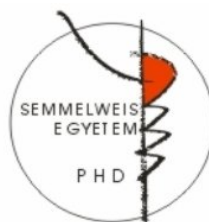


Recurrent diseases following orthotopic liver transplantation

Ph.D. Doctoral Thesis

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Introduction

Due to the growing number of transplanted patients and longterm follow-up, disease recurrence have become the main challenge after OLT. My research was based on the recurrence of chronic liver diseases after orthotopic liver transplantation (OLT), in which diagnostical and therapeutical challenges are still an ongoing problem after OLT.

We have studied the following liver transplanted patient groups for the following reasons: 1. HCV positive patients, in whom the recurrence of hepatitis C virus (HCV) infection significantly affects mortality and it is still the main therapeutical challenge after liver transplantation. 2. Patients with primary sclerosing cholangitis (PSC), in whom disease recurrence means mainly differential diagnostical difficulties after OLT. We have also studied features and activity of PSC associated inflammatory bowel disease (IBD) in the context of liver transplantation in these patients. 3. Patients with hepatocellular carcinoma (HCC), in whom the recurrence of HCC is a life-threatening condition associated with very low survival rates in the short-term.

HCV induced chronic liver failure is the leading indication of OLTs. The recurrence of HCV infection is universal after OLT. High rate of mortality, graft loss and therapeutical difficulties associated with HCV recurrence have emerged as a major concern. Therefore, clinical and molecular biological studies have expansively performed regarding the factors associated with early HCV recurrence and rapid progression. The shortage of suitable donor organs has led transplant centers to accept liver donors with higher risk of postoperative graft dysfunction, so-called extended criteria donors (ECDs) for liver transplantation. This is a widely accepted alternative to expand the donor pool, however, ECDs (older donors, those

with liver steatosis) could favor viral replication postOLT. New onset diabetes after transplantation (NODAT) is a common complication after liver transplantation and it is strongly associated with hepatitis C virus infection. It is known that HCV induces insuline resistance (IR) and it is independent of body-mass index (BMI) and other metabolic factors. Moreover, NODAT accelerates the progression of HCV recurrence.

Recently, a number of cellular factors required for HCV infection have been determined. HCV particles reach hepatocytes in association with lipoproteins via the sinusoidal blood. Hepatocytic entry of the virus is a complex process requiring the interaction of several viral and host cell factors. After non-specific attachment to the cell surface molecules such as Low-Density Lipoprotein (LDL)-receptors and glycosaminoglicans, HCV particles are consecutively bound to a complex formed by scavenger receptor type B1 (SR-B1) and CD81. A virus associated with CD81 would then be transferred into tight junctions (TJ), where HCV would interact with CLDN-1 and OCLN to enter the cell via clathrin mediated endocytosis. It is suggested that the expression of CLDN-1 and OCLN proteins is increased in HCV-infected liver compared with normal liver tissue. It was also shown HCV recurrence after OLT have been associated with increased levels of CLDN-1 and OCLN proteins. However, there was no correlation between mRNA and protein levels of these receptors. These data suggest that CLDN-1 and OCLN expression may be influenced at posttranscriptional level in the presence of HCV. microRNAs (miRs) are small endogenous non-coding ~22 nucleotid long RNAs that regulate gene expression at posttranscriptional level. miRs are in the focus of interest in many research area due to their role in disease prediction and therapy. Dysregulated expression of miRs a play role in the development of many diseases including HCV infection. For example, the liver-specific microRNA, miR-

122 is known to be a positive co-factor in the HCV replication cycle *in vitro*. According to the literature, there was no available study on the expressional regulation of HCV receptors by miRs.

Despite the excellent short-, and longterm survival rates, impaired graft survival have been detected in patients with PSC. The diagnosis of recurrent PSC (rePSC) is difficult because non-anastomotic extra-, or intrahepatic biliary stricturing is an aspecific reaction for many insult affecting the new graft. The presence of PSC associated IBD (PSC-IBD) at OLT have been proposed to be the main risk factor of rPSC. PSC-IBD has been reported as a unique entity of IBD due to it's specific manifestation compared to IBD alone. PSC-IBD patients appear to have quiescent colonic disease, increased incidence of pancolitis and right-sided colitis, rectal sparing, back-wash ileitis and colorectal cancer. The symptoms of IBD can improve after OLT, however, there is a group of patients with more severe colonic disease despite the immunosuppressive therapy.

