In vivo and in vitro study of autophagy and microRNA expression in chronic and neoplastic liver diseases

Doctoral (Ph.D) Theses

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1. Introduction

From the various kinds of diseases that affect humans worldwide, liver diseases are of great importance owing to their high incidence and mortality.

Chronic hepatitis (CH) and cirrhosis are among the most common risk factors in the development of hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC). Better knowledge of the cellular and molecular processes improves our understanding of the pathomechanism of these diseases and may add to the development of better therapeutic strategies and prediction of disease outcome.

The roles of microRNA regulation and autophagy have been in the focus of several recent studies, which could have significance in several liver diseases. Both processes play an important role in the normal liver function and alterations of varying degrees have been demonstrated in association with inflammation, increased connective tissue accumulation, architectural changes and malignant transformation.

It has been shown in chronic hepatitis C (CHC) that autophagy participates in hepatitis C virus (HCV) replication, the survival of infected cells as well as in the progression of hepatitis caused by the virus. By contrast however, very few data are at hand concerning the role of autophagy in autoimmune hepatitis (AIH).

Altered autophagy and accumulation of damaged mitochondria have been demonstrated in benign and

malignant liver tumors of hepatocytic origin leading to progression through increased ROS production. Other data have proved that autophagy has important role in CC too, the exact mechanisms and association with other cellular events however have not been clarified as yet.

2. Aim of the study

Based on the above, our main goal was to gain better knowledge and understanding of the molecular basis of liver diseases, with special regard to the different forms of chronic hepatitis, such as CHC, AIH and certain focal liver lesions including benign (focal nodular hyperplasia – FNH) and malignant (HCC, CC) tumors. The molecular mechanisms studied in both diffuse and focal liver lesions included the regulation of autophagy and microRNA expression.

Our study involved human liver needle biopsies and surgical resection materials, which were followed by analyses in vitro. CC- and HCC-derived cell lines were studied from the viewpoint of changes in autophagy and microRNA expression.

Taking the above into consideration, the following questions were raised:

1. Are there any differences in autophagy, mitochondrial mass and microRNA expression in case of CH of different etiology – CHC and AIH – and are any of these changes associated with steatosis, stage of fibrosis and grade of necroinflammation?

2. Is there a difference in microRNA expression between non-tumorous, premalignant, benign and malignant liver alterations, is there a typical pattern which characterizes the lesions?

3. Are there any differences in the expression of autophagic proteins between CC and HCC as compared to the surrounding non-tumorous liver? Is there an association with the mitochondrial dysfunction?

4. Do the autophagic markers and mitochondrial proteins have a typical pattern characterizing the different subgroups of CC – intrahepatic (iCC), perihilar (pCC) and distal (dCC) – based on anatomic localization and grade of differentiation?

5. Is there an association between the overall survival (OS) of patients and the expression of autophagic protein pattern?

6. Are there any differences in mitochondrial morphology and fluorescent-labeled autophagosomes between the iCC, extrahepatic CC (eCC) and HCC-originated cell lines?

7. Can autophagy be induced by Rapamycin or by chemotherapeutic agents in cell lines of different origin? Can proliferation activity be influenced by using autophagy-inhibitors in cell lines of different origin?

3. Material and methods

Patients and tissue samples

A total of 279 formalin-fixed paraffin-embedded (FFPE) surgically obtained resection samples and liver biopsy tissues were selected from the archives of the 1st Department of Pathology and Experimental Cancer Research as well as the 2nd Department of Pathology, Semmelweis University.

Of the 63 biopsy samples analyzed, 45 cases were diagnosed with chronic hepatitis C (CHC) and 18 cases with autoimmune hepatitis (AIH). To examine microRNA expression, tissue samples were collected from 22 FNHs, 45 cirrhosis cases, 24 HCCs, and 15 normal liver tissues. Tissue microarrays (TMA) were prepared using 70 cholangiocarcinoma (CC) samples and 31 non-tumorous surrounding liver tissues and 9 hepatocellular carcinoma (HCC) samples.

CC tissue samples were divided into intrahepatic, perihilar and distal categories by repeated reviews.

miRNA expression

To determine microRNA expression, total RNA isolation was performed from tissue samples using the RNeasy FFPE kit (Qiagen, Venlo, Germany) according to the manufacturer's recommended instructions, optimized for microRNA. Relative expression was determined by reverse transcription using a real-time quantitative PCR reaction based on the TaqMan MicroRNA Assay.

