogenesis and tumor progression. Tumors reveal tumor specific changes of the claudin expression pattern as previously shown by our group. This changed claudin expression might allow identification of the tumor and might also provide prognostic and predictive information on the specific tumor

type. Altogether, the specific miR and tight junction component (claudin) expression profile carries important information regarding primary and metastatic liver lesions and might help to distinguish primary and metastatic liver lesions and provide therapeutic targets for persionalized treatment. Primary and metastatic tumors of the liver take significant share in cancer morbidity and mortality worldwide. The incidence of hepatocellular carcinoma has been constantly rising in the past decades. One of our focuses was the liver metastasis of colorectal tumors and the role of the altered expression of the tight junction components and their possible regulation by microRNA expression. The other major line of research was to discover the role of altered microRNS expression in cirrhosis and to compare this with the morphologically similar focal nodular hyperplasia (FNH). However, FNH does not lead to hepatocellular carcinoma, while cirrhosis is a known risk factor for hepatocarcinogenesis.

Hepatocellular carcinoma (HCC) is one of the leading cancers in the world, ranking fifth in malignant morbidity and representing the third most frequent cause of cancer-related death [1]. The incidence of HCC is rising, especially in the Western population [2], which is explained by the increasing prevalence of hepatitis C virus (HCV) infection. Claudins have been reported to be differentially regulated in malignancies and implicated in the process of carcinogenesis and tumor progression. The question arises whether and how claudin expressions are altered in HCV-related and -unrelated cirrhosis and HCC, and whether these changes are attributable to the development of cirrhosis or rather to viral infection. Claudin-1 has been described as key factor in the entry of hepatitis C virus (HCV) into hepatocytes and as promoter of epithelial mesenchymal transition in liver cells. The aim of our research was to characterize claudin expression (claudins-1, -2, -3, -4 and -7) in hepatocellular carcinoma (HCC) as well as HCC-surrounding and normal liver samples with respect to cirrhosis and HCV infection. Expression of claudins-1, -2, -3, -4, and −7 was measured by morphometric analysis of immunohistochemistry, and Western blotting in 30 HCCs with 30 corresponding nontumorous tissues and 6 normal livers. Claudin-1 and −7 protein expressions were found to be significantly elevated in cirrhosis when compared with non-cirrhotic liver. HCCs developed in cirrhotic livers showed even higher expression of claudin-1 contrary to decreased claudin-7 expression when compared with cirrhosis. With reference to HCV status, HCCs or surrounding livers of HCV-infected samples did not show significant alterations in claudin expression when compared with HCV-negative specimens. Cirrhotic transformation associates with elevated claudin-1 and -7 expressions in both nontumorous liver and HCC. The fact that no significant differences in claudin expression were found regarding HCVpositivity in our sample set

suggests that HCV infection alone does not induce a major increase in the total amount of its entry co-factor claudin-1. It was concluded that increased expression of claudin-1 seems to be a consequence of cirrhotic transformation and might contribute to a more effective HCV entry and malignant transformation (***Holczbauer Á, Gyöngyösi B, Lotz G, Törzsök P, Kaposi-Novák P, Szijártó A, Tátrai P, Kupcsulik P, Schaff Zs, Kiss A: Increased expression of claudin-1 and claudin-7 in liver cirrhosis and hepatocellular carcinoma. Pathol Oncol Res 20: 493-502, 2014, IF: 1,855).***