The incidence of HCC increasing worldwide and has become a leading cause of cancer related death. Despite the improvements in systemic therapy (Sorafenib), surgical resection (resection, liver transplantation) has still emerged as the primary treatment of HCCs. Early experiences with OLT for HCC had very poor outcome due to high rate of tumor recurrence, reflecting the inclusion of patients with advanced stage HCC. Since then, patients eligible for liver transplantation include those within distinct selection criteria identifying early stage HCC. The Milan criteria for the selection of liver transplant candidates has been the gold standard for decades reporting recurrence rate of <15% and comparable outcomes after OLT of HCC patients and other indications of OLTs.

Aims

We aimed to study factors associated with disease recurrence after OLT in the Hungarian liver transplant population. The following issues have been concerned:

1. HCV recurrence:

- a. expression of microRNAs that either have one of the HCV receptors among their target mRNAs according to target prediction software, or have a role in HCV infection in HCV positive liver transplant recipients before and after antiviral therapy
- b. clinical features of HCV recurrence and outcome of OLTs in the past 10 years
- c. impact of ECDs on the outcome of OLTs, time and progression of HCV recurrence
- d. incidence of NODAT, its association with timing and progression of reHCV

2. Recurrence of PSC and PSC associated IBD after liver transplantation:

- a. clinical features and activity of PSC-IBD before and after liver transplantation
- b. incidence of recurrent PSC and its association with clinical factors

3. Hepatocellular carcinoma and liver transplantation:

- a. outcome of liver transplantations, recurrence rate in HCC patients
- b. impact of Milano criteria on recurrence rate of HCC and postoperative outcome
- c. impact of preOLT down-staging techniques on recurrence rate of HCC and postoperative outcome

Methods

Prospective study on microRNA expression:

Twenty-eight liver needle biopsies of HCV-positive adult liver transplanted patients were included. Liver biopsies were taken twice: at the time when HCV recurrence was observed following liver transplantation and after the end of antiviral therapy. Normal liver samples (N=13) were obtained from deceased donors during organ receiving, just before ligation of the abdominal aorta and reperfusion. Liver samples were fixed in 10% buffered formalin and embedded in paraffin. All selected patients received the combination of IFN/RBV for 12 months without interruption. Six patients (21%) achieved SVR (HCV was undetectable in the sera using RT-PCR six months following the completion of IFN/RBV therapy). Patients were defined as being non-responders if their sera were positive for HCV RNA during either examination (22 patients, 79%). All patients had HCV genotype 1b infection. *In silico* identification of microRNAs that may bind to any mRNAs of HCV receptors CLDN1, OCLN, SCARB1 and CD81 was performed using microRNA.org (<http://www.microrna.org>) target prediction database and software application developed by Tömböl and coworkers. The latter is capable of merging three target prediction databases such as TargetScan 6.0 (<http://www.targetscan.org>), PicTar (<http://pictar.mdc-berlin.de>) and MicroCosm Targets Version 5 (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>). The following microRNAs were selected to modulate HCV receptor expression by target prediction and based on sequence homology: CLDN1: **21, 34a, 96, 194**, 195; OCLN: **122**, 194, 224; CD81: 23a, 125b, **194**; SCARB1: **96**, 99a*, **125b, 195** (Letters in bold indicate consensus in all three databases). microRNA expressions were measured by real-time PCR. Relative expression level in samples was

determined by $2^{\Delta Cq}$ method ($\Delta Cq = Cq_{ref} - Cq_{sample}$) using the mean Cq value of miR-23a and miR-34a as reference.

Retrospective clinical studies:

In **HCV** positive patients the following data were analyzed: time of HCV recurrence (histologically proven), HCV-PCR levels, HCV RNA „cut-off” level: 8.78×10^6 copy/ml, HAI (Histology Activity Index, modified ISHAK score /0-18/) and Fibrosis score /0-6/. *ECD grafts* were defined by the authors previously reported criteria. *NODAT* was defined if the patient had ≥ 126 mg/dl fasting plasma glucose permanently after the third months after the liver transplantation, and/or sustained antidiabetic therapy was given.