Immunohistochemistry

Immunohistochemical reactions were performed on needle biopsy FFPE samples obtained from AIH and CHC cases. TMAs were prepared from HCC, CC cases and the surrounding non-tumorous liver tissues using an automated TMA Master Device from 3D Histech (Budapest, Hungary).

The primary antibodies used were Beclin1 (polyclonal rabbit, Santa Cruz), LC3 (polyclonal rabbit, Novus Biologicals), p62 (monoclonal mouse, AbCam), TOMM20 (monoclonal mouse, Santa Cruz), COX4 (monoclonal mouse, Santa Cruz) and Ki-67 (monoclonal mouse, DAKO).

Immunohistochemical reactions were performed on Ventana Benchmark XT (Ventana Medical Systems Inc., Tucson, AZ, USA) automated immunostaining machine using the HRP Multimer-based biotin-free detection technique according to the manufacturer's protocol.

The intensity (0-5 points) and extent (0-5 points) of immune responses of Beclin1, LC3, p62, TOMM20, COX4 to cytoplasmic expression were evaluated semiquantitatively. The extent of the nuclear-appeared Ki-67 positive reaction was evaluated as a percentage.

Cell cultures

To study mitochondrial network and autophagy, 3 cell lines were used: HuH-28 (iCC-derived); TFK-1 (eCC-derived) and HepG2 (HCC-derived) provided by the

University of Heidelberg, Institute of Pathology (Stephanie Roessler, PhD).

Treatments and in vitro studies

Detection of mitochondrial morphology and autophagy in the above cell lines was performed by immunofluorescence technique. Mitotracker Orange CMTMRos (Life Technologies Eugenes, Oregon, USA) and Mitoview Green (Biotium Inc., Fremont, CA, USA) were used for fluorescence determination of mitochondria. Cells were also treated with 50 μM Chloroquin (CQ, Sigma-Aldrich Saint Louis, Missouri, USA) for 24 h to detect autophagy and Monodansylcadaverine (MDC, Sigma-Aldrich) was used for microscopic imaging. To examine the inducibility of cells to autophagy by Rapamycin (Rapa, Sigma-Aldrich) as an mTOR inhibitor cell lines were incubated for 24 h with 0.2 μM Rapa, 50 μM CQ, and a combination of the two agents. To examine the inducibility of cells to autophagy by chemotherapeutic agents, cell lines were incubated with 5-Fluorouracil (5-FU, Sigma-Aldrich), (0, 10, 50, 200, 400 μM concentrations) and Sorafenib (Santa Cruz Biotechnology, Heidelberg, Germany) (0, 5, 10, 15, 20 μM concentrations). After treatments, expression of LC3 I, LC3 II and p62 proteins was examined by Western blot technique.

The viability of cell lines (sulforhodamine B) was examined by treatment with 5-FU (10–400 μM) and Sorafenib (5–25 μM). By treatment with

chemotherapeutic agents supplemented with the autophagy inhibitor, CQ, the proliferation of cell lines was examined.

Statistical analysis

Non-parametric tests were used: the Mann-Whitney U test to compare the two groups, and the Kruskal-Wallis test and post hoc analysis to compare several groups. The interaction of proteins, the relationship between protein and microRNA expression, and the relationship between immunoreactions and clinicopathological features were examined using the Spearman Rank correlation test. Survival analysis (OS, overall survival) was performed by the Kaplan-Meier method, and a Log-rank test was chosen to compare the survival curves obtained.

For in vitro studies, the IC50 values of the treatments were determined by non-linear regression. Statistical analyses were performed using GraphPad Prism (version 5.01; California, USA) and Statistic v.13 (Stat-Soft Inc., Tulsa, Oklahoma, USA). The results were considered statistically significant if $p \leq 0.05$.

4. Results

Autophagy, mitochondrial mass and microRNA expression in CHC and AIH

Significantly increased LC3 ($p < 0.0001$) and TOMM20 ($p < 0.0001$) expressions were detected in AIH as compared to CHC by immunohistochemistry. There was a significant difference in protein expression between the severe and mild grade inflammation in case of AIH.

