Cancer incidence and mortality, including liver cancer are increasing worldwide despite the great efforts made in the field (ref. 4,15). In the more recent years several new biomarkers were detected and even used in target oriented therapy. Except for the multikinase inhibitor sorafenib, which might be used as predictive marker too, no such therapy exists for treatment of liver cancer (ref.1). For this reason, several research groups including ours have focused on the discovery of new mechanisms of cancer development, which steps might be characterized by molecular markers. Detection of such markers in premalignant, benign, malignant liver alterations like hepatoblastoma, hepatocellular carcinoma (HCC) and especially cholangiocarcinoma (CC) could have prognostic and diagnostic significance and be target for therapy.

In our previous research the role and significance of **tight junction (TJ)** proteins seemed to be important in the carcinogenesis of several tumors, such as gynecological, breast, thyroid, pancreatic and gastrointestinal cancers. In the frame of the recent project, involvement of TJ proteins was a central issue; TJ changes and alterations in liver differentiation and in liver tumor development. In addition the expressional changes of TJ proteins have been associated with treatment outcome and survival **(1st PART of the Report).**

In the second part of our studies **microRNAs**, the short regulating RNA molecules which interfere with gene expression at posttranscriptional level, were analysed. Deregulated microRNA expression has been found in many disorders including liver diseases. We studied the expressional changes in premalignant liver alterations, such as cirrhosis, liver fibrosis, benign and malignant liver tumors and in association with treatment **(2nd PART of the Report).**

The previous studies led us to the study of **mitochondria and autophagy.** Energy production in mitochondria and removal of damaged cell components (autophagy) are essential processes in cell survival. Proliferation may impose increased energy production on tumor cells; in turn, a correlation between increased mitochondrial function and autophagy would be expected. Our aim was to explore these cell biological processes in human cholangiocarcinoma (CC) **(3rd PART of the Report).**

1st PART

1. Expression profiling of hepatocyte-like cells differetiated from human embryonic stem cells (ref. 8)

Expression of TJ components was studied in human embryonic stem cells differentiated in vitro by addition of morphogens and growth factors (in collaboration with the Inst. Enzymology, Res. Center, Hung.Acad.Sci). The stem cells were differentiated into endoderm-like cells and further into hepatocyte-like cells. Oct3/4, Nanog, alpha-fetoprotein, albumin, cytokeratins (CK7, CK8, CK18, CK19) and various TJ components (claudin-1,-2,-4,-5,-7 and tricellulin) as well as the extracellular matrix component agrin were analysed during the differentiation by real-time quantitative PCR and immunohistochemistry. The stem cell markers significantly and gradually decreased, while the liver-associated genes increased. The endoderm-like cells expressed claudin-1, which later became declined. The expression of cholangiocyte markers (claudin-4, CK7, CK19) gradually increased and was the highest at the end stage of differentiation, while claudin-7, CK8 and tricellulin expression could not be detected. *The study proved that at the end of the differentiation the cells revealed both hepatocytic and cholangiocytic characteristics.*

2. Expression profiling of TJ protein tricellulin in hepatoblastoma (ref. 5)

The expression of tricellulin, a recently described TJ protein was studied in the hepatoblastoma (HB) samples and was found to correlated with the differentiation of the tumor. Similarities and differences were also observed in the tricellulin and EZH2 (enhancer of zeste homolog 2) expression of hepatoblasts as compared with cholangiocytes and cholangiocarcinoma (CC) cells. Cholangiocytes strongly expressed tricellulin, however, EZH2 was not detected by immunohistochemistry, whereas tricellulin expression was weak in hepatoblasts, although showing increase with progression of differentiation. Tricellulin expression of cholangiocarcinoma cells was notably increased as compared with normal liver, hepatoblastomas and hepatocellular carcinomas, but was found to be inversely proportional to the stage of CC. EZH2 showed strong expression in CC when compared with ductular reaction and normal biliary ducts.

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 while the embryonal component displayed significantly increased nuclear EZH2 positivity, in comparison to other epithelial subtypes and non-tumorous surrounding hepatocytes. Strong nuclear β-catenin staining was notably more

frequent in embryonal than in fetal types. High tricellulin expression was associated with significantly increased overall survival (P=0.03), while elevated EZH2 expression was linked to the presence of distant metastases (P=0.013). Conclusions: *Our data indicate that patients with treated HBs showing high expression of tricellulin have significantly better overall survival, independent of histological subtype. Increased nuclear expression of EZH2 was associated with the presence of distant metastases.*

2nd PART

1. MicroRNA expression pattern in altered non-tumorous liver (ref. 3,9,10)

(ref. 3) 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-fxed 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 expression of miR-33a and miR-224 were elevated in CHC-Steatosis and Steatosis in comparison to control tissue ($P<0.01$). 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, 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 fbrosis stage. Conclusion**:** *Differences 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.*

