ANALYSIS OF MICRO-RNA AND CLAUDIN EXPRESSION IN HUMAN HEPATOCELLULAR CARCINOMA

Doctoral (Ph.D.) theses

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1 - INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary, malignant liver tumor of hepatocyte origin, currently the sixth most common cancer affecting 782,000 people worldwide. Although much knowledge has been gathered about this tumor in recent decades, HCC remained one of the few cancers with increasing mortality in the US and Canada. Besides, HCC is the second most common cause of cancer-related death worldwide. HCC is a very aggressive cancer, with a median patient survival between 6-9 months and a 5-year survival rate of 20%. As in other malignant tumors, prevention and early diagnosis would be desirable for the best possible outcomes, but because of the lack of symptoms, patients usually present at a late and advanced stage.

The pathogenesis of HCC relies on many altered signal transduction pathways that also might serve as targets for molecular therapies. Although dynamic imaging modalities are in the first line of diagnostic tools, pathological diagnosis from biopsy material is still the gold standard in questionable cases. In case of early diagnosis, potentially curative surgical treatments are available, however, only a minority of patients with localized small sized tumors are eligible for these therapeutic options (15-35%). Unfortunately, a great percentage of patients are diagnosed at an advanced stage of disease, for whom only limited therapeutic choices are available. Sorafenib, a recently introduced systemic, molecular-targeted, multi kinase inhibitor is the first approved treatment that significantly prolongs overall survival in patients with advanced HCC. Although sorafenib was proved to significantly prolong overall survival, it provides only limited clinical benefit due to the heterogeneity of the treated patients and the lack of validated molecular marker. Moreover, stratification of HCC patients according to sorafenib responsiveness and predicting patient outcome

is still an unmet medical need because there is no consensus regarding the utility of potential clinical or molecular markers.

MicroRNAs (miRNAs, miRs) are small (19-25 nucleotide), noncoding, posttranscriptional regulators of gene expression commonly dysregulated in tumors. They function through binding to the 3' untranslated region of messenger RNAs resulting in degradation of the target mRNA or inhibition of translation. MicroRNAs target both tumorsuppressor and oncogenic pathways and their altered expression carries great diagnostic and prognostic potential. In addition, miRNAs are highly stable and are reliably detected in archival clinical samples and cytology specimens, and are therefore considered to be ideal biomarker candidates. We analyzed a panel of miRNAs that are frequently dysregulated in HCCs or sensitize cells to sorafenib treatment.

Beside the not full understanding of the biological behavior of an already developed HCC, there is limited knowledge on the process of HCC pathogenesis, too. It is known that HCCs predominantly develop in cirrhotic livers on chronic hepatitis background. One of the hallmarks of cancers is loss of cell polarity and change in cell-cell contacts, and cirrhotic change alone can lead to parenchymal rearrangement and disturbed intercellular junctions. Claudins are transmembrane proteins constituting the backbone of the intercellular tight junction protein complex. Their altered expression has been associated with altered cell adhesion, tumor progression, moreover, they can serve as entry co-factors for certain viruses, like hepatitis C virus (HCV). It is not yet fully understood, how claudin expression changes in cirrhosis and HCC, and how it correlates with hepatitis C infection.

2 - AIMS

Sorafenib represents the first effective therapy for advanced stage hepatocellular carcinoma providing significant survival benefit through molecular-targeted mechanisms. However, adequate patient stratification regarding sorafenib-responsiveness is still missing due to the lack of reliable and validated biomarkers.

It is not yet fully understood, either, how expression of claudins, which constitute the backbone of tight junctions, changes with the development of HCC, further, how claudins change in HCV-related and -unrelated cirrhotic rearrangement of liver parenchyma.