The **liver** is one of the most common sites of **metastasis** and also gives rise to highly malignant primary cancers. Colorectal and pancreatic carcinomas are common sources of hepatic metastases, which are approximately 20 times more frequently found than the most common primary liver cancer, hepatocellular carcinoma (HCC) (Kadry et al. 2001). Given the high prevalence and mortality rates of these focal lesions, a better molecular characterization is of great importance, especially because these lesions might also represent differential diagnostic problems. Tight junction proteins, including claudins, are often dysregulated during carcinogenesis and tumor progression. Moreover, the claudin expression pattern usually varies between different tumor entities. Our goal was to investigate claudin expression profiles of primary and metastatic liver malignancies. Claudin-1, -2, -3, -4, and -7 expression pattern was analyzed by quantitative immunohistochemistry and real-time RT-PCR, respectively. Twenty hepatocellular carcinomas (HCCs) and liver metastases of 20 colorectal adenocarcinomas (CRLMs) and 15 pancreatic adenocarcinomas (PLMs) were studied together with paired surrounding nontumorous liver samples and 5 normal liver samples. Strong claudin-3 and -7 immunohistochemical positivities were detected in CRLM samples, each with significantly stronger staining when compared with HCC and PLM groups. Claudin-1 protein was found highly expressed in CRLM, in contrast to lower expression in PLM and HCC. CRLMs and PLMs also were strongly positive for claudin-4, while being virtually undetectable in HCC. Claudin-2 showed strong positivity in non-tumorous liver tissue, whereas significantly weaker positivity was observed in all tumors. Differences in mRNA expression were mostly similar to those found by immunohistochemistry. In conclusion, HCC and both CRLM and PLM display distinct claudin expression profiles, which might provide better understanding of the pathobiology of these lesions and might be used for differential diagnosis ***(Holczbauer Á*, Gyöngyösi B*, Lotz G, Szijártó A, Kupcsulik P, Schaff Z, Kiss A: Distinct claudin expression profiles of hepatocellular carcinoma and metastatic colorectal and pancreatic carcinomas. J Histochem Cytochem 61: 294-305, 2013, IF: 2,403).***

Hepatoblastoma (HB) is the most common primary liver malignancy in childhood in western countries, representing approximately 1–4% of paediatric solid tumors. In Europe HB shows increasing incidence, with 1.2–1.5 cases per million children. Several different histological types of HB are known, the most common being the epithelial type, which can be divided further into pure fetal, mixed embryonal/fetal, macrotrabecular and small cell undifferentiated subtypes. There are data to suggest that prognosis depends upon the proportion of different epithelial – fetal and embryonal – components, however, the molecular mechanisms or activating pathways leading to these heterogeneous phenotypes have not yet been defined clearly. The more differentiated fetal component of hepatoblastoma (HB) is characterized by increased expression of tight junction proteins claudin-1 and -2 when compared with the embryonal component. MicroRNA (miRNA) expression patterns, as well as the recently identified tight junction protein, tricellulin and epigenetic regulator enzyme EZH2 were investigated in epithelial subtypes of HB to relate them to survival. Twenty cases of epithelial HBs were subtyped as pure fetal components (n=12) and embryonal components (n=8), along with 15 nontumorous surrounding liver samples which were analysed by immunohistochemistry for tricellulin, β-catenin and EZH2 expression. Relative expressions of miR-17-5p, miR-18a, miR-21, miR-34a, miR-96, miR-122, miR-181a, miR-195, miR-210, miR-214, miR-221, miR-222, miR-223 and miR-224 were determined by TaqMan MicroRNA Assays applying miR-140 as reference.

No significant differences were revealed in overall survival between fetal and embryonal/fetal types of HBs. The fetal component, however, showed considerably increased tricellulin expression, whereas the embryonal component displayed significantly increased nuclear EZH2 positivity in comparison to other epithelial subtypes and nontumorous surrounding hepatocytes. Strong nuclear β-catenin staining was notably more frequent in the embryonal than the fetal type. High tricellulin expression was associated with significantly increased overall survival ($p=0.03$), while elevated EZH2 expression was linked to presence of distant metastases ($p=0.013$). Elevated level of miR-18a ($p<0.01$) was found in the embryonal component when compared with fetal component. Decreased miR-17-5p, miR-195, miR-210, miR-214 and increased miR-221 levels were detected in the fetal component ($p<0.02$) in comparison with SL samples, whereas decreased miR-122 level was observed in the embryonal component ($p<0.003$). High miR-21, low miR-222 and low miR-224 levels proved to be independent prognostic factors for HB and were associated with significantly increased overall survival

($p<0.03$). Our results led to the conclusion that treated HBs showing high expression of tricellulin reveal significantly better overall survival independent of histological subtype. Increased nuclear expression of EZH2 was associated with the presence of distant metastases. Furthermore, miR-21, miR-222 and miR-224 levels could serve as valuable tools to predict overall survival of HB patients regardless of epithelial subtype. Our research project resulted in two publications carried out on hepatoblastoma samples: ***Schlachter K, Gyugos M, Halász J, Lendvai G, Baghy K, Garami M, Gyöngyösi B, Schaff Z, Kiss A.: High tricellulin expression is associated with better survival in human hepatoblastoma. Histopathology 65: 631-641, 2014, IF: 3,453; 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 Arch 464: 419-427, 2014, IF: 2,651).***