(ref. 9,10) Expression levels of several microRNAs (miR-21, miR-122, miR-214, miR-221, miR-222, miR-224) were analysed in liver fibrosis and cirrhosis. It was found that miR-122 negatively correlated with fibrosis in 52 patients representing various etiological backgrounds of fibrosis as detected by histology and FibroScan. Liver stiffness was found to be associated

with Metavir fibrosis scores and digital morphometric analysis in fibrotic liver diseases. Histological and non-invasive methods for detection of liver fibrosis were analysed. *The data obtained by digital morphometry, liver stiffness measurement and APRI score in fibrotic liver diseases were compared. The non-invasive methods showed good correlation with histological methods; liver stiffness proved to be more accurate than APRI.*

2. MicroRNA expression pattern in liver tumors (hepatoblastoma, hepatocellular carcinoma, cholangiocarcinoma) (ref. 1,2,6,14,16,17,18)

(ref. 1) Correlation between pretreatment microRNA levels and outcome was found in Sorafenib-treated hepatocellular carcinoma (HCC). Total RNA was isolated from diagnostic fine needle aspitration biopsy cytological smears. A total of 14 frequently deregulated miRNAs were detected. *Survival analysis revealed that miR-224 expression was associated with increased progression-free and overall survival. Using the same methodology, analysis of cytological samples from CC cases was performed.*

(ref. 16,17) 275 samples were analysed, including 43 HCCs and matching surrounding liver tissues, 63 cholangiocarcinomas (CC) and 51 matching surrounding tissues, 13 hepatocellular adenomas (HCA), 15 focal nodular hyperplasias (FNH) and 47 normal tissues. A total of 12 tissue multi-arrays from these were created. The miR-224 expression was determined by *in situ* hybridization using a 5' digoxigenin-tagged LNA probe (Exiqon) on the established tissue microarrays (in collaboration with the Department of Medicine (DIMED), Surgical Pathology Unit, University of Padua, Italy, publication in progress). The intensity of cytoplasmic miR-224 expression was semi-quantitatively categorized as negative, low, moderate (expression in <50% of cells) and high (from moderate to strong expression in most of the cells). For statistical analyses, nonparametric Mann-Whitney test and Wilcoxon rank test were applied along with Kaplan-Meier method for generating survival curves. The curves were computed using the Kaplan-Meier method. In comparison to normal liver, increased miR-224 level was found in HCC, HCA and FNH ($p<0.01$). Elevated miR-224 expression was detected in CC when compared with normal and surrounding tissues ($p<0.01$). CC showed the highest miR-224 expression when compared with the other investigated liver lesions ($p<0.01$). Regarding etiology, miR-224 expression was significantly different in hepatitis C virus-associated HCC and HCC of unknown origin as compared to their corresponding surrounding liver. Furthermore, high miR-224 expression level was found to be associated with shorter overall survival in CC patients as compared with patients with low miR-224 levels. Conclusions: *The highest miR-224 expression detected in CC when compared with HCC, HCA and FNH*

suggests that the association between high level of miR-224 and shorter survival might play important role as a regulator determining the prognosis of CC.

(ref. 2) MicroRNA (miRNA) expression patterns of the main subtypes of epithelial hepatoblastoma (HBs) were analysed to reveal differences and relate them to survival. We studied 20 cases of epithelial HB, subtyped as pure fetal (n=12) or embryonal/fetal (n=8) and 15 samples of nontumorous surrounding liver (SL). 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, miR-224 were determined by TaqMan MicroRNA Assays applying miR-140 as reference. A higher level of miR-18a ($p<0.01$) was found in embryonal samples than in fetal samples. Lower miR-17-5p, miR-195, miR-210, miR-214, and higher miR-221 levels were detected in fetal samples ($p<0.02$) in comparison with SL samples, whereas lower miR-122 level was observed in embryonal samples ($p<0.003$). Histological subtype did not correlate with survival, however high miR-21, low miR-222 and low miR-224 levels proved to be independently prognostic for HB with significantly increased overall survival ($p<0.03$*). The fetal and embryonal components of epithelial HB, as well as SL, revealed different miRNA expression patterns. Furthermore, miR-21, miR-222, and miR-224 levels predict overall survival of HB patients regardless of epithelial subtype.*

(ref. 6,14) The liver disease focal nodular hyperplasia (FNH) has several histological features that resemble hepatic cirrhosis. Since cirrhosis may develop further into hepatocellular carcinoma (HCC) or cholangiocarcinoma (CC) contrary to FNH, the aim of our studies was to identify microRNAs (miRNA), which, by their altered expression levels, are associated with the benign, tumor-like nature of FNH and characterize HCC and CC. Altogether 106 surgically removed formalin-fixed paraffin-embedded liver samples were selected, including 22 FNH, 30 cirrhosis, 24 HCC, 15 tumor surrounding cirrhosis (TS cirrhosis) and 15 normal liver tissues. Etiology of the cirrhosis and HCC cases included hepatitis C and alcoholism and the HCC cases developed in cirrhotic livers. Relative expression levels of 14 miRNAs were determined using TaqMan MicroRNA Assays. In comparison to normal liver, elevated levels of miR-34a and miR-224 were found not only in FNH but also in cirrhosis and HCC. Expression of miR-21 and miR-18a was increased in cirrhosis and miR-21, miR-222 and miR-18a in HCC compared with FNH, whereas miR-195 and miR-210 levels were decreased in FNH compared with cirrhosis. In conclusion, the *elevation of miR-34a and miR-224 may be associated with both benign and malignant proliferative processes, nevertheless the increased*

expression of oncomiRs miR-21 and miR-222 in cirrhosis and HCC but not in FNH may be related to malignant processes of the liver. The decreased levels of miR-18a, miR-195 and miR-210 may further differentiate FNH from cirrhosis, reflecting the different pathogenesis of these two entities contrary to some histologically similar features.