PSC recurrence was diagnosed by the diagnostic criteria of Graziadei et al. Out of 49 OLT, 24 cases were excluded due to hepatic artery thrombosis/stenosis (N=8), biliary anastomotic stricture (N=5), non-anastomotic biliary stricture within 90 days after OLT (N=1), established ductopenic rejection (N=3) and lacking follow-up interval mainly due to early death (N=7). Based on radiological, histological and clinical features, six patients had recurrent PSC (rPSC) (24 %), the remaining 19 patients considered as controls (no-rPSC, 76%). Activity, localisation and therapy of IBD, atypical (p)- ANCA were measured in PSC patients. We used Mayo score (Disease Activity Index) to assess the severity of ulcerative colitis (UC) before and after OLT.

545 **HCC** patients were studied, including 64 in which the tumor was the indication of OLT and 9 in which it was incidental finding. The following data were recorded: size of tumor (mm), number of foci (>7: multifocal HCC), grading, hilar lymph node propagation, tumor relapse, de novo tumor occurrence, impact of Milano criteria (1 tumor <5 cm or up to 3

tumors, with the largest <3 cm) and preOLT down-staging technics (FRA, TACE) on postoperative outcome.

Statistical analysis

SPSS 15. version (SPSS, Inc., Chicago, Ill) was used for the statistical analysis. Continuous variables are shown as mean values and standard deviations. Student's *t*-test, ANOVA test with Scheefe and Bonferroni posthoc tests, as well as Mann-Whitney *U*-test were utilized for univariate analyses, after examining population homogeneity of the variables (Levene test). We used ANOVA method to compare microRNA expression before and after IFN therapy when therapy response (SVR versus NR) was taken into account. The connections between continuous variables were evaluated by correlation analysis, using Pearson correlation coefficient. Cumulative graft- and patient survivals were examined by Kaplan-Meier analysis. The differences were considered to be significant when $P < 0,05$.

Results

MicroRNS expression profile before and after antiviral therapy in HCV positive liver transplant recipients:

High viral load (above median 3.4×10^6 /ml) at the time of HCV recurrence was associated with higher expression level of miR-122. The transcriptional levels of miR-99a* and miR-224 were significantly increased, while miR-21 and miR-194 were decreased in liver samples obtained at HCV recurrence as compared with the levels measured in normal liver tissue. To examine whether IFN/RBV therapy has an impact on microRNA expression after OLT, we compared paired liver samples of patients obtained before and after antiviral treatment. In comparison to pretreatment expression levels, increased expressions were found for miR-221, miR-224 and miR-217 in samples taken after administration of antiviral treatment. MicroRNA expression levels were also investigated in relation to therapy response. Due to the fact that only SVR is associated with long term clinical improvement, we focused on this patient group (N=6.21%). Significantly increased miR-96, miR-99a*, miR-122, miR-181a-2*, miR-217 and miR-221 expression levels were found after antiviral therapy in samples of patients having reached SVR in comparison to non responders. Upon comparing the patients who reached or did not reach SVR after treatment, however, no significant difference in microRNA expression was detected at HCV recurrence. SVR patients after antiviral therapy showed significantly elevated levels of miR-221 and miR-122 when compared with samples before antiviral therapy.

Results of clinical studies on HCV recurrence:

Worse patient and graft survival were observed in HCV positive patients in comparison to hepatitis C virus negative recipients. HCV recurrence was observed in the majority of the patients (132 patients, 85%), mainly within the first year (83%). The authors observed recurrence within 6 months in 71 patients (54%), and within 3 months in 26 patients (20%). The mean hepatitis C virus recurrence free survival was 243 days (21-1159). Despite the immediate start of antiviral treatment, early recurrence (< 3 months) had a significant negative impact on patient survival. In recent years, viral recurrence was observed earlier in comparison to our previous results. HCV recurrence was observed earlier, and higher Knodell-, and fibrosis score were measured at time of recurrence in HCV patients with ECD grafts. Further, the use of ECD grafts had a negative impact on patient-, and graft survival in HCV patients. NODAT occurred in 63 patients (20%) after OLT. Worse patient and graft survival rate were found in NODAT patients when compared with the control group. Early virus recurrence (within 4-6 months) was associated with the development of NODAT. Fibrosis score and Knodell score were higher in the NODAT group after the antiviral therapy. In case of early recurrence, NODAT have developed in the majority of HCV positive patients.