Based on these, our objectives were the following:

- To analyze miRNA expression of diagnostic, pretreatment fine needle aspiration biopsy samples of sorafenib-treated HCC.
- To study possible associations between miRNA expression and clinicopathological features of HCC patients.
- To analyze the association between the pretreatment miRNA profile of HCC and patient survival under sorafenib treatment, particularly miRNAs that could sensitize cells to sorafenib *in vitro*.
- To characterize claudin-1, -2, -3, -4 and -7 protein expressions in resected HCC and its surrounding cirrhotic or non-cirrhotic, non-tumorous liver parenchyma.
- To investigate associations between claudin expression and tumor grade, patient survival and HCV status, both in tumorous and non-tumorous samples, with special interest in claudin-1 as a known HCV entry co-factor.

3 - MATERIALS AND METHODS

We studied a total of 50 HCC samples from the archives of the 2nd Department of Pathology, Semmelweis University, Budapest. The studied material was obtained with permission from the Regional Ethical Committee of Semmelweis University (#172/2003, #137/2008, #35/2011).

For miRNA studies, diagnostic, pretreatment, fine needle aspiration biopsy samples of twenty advanced stage HCC patients were used. The presence of >2000 well-preserved and well-visualized HCC cells was appointed as minimum accepted cellularity. Only slides with a proportion of tumor cells >50% and without massive necrotic debris were selected. Median patient age was 68 years (52–82 years), male:female ratio was 16:4. Patients mostly had good liver functions (16/20 Child-Pugh A), and favorable clinical performance (13/20 ECOG 0), etiology was mostly chronic viral hepatitis (11/20), median tumor size was 5 cm (1.5–20 cm), tumors were mostly multinodular (14/20), with elevated AFP levels in 13/20 cases. Median follow-up time was 33.6 months. All patients in this study met the following criteria: no prior surgery, loco-regional or systemic treatment, no subsequent loco-regional treatment, more than 3 months survival after diagnosis and available follow-up data.

Total RNA isolation was performed with RNeasy FFPE kit, optimized for miRNA extraction. After reverse transcription, miRNA levels were quantified using TaqMan MicroRNA Assay and real time PCR. Relative expression values were correlated with clinicopathological data and survival data using Fisher exact test, Kaplan-Meier method and log-rank test.

For claudin studies, formalin-fixed, paraffin-embedded surgical resection specimens of thirty HCC cases with thirty corresponding non-

tumorous surrounding liver samples, and six normal control liver samples were investigated. Median age was 63 years (44–78 years), male:female ratio was 20:10. Mean tumor size was 7.1 cm (2–22 cm), tumors were mostly unifocal (24/30). Seven tumors showed grade 1, 10 grade 2, 13 grade 3 differentiation. Seventeen HCC patients were serologically HCV positive. In 11/30 cases, HCC developed on cirrhotic background (score 5–6 per the modified fibrosis/cirrhosis index) and 11/30 showed chronic hepatitis (CH) with fibrosis (score 3 or 4) but no cirrhosis. A total of 6/17 HCV positive cases coincided with cirrhosis while 11/17 with CH and fibrosis. Five out of the thirteen HCV-negative HCC patients developed cirrhosis of alcoholic etiology, whereas the other patients of this group did not show CH or fibrosis.

We investigated the normal – cirrhosis – HCC axis comparing normal liver (NL), non-cirrhotic surrounding liver (ncSL), cirrhotic surrounding liver (cSL), non-cirrhotic HCC (ncHCC) and cirrhotic HCC (cHCC).

Immunohistochemical detection of claudins was performed on 3-5 μm thick sections of FFPE tissue blocks, using anti-claudin-1, -2, -3, -4, and -7 primary antibodies (Zymed) with Ventana ES automatic immunostainer. The immunohistochemical reactions detecting claudins were photodocumented using light microscopy (200x magnification). Ten-fifteen randomly selected areas were assessed, and stained area percentages were quantified using Leica QWin morphometrical software (Leica Microsystems Imaging Solutions Ltd.) For the confirmation of the specificity of the results, Western blot analysis was performed on representative fresh frozen samples, following protein isolation. Results of immunohistochemistry were compared with tumor grade, patient survival and HCV status, utilizing Mann-Whitney U test, Kruskal-Wallis and post hoc tests, Kaplan-Meier method and log-rank test.