3rd PART

Mitochondral mass and autophagy in liver tumors in vivo and in vitro (ref. 7,11,12,13, 15,19)

In vivo studies: The previous data led us to extend our research to the manner in which tumor cells differ from normal cells in energy supply and elimination of damaged cellular components during carcinogenesis. Several data prove that mitochondria are key organelles in carcinogenesis and play important role in the regulation of apoptosis and autophagy. Mitochondria not only provide biochemical energy for cells but either actively (through apoptosis) or passively (through reactive oxygen species – ROS) have significant influence on cellular metabolism and have impacts on cellular homeostasis. Significance of mitochondrial dynamism (mitochondrial fusion and fission) and constant remodeling is highly recognized to have central role in driving and correction of cellular functions, including mitophagy. The role of autophagy is controversal in the regulation of cell death and survival and it has been shown that reduced or inhibited autophagy have significant effect in several steps in tumorigenesis. Autophagy acts as tumor suppressor by preventing genetic instability before cellular transformation in non-tumorous cells, on the other side, after malignant transformation, autophagy promotes cancer cell survival and facilitates tumor progression. Increase of autophagy-associated proteins like LC3, beclin-1 and p62 was found in premalignant or early stages of carcinogenesis.

We performed studies on paraffin embedded tissue microarrays prepared from 70 CC cases [28 intrahepatic (iCC), 19 perihilar (pCC) and 23 distal (dCC) cases (according to the new classification – ref.15)] with 31 adjacent non-tumorous tissues. The transporter of outer mitochondrial membrane (TOMM20) was used to characterize the mitochondrial mass, Beclin1 (BCLN1), microtubule-associated protein light chain 3 alpha (LC3A) and p62 for demonstration of autophagic activity. The mRNA expression levels of TOMM20, BCLN1, LC3A and p62 were detected using real-time PCR, protein expression and Ki-67 were determined by immunohistochemistry. For statistical analyses, Mann-Whitney test and Spearson's rank test were applied along with Kaplan-Meier method for generating survival curves.

Elevated TOMM20, LC3 and p62 expressions were detected in CC when compared with surrounding tissues ($p<0.05$). According to the anatomical localization of the tumors no significant differences were found in the markers of autophagy. The Beclin1 level was negatively correlated with p62 expression and tumor size, and the higher Beclin1 expression showed significantly longer survival in dCC patients.

We concluded that *increased amount of mitochondria and markers of autophagy characterize the tumors reflecting the elevated metabolic activity. Beclin1 may be a prognostic factor in dCC. The data further prove the importance of mitochondria and autophagy in CC not related to the localization* (after presentations at local and international conferences, a manuscript is under preparation).

In vitro studies: Human cholangiocarcinoma cell lines were used – two of extrahepatic origin **(TFK-1, EGI),** one intrahepatic cholangiocarcinoma cell line **Huh28,** and one HCC cell line (**HLF)**. Each cell line listed originally derived from American Type Culture Collection (ATCC). Our laboratory obtained all these cultures from Stephanie Roessler`s laboratory (Dept. Pathol., Univ. Heidelberg, Germany) given as a kind gift.

The cells were plated at appropriate density (5000-10000 cell/well) in 96-well plates for cell proliferation assay. An immunoblotting analysis was performed. Claudin-4,-18 and CK7, 19, Beclin1, LC3, p62 detection was performed by immunofluorescence. qRT-PCR was done from the different cell lines.

After characterization of each cell line for the markers listed above, we studied sorafenib induced cytotoxicity and cell proliferation, which decreased in all examined CC (intrahepatic - Huh28, extrahepatic - EGI, TFK-1) and HCC (HLF) cells. The most effective decrease in cell proliferation activity induced by sorafenib was detected in HLF hepatocellular carcinoma cells when compared to cholangiocarcinoma cells. Significant decrease in cell number was measurable already at lower concentration of the drug (2,5-5 μM) when we compared the effectivity of sorafenib with cisplatin and 5-Flurouracil (effective dose ~ 10-30 μM). Cisplatin was effective at low concentration only in HCC cells, and showed less intensive effect on cell proliferation on TFK-1 and EGI cholangiocarcinoma cells than on HCC cells. All these data were represented as mean ±SD (*$P<0.05$), vs DMSO (=control group). After optimization of cell culture assays we further studied the effect of autophagy inhibition in the cells under drug administration. A manuscript from these studies is under preparation in collaboration with the Dept. Pathol. of Univ. Heidelberg.