The microRNA profile in liver biopsies taken from **hepatitis virus C infected chronic hepatitis patients with or without steatosis** was studied in the frame of the current project. Further, biopsies from steatotic patients without chronic hepatitis were investigated as well. Briefly: microRNA expression in chronic HCV liver diseases that can progress into hepatocellular carcinoma. In our study, differences were found in expression of selected microRNAs in biopsy samples of steatotic liver, CHC-infected, and steatotic CHC-infected liver as compared with control samples. Interestingly, the levels of miR-224, which are increased in hepatocellular carcinoma, were elevated in both types of steatotic liver when compared with normal or only CHC-infected liver tissues, and may be an indicator of a precancerous state. Our aim was to assess the expression of selected microRNAs (miRNA) in hepatitis C, steatotic hepatitis C, noninfected steatotic and normal liver tissues. The relative expression levels of miR-21,miR-33a, miR-96, miR-122, miR-125b, miR-221 and miR-224 were determined in 76 RNA samples isolated from 18 non-steatotic and 28 steatotic chronic hepatitis C (CHC and CHC-Steatosis, respectively) cases, 18 non-infected, steatotic liver biopsies of metabolic origin (Steatosis) and 12 normal formalin-fixed paraffin-embedded liver tissues using TaqMan MicroRNA Assays. All CHC biopsy samples were obtained prior to initiating therapy. Patients' serum biochemical values, which included glucose, triglyceride, cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl-transferase (GGT), alkaline phosphatase (AP), were obtained and correlated with relative miRNA expression. When compared with control non-infected liver samples, miR-122 and miR-221 levels were reduced in CHC-Steatosis ($P < 0.03$) and in CHC, CHC-Steatosis and

Steatosis ($P < 0.01$). Alternatively, the expressions of miR-33a and miR-224 were elevated in CHC-Steatosis and Steatosis in comparison to control tissue ($P < 0.01$). The levels of miR-33a and miR-224 in CHC-Steatosis ($P < 0.02$) and miR-224 in Steatosis ($P < 0.001$) were increased in comparison to CHC samples. By contrast, the expression of miR-21 did not differ statistically between diseased and normal liver samples. Levels of miR-33a correlated negatively with serum AST and AP levels in Steatosis as well as with necroinflammatory grade in CHC, whereas miR-21 correlated positively with AST in Steatosis and displayed negative correlation with triglyceride level in CHC-Steatosis. In contrast, miRNA levels were not correlated with ALT, GGT, cholesterol levels or fibrosis stage. In conclusion it could be stated that diferences in miRNA expression were observed between CHC and steatotic CHC, CHC and steatotic liver, but not between steatotic CHC and steatotic liver of metabolic origin ***(Lendvai G, Jármay K, Karácsony G, Halász T, Kovalszky I, Baghy K, Wittmann T, Schaff Z, Kiss A: Elevated miR-33a and miR-224 in steatotic chronic hepatitis C liver biopsies. World J Gastroenterol 20: 15343-15350, 2014, IF: 2,369).***

The **microRNA profile** was investigated in fine needle aspiration biopsies derived from **Sorafenib treated hepatocellular carcinoma** patients before initiation of therapy. Our goal was to get insight into the possible association between pretreatment microRNA profile and the outcome of the treatment. This possible predictive value was investigated. Briefly: Sorafenib represents the first effective targeted therapy for advanced stage hepatocellular carcinoma (HCC); however, adequate patient stratification regarding sorafenib-responsiveness is still missing. Our aim was to analyse the association between the pretreatment microRNA profile of HCC and patient survival under sorafenib treatment. Total RNA was extracted from diagnostic fine-needle aspiration biopsy (FNAB) cytological smears of 20 advanced stage HCC patients collected between June 2008 and July 2012. All patients underwent sorafenib administration after FNA. Clinicopathological and survival data were recorded. Fourteen frequently deregulated miRNAs in HCC (miR-17-5p, miR-18a, miR-21, miR- 34a, miR-122, miR-195, miR-210, miR-214, miR-221, miR-222, miR-223, miR-224, miR-140, miR-328) were tested by qRTPCR. NormFinder software was used to select proper miR (mir-140) as a reference. Satisfactory amount of total RNA was obtained from all the considered samples (mean 10.8 $\pm$ 9.3 μg, range 0.2‑32.2 μg). Among the analysed miRNAs, high miR-214 expression was associated with smaller tumor size (p=0.019), whereas high miR-17-5p expression correlated with better Eastern Cooperative Oncology Group performance status