PSC recurrence and PSC associated IBD after liver transplantation:

Approximately 60% of PSC-IBD patients had pancolitis and the majority of patients (95%) had inactive or mild colitis treated with 5-ASA medications before transplantation. On the contrary, moderate or severe colitis activity was observed in 60% of patients after transplantation. Worse graft survival rate was found in PSC-IBD patients when compared with the control PSC group. Using strict exclusion criteria, clinical data of 25 PSC patients (16 male and 9 female) were analysed. Six patient had recurrent PSC (rPSC)

(24 %), the remaining 19 patient considered as controls (no-rPSC, 76%). Younger recipient age, higher donor body mass-index (BMI), worse patient and graft survival were observed in rePSC group in comparison to control PSC group. In the rPSC group, none of the patients had colectomy prior to OLT, but two colectomies were indicated due to severe, therapy resistant colitis after OLT. In contrary, there were four colectomies out of the 19 patients in the control group during the preOLT setting. Progressively increasing p-ANCA titers were measured in the majority of PSC patients after OLT, especially in the rePSC group.

Liver transplantation for HCC

Out of 59 HCC patients, 38 (64%) were transplanted within the Milano criteria, 21 patients exceeded criteria. PreOLT down-staging technics were performed in 25 patients: RFA in 11 cases, TACE in 13 cases and alcohol injection in 4 cases. Recurrence of HCC was diagnosed in 9 (15%), mainly HCV positive patients. All of the patients had recidive HCC exceeded Milano criteria. 3 hepatic, 3 multiplex, 1-1 lymphoglandular and osseal recidivism were observed. Surprisingly, recurrence rate was higher in those patients in whom preOLT down-staging was performed. PreOLT down-staging technics had no significant impact on patients and graft survival. Worse patient and graft survival were found in HCC group in comparison to other indications of OLTs. Within HCC group, patients exceeded Milano criteria had significantly higher mortality than those within criteria. However, there was no significant difference between HCC patients within Milano criteria and no HCC patients regarding cumulative patient survival.

Conclusions

1. HCV induced liver cirrhosis is the leading indication of liver transplantation in Hungary. Poor outcome of OLTs in HCV positive patients is still a challenge. Viral recurrence has been occurred earlier recently in comparison to our previous observation. Early recurrence (<3 months). Despite an immediate start of antiviral treatment, early recurrence has a significant negative impact on the outcome of transplantations. Use of ECD liver grafts is an effective alternative to expand the donor pool, however, these grafts should be avoided in Hepatitis C virus positive recipients.
2. New onset diabetes (NODAT) has a negative impact on patient survival. The occurrence of NODAT in hepatitis C positive patients is associated with early recurrence, more severe viraemia and fibrosis after antiviral therapy.
3. Liver biopsies of the hepatic allograft at recurrence of HCV and after antiviral therapy revealed different expression profile of HCV-related microRNAs and those potentially targeting mRNAs of HCV receptors. In particular, miR-194 and miR-21 might be involved in expressional regulation of HCV receptor protein CLDN-1 and OCLN during HCV infection and antiviral therapy. High HCV titer at recurrence was associated with higher level of miR-122.
4. Excellent results have been shown after liver transplantation in patients with PSC. However, recurrence of the disease have a significant negative impact on longterm graft survival. Younger age at OLT and severe active IBD may be associated with PSC recurrence. p-ANCA seropositivity might be helpful in the diagnosis of recurrent PSC.
5. PSC associated IBD has a negative impact on postoperative outcome in PSC patients. Pancolitis and quiescent course of IBD have been shown in the majority of PSC-IBD patients before liver transplantation. After OLT, the

activity of inflammatory bowel disease worsens in the majority of patients after liver transplantation. Therefore, total colectomy prior to OLT or early after liver transplantation is a life-saving intervention and it seems to have a protective effect for recurrent PSC.

6. Patients with hepatocellular carcinoma (HCC) have poor prognosis after liver transplantation. Application of Milan criteria for the selection of patients with HCC associated with lower rate of HCC recurrence and better survival after OLT.

Publications

I. Publications related to dissertation:

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II. Publications not related to dissertation:

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