No statistically significant differences were observable in the expressions of miR-224, -155 and -204 between CHC and AIH, however, miR-101 was elevated in CHC as compared to AIH ($p < 0.0001$). MiR-224 correlated positively with steatosis in CHC ($p < 0.05$). In case of CHC, increased miR-155 expression was detected in higher fibrosis stage as compared to lower stage ($p < 0.05$).

MicroRNA expression in FNH, cirrhosis, HCC and surrounding non-tumorous liver

miR-34a and miR-224 were elevated in FNH, cirrhosis and HCC as compared to the surrounding liver ($p < 0.001$). Decreased miR-17-5p, miR-18a and miR-210 expressions were observed in FNH ($p < 0.03$), miR-17-5p and miR-221 showed decreased levels in cirrhosis ($p < 0.01$) and miR-223 in HCC as compared to the surrounding liver ($p < 0.0001$). Expressions of miR-18a, miR-21 and miR-222 were decreased in FNH as compared to cirrhosis and HCC ($p < 0.01$), miR-195 and miR-210

showed decreased levels as compared to cirrhosis ($p < 0.04$) and miR-17p as well as miR-221 levels were also decreased when compared to HCC ($p < 0.04$). Increased miR-195 and decreased miR-221 expressions were detected in cirrhosis as compared to HCC ($p < 0.02$). Decreased expressions of miR-18a, miR-21 and miR-222 were seen in FNH as compared to HCV-associated cirrhosis ($p < 0.04$).

Autophagy and mitochondria in human CC and HCC

LC3, p62, TOMM20 significantly differed in iCC ($p < 0.001$), however only TOMM20 and LC3 were elevated in eCC as compared to the surrounding liver ($p < 0.05$). Decreased p62 and increased TOMM20 expressions were detected in eCC as compared to iCC ($p < 0.05$). TOMM20 was elevated in eCC as compared to HCC ($p < 0.05$). Further division of the subgroups showed increased TOMM20 in pCC ($p < 0.05$) and dCC ($p < 0.01$), and a decreased level of p62 was found in dCC ($p < 0.05$) as compared to iCC.

By Ki67 immunohistochemistry, iCC showed a positivity of 15%, pCC and HCC of 25%, whereas a positivity of 70% was found in case of dCC. Further classifying the dCC group into high and low Ki67-expressing groups, the high Ki67 group was found to be associated with low Beclin1 ($p < 0.05$), LC3 ($p < 0.01$) and COX4 ($p < 0.05$) expressions and increased p62 expression ($p < 0.001$) as compared to the low Ki67 expressing group.

A positive correlation was found between LC3 expression and grade of differentiation in the pCC cases ($p < 0.05$).

Increased Beclin1 and low Ki67 levels correlated with better overall survival (OS) in dCC ($p < 0.05$), while no correlation was found in other subclasses of CC and the studied proteins.

Mitochondrial morphology and autophagy in vitro in iCC-, eCC- and HCC-derived cell lines

A difference in morphology was found regarding the mitochondria studied by immunofluorescence between the iCC (HUH-28), eCC (TFK-1) and HCC (HepG2) cell lines using Mitotracker Orange CMTMRos (redish, orange) and Mitoview Green (green) reactions. Redish and green tubular mitochondria were seen in HepG2. A different type of meshwork composed of shorter mitochondrial units was seen in the HuH-28 and TFK-1 cell lines.

The autophagic vacuoles detected by Monocadaverin (MDC) fluorescent staining demonstrated a cytoplasmic, point-like fluorescent signal. The reaction was diffuse in the control HuH-28 and TFK-1 cells, which was rather granular in distribution after CQ-treatment. The reaction was diffuse in the untreated HepG2 cells, however, after CQ-treatment diffuse, and in certain areas, point-like reaction was observable.

Effect of Rapamycin (Rapa) on autophagy

The level of LC3 II was compared in case of initial (Control+CQ – Control) and induced (Rapa+CQ – Rapa) autophagy. The difference was larger in HepG2 and HuH-28 cells (5.2 and 6.9 times, respectively) after treatment, while the difference was less pronounced in TFK-1 cells. The level of p62 was increased in HepG2 after Rapa+CQ treatment (1.4x), in HuH-28 the increase was 1.7 fold, while being only 1.5 fold after CQ-treatment, and the same finding was observed in case of the TFK-1 cells (1.5x).