(p=0.003). The survival analysis revealed that high miR-224 expression was associated with increased progression-free and overall survival (PFS p=0.029; OS p=0.012). Search for potential target indicated platelet-derived growth factor receptor beta (PDGFR-β), a known target of sorafenib, as the potential target of miR-224. Our research on Sorafenib treated HCC concluded that pretreatment microRNA profiling, especially miR-224 expression, might serve as an ancillary tool for the better assessment of expected survival rates for patients under sorafenib treatment **(Gyöngyösi B, Végh E, Járay B, Székely E, Fassan M, Bodoky G, Schaff Z, Kiss A: Pretreatment MicroRNA Level and Outcome in Sorafenib-treated Hepatocellular Carcinoma. J Histochem Cytochem 62: 547-555, 2014, IF: 1,959).**

Two further projects were finalized in the extension period of our OTKA funded research. I would like to thank OTKA for granting the extension which enabled us to finish our planned investigations. Therefore, we were able to manage the delays mainly caused by the mobility of the researchers participating in our projects.

Hepatic cirrhosis is the eighth leading cause of mortality worldwide. The most common etiology of cirrhosis includes chronic viral hepatitis (predominantly hepatitis B and hepatitis C viruses), alcoholic liver disease (ALD), metabolic disorders (non-alcoholic steatohepatitis) and autoimmune liver diseases. Histologically, cirrhosis is characterized by rearrangement of the parenchyma into nodules, excess deposition of extracellular matrix and production of fibrotic tissues. The histologic appearance of a benign focal lesion, **focal nodular hyperplasia** (FNH) may look very similar to a part of cirrhosis under the microscope, however, by gross examination, FNH appears as a tumor-like hepatic lesion and develops in noncirrhotic liver. Thus, FNH never progresses into **hepatocellular carcinoma** (HCC), whereas cirrhosis is regarded as a premalignant lesion preceding HCC. The pathogenesis of FNH is unclear, yet FNH is hypothesized to result from congenital or acquired vascular abnormalities leading to either arterial or portal venous thrombosis. In contrast, HCC develops predominantly on the basis of cirrhosis. Hepatic cirrhosis and focal nodular hyperplasia (FNH) have several similar histological features despite the differences in gross appearance. Since cirrhosis may develop further into hepatocellular carcinoma (HCC) contrary to FNH, the aim of the present study was to identify microRNAs (miRNA) that had changed their expression in these liver diseases as an

indication of carcinogenesis as compared with normal liver, and to detect miRNAs that might differentiate diffuse cirrhosis from FNH. Altogether 106 surgically removed formalin-fixed paraffin-embedded liver samples were selected, including 45 cirrhosis, 24 HCC, 22 FNH and 15 normal liver tissues. The cirrhosis and HCC cases originated from hepatitis C and alcoholic etiologic backgrounds and the HCC cases developed in cirrhotic livers. Relative expression levels of 14 **miRNAs** were determined using TaqMan MicroRNA Assays

Elevated miR-34a, miR-224 levels were found in cirrhosis, HCC and FNH in comparison to normal liver, miR-21, miR-222 showed increased levels in cirrhosis, while miR-21, miR-221, miR-222 showed increased expression in HCC as compared with FNH. Furthermore, miR-195, miR-214, miR-223 were decreased in HCC, miR-18a, miR-195, miR-210 in FNH in comparison to cirrhosis, and miR-17-5p, miR-18a were decreased in FNH as compared with HCC. Taken together, the elevated expressions of miR-21 and miR-222 were associated with cirrhosis and HCC but not with FNH, therefore, it may be indicative of hepatocarcinogenesis. Further, reduced expression of miR-18a, miR-195 and miR-210 may differentiate FNH from cirrhosis, reflecting the different pathogenesis of these two entities contrary to some histologically similar features.

miR and claudin expression profiling of colorectal cancers and their liver metastases