Effect of chemotherapeutic agents on autophagy

After 10 uM 5-FU treatment the level of LC3 II/I showed a 3.8 fold increase as compared with the control in HuH-28 cells after 72 hrs, a 3 fold increase in HepG2 cells after 72 hrs and an increase of 3.9 folds after 48 hrs. P62 showed a minimum of 1.6 fold decrease in HuH-28 cells after 72 hrs treatment, while it showed no changes in TFK-1 and HepG2 cells.

Sorafenib treatment with increasing concentrations from 5 uM to 20 uM resulted a dose-dependent increase of LC3 II/I in HuH-28 cells after 72 hrs, while a decrease was observable in HepG2 and TFK-1 cells after 48 and 72 hrs. The level of p62 did not change in HuH-28, while it showed a 2 fold increase in TFK-1 after 72 hrs of Sorafenib treatment and an increase of 1.5 folds in HepG2 cells after 48 and 72 hrs.

Effect of chemotherapeutic agents on cell proliferation

Decreased IC50 was noted after combination of 5-FU and Sorafenib with CQ in HuH-28 after 48 and 72 hrs. A decrease was observed in TFK-1 after 5-FU+CQ treatment, however no changes were seen after 72 hrs. A minimal decrease was noted in HepG2 cells after 5-FU+CQ treatment compared with single 5-FU treatment in HepG2 cells after 72 hrs.

5. Conclusions and new findings

1. Our findings are the first to show differing autophagy (LC3, p62) and mitochondrial mass (TOMM20) in CH of various etiology (CHC and AIH), which reflects upon the different etiopathogenesis of the two etiological forms of CH.
2. We are the first to observe an association between inhibited autophagy (increased p62 expression) and the severity of necroinflammation in AIH, resulting in extended inflammation, significantly increased activated immune reaction and necrosis.
3. We detected a relationship between microRNA regulation and progression of CHC based on the association found between miR-224 level and degree of steatosis, as well as miR-155 expression and stage of fibrosis.
4. Varying microRNA patterns were seen in focal nodular hyperplasia (FNH), cirrhosis and HCC samples. The levels of miR-34a and miR-224 were elevated in cirrhosis, HCC and FNH, which finding is indicative of their involvement in the benign and malignant proliferative processes of the liver.
5. We demonstrated that autophagic and mitochondrial proteins showed different patterns in the CC subgroups – intrahepatic (iCC), perihilar (pCC) and distal (dCC). The level of LC3 was associated with the grade of differentiation of the tumor in pCC.
6. As a results of altered autophagy, LC3 and p62 were elevated in iCC and increased p62 levels were seen in pCC

as compared with the surrounding liver. The expression of TOMM20 was associated with Beclin1 and LC3 in iCC and pCC, which might suggest an association between mitochondrial damage and autophagy.

7. The dCC cases showing high-level Ki67 and low level Beclin1 had worse overall survival (OS), which is suggestive of a prognostic role of these proteins in dCC, and they might be valuable markers bearing prognostic significance.
8. The in vitro studies using Western blot analysis revealed varying mitochondrial morphology and basal autophagy in the iCC-, eCC- and HCC-derived cell lines.
9. Treatment with the mTOR inhibitor Rapamycin induced autophagy in iCC (HuH-28) and HCC (HepG2). Autophagic activity was induced by treatment with Sorafenib and 5-FU in case of HuH-28 and by 5-FU treatment in HepG2. Furthermore, our data showed that the viablility of cells became decreased by inhibition of the process. In case of the TFK-1 (eCC) cell line, autophagy induced by Rapamycin and certain chemotherapeutic agents resulted in loss of autophagy. This finding suggests that the signalling pathways of autophagy differ in the CC cell lines of varying origin.

6. List of publications

Publications related to the Dissertation*:*

Szekerczés T., Gógl A., Illyés I., Mandl J., Borka K., Kiss A., Schaff Zs., Lendvai G., Werling K. (2020) Autophagy, mitophagy and microRNA expression in chronic hepatitis C and autoimmune hepatitis. Pathology and Oncology Research

https://doi.org/10.1007/s12253-020-00799-y

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***** expected impact factor values