The other project targeted the role of altered expression of claudins and the possible regulatory function of microRNAs in colon cancers and in their liver metastasis. The expression pattern of claudins and their expressional regulation might show significant changes in carcinogenesis and in tumor progression. miRs might also play role in these processes and may influence tumor cell invasion and metastasis formation. Surgical resection specimens of primary and metastatic tumors from the same patients were collected. Altogether, a set of more than 100 samples from identical patients were collected and tissue microarrays were created containing samples from the tumor surrounding **normal colon mucosa (COL), primary colorectal carcinoma (CRC) and liver metastasis (MET)** as well as metastasis surrounding normal liver. Claudin expression was studied by immunohistochemistry (IHC) and tissue microarrays (TMA) were created from samples sets derived from 96 patients. The immunohistochemical reactions (IHC) were carried out using Benchmark XT automation system. The IHCs were evaluated by both quantitative and qualitative analyses. Possible negative correlations between miR and claudin

expression were also analysed. Further, low and high levels of miR and claudin expressions were compared with survival data. Immunohistochemistry of selected claudins was carried out on tissue microarrays and isolation of total RNAs are still in progress. We aimed to investigate the expression pattern of claudins-1, -3, -4, -7 parallel with the study of the expression pattern of miR-22, miR-29b, miR-24, miR-27a, miR-155, miR-455-3p, miR-596, miR-149, miR-665, miR-342-5p. These miRs are suggested to target the investigated claudins based on comparative analysis of several miR databases, miR-345 was used as reference RNA. The total number of patients eligible for complete statistical analysis was 47 out of the 100. IHC of claudins-1, -3, and -7 showed significantly lower levels in CRC and MET when compared with COL. Further, the expression of claudin-1 protein was also detected to be decreased in MET in comparison to CRC. No difference was found in claudin-4 expression between the groups of COL, CRC and MET. The expressions of miR-22, miR-29b, miR-24, miR-27a, miR-155 did not differ between the investigated groups. However, the expression levels of miR-596, miR-149, miR-665 és miR-342-5p were decreased in MET in comparison to COL. Moreover, expressions of miR-665 and miR-342-5p were found to be decreased also in CRC. Further, miR-455-3p was found significantly increased in CRC and MET when compared with COL, while claudin-3 showed significantly reduced expression in the same comparison. The high or low expression of claudins or the investigated miRs did not show association with survival. In concluson, the expression of the investigated claudins showed decreased levels in CRC and MET when compared with COL. miR-455-3p revealed negative correlation with claudin-3 protein expression, therefore, the possible regulatory role of miR-455-3p on claudin-3 expression might be raised. On the other hand, the expression of claudins did not reveal significant changes between CRC and MET, except for claudin-1 which was found to be decreased in MET in comparison to CRC. The investigated miRs and claudins did not show correlation with survival.

Finally, in the last phase of the extension of the OTKA research project the regulatory role of microRNAs on the expression of claudins and growth factors in hepatic and colorectal carcinogenesis was investigated. In several colorectal cancer cell lines (SW1417 (adenocarcinoma), DLD1 (adenocarcinoma), HT-29 (adenocarcinoma), HTC116) we investigated the effect of the inhibition of miR-455-3p by a miRNA-specific Exiqon miRCURY LNA Power Inhibitor on the expression of claudin-3. On the other hand, we studied the inhibition of miR-224 by a miRNA-specific Exiqon miRCURY LNA Power Inibitor on the expression of PDGFR-β in hepatoma and HCC cell lines: HepG2, HLE (HCC – hepatoma, epythelial like), HLF (HCC – hepatoma, epythelial like), PLC (HCC – hepatoma, epythelial like). Our results revealed a moderate increase of claudin-3 protein expression (1.3-times higher

in comparison to controls) when the negative regulatory effect of miR-455-3p on claudin-3 was reduced by inhibition in the four investigated cell lines. Further, PDGFR-β protein expression was increased about 8.3 times in HepG2 and HLE cells when compared with the controls under reducement of the negative regulatory effect of miR-224 on PDGFR-β by inhibition. These data indicate that these miRNAs actively participate in the regulation, however, they are not the only miRNAs regulating the levels of these proteins.

Overall, our OTKA funded research project #101435 resulted in 6 publications, with a total Impact Factor of 14,69 and so far 61 citations.