4 - RESULTS

4.1 - miRNA studies on HCC FNAB samples

4.1.1 - Availability of miRNA profile in HCC FNAB samples

A satisfactory amount and quality of total RNA was obtained from all cytology samples. The mean ± SD RNA yield from the smears was 10.8 ± 9.3 μg (range, 0.2 – 32.2 μg) with an OD 260/280 ratio of 2.0 ± 0.1, as measured by NanoDrop.

4.1.2 - Relationship between Pretreatment miR Expression and Clinicopathological Features

For the study of possible associations between miR expression and patient characteristics, 2x2 contingency tables were used. Clinicopathological parameters, such as age (<68 years / ≥68 years), gender (male / female), etiology (viral / other), tumor number (single / multinodular), tumor size (≤5cm / >5cm), Child-Pugh status (A / B), ECOG status (0 / 1) and AFP levels (<10 ng/ml / ≥10 ng/ml), were analyzed and patients were dichotomized into "high" and "low" miR expression groups based on the median relative miR expression.

High miR-214 expression was more frequent in patients with smaller size tumors (≤5 cm) than in patients with larger size ones (>5 cm) (80% vs 20%, p=0.019). High miR-17-5p expression was more frequent in patients with better performance status than in patients with an ECOG score of 1 (77% vs 0%, p=0.003). All other analyzed miRs did not reveal significant correlations with patient characteristics.

4.1.3 - Relationship between Pretreatment miR Expression and Outcome under Sorafenib Treatment

From the twenty sorafenib-treated patients, 15 died by the end of the study (75%). The median overall survival (OS) and progression-free survival (PFS) for all patients was 6.2 months (95% CI 2.7 – 9.3 months) and 5 months (95% CI 3.5 – 6.5 months), respectively.

We found significant association between pretreatment miR-224 levels and patient survival under sorafenib treatment. The median expression difference between "high miR-224" and "low miR-224" groups were more than 20-fold (21.7x), showing the significantly altered expression of this miR in these tumors. We found that both OS and PFS rates were better in the "high miR-224" group than in the "low miR-224" group. Median OS in the "high miR-224" group was 39.7 months, while 5.7 months in the "low miR-224" group (log-rank p=0.012). The hazard ratio was 0.24 [95% CI: 0.07 – 0.79]. Median PFS in the "high miR-224" group was 13 months, contrary to 4.5 months in the "low miR-224" group (log-rank p=0.029). The hazard ratio was 0.28 [95% CI: 0.09 – 0.92]. The other analyzed miRs did not show any significant associations with either PFS or OS of sorafenib-treated patients in our sample set.

4.2 - Claudin studies on resected HCC samples

4.2.1 - Claudin-1 – Elevated expression in cirrhosis and HCC

Claudin-1 reaction invariably resulted in plasma membrane staining. In normal and non-tumorous livers, strong apical staining appeared on the biliary epithelial cells and weak to moderate staining at the junction lines of hepatocytes. Hepatocytes in cirrhotic nodules showed moderate to strong expression, and unlike on normal hepatocytes, the membranous staining often appeared on the entire

circumference of the cells, though with uneven intensity. In seven cirrhotic cases, intense membrane staining was detected in dysplastic hepatocytic nests as well. HCCs showed variable degree of immunostaining, with the majority of tumor cells exhibiting moderate to strong membranous reaction.

In quantitative terms, claudin-1 expression was significantly elevated in cirrhotic surrounding livers (cSL) relative to non-cirrhotic surrounding livers (ncSL). All HCCs, including those formed in cirrhotic and non-cirrhotic livers, showed increased claudin-1 expression relative to NL and ncSL, yet the difference was significant only in case of cirrhosis-based HCCs (cHCC). cHCC reacted even stronger than cSL and was also stronger than in non-cirrhosis-based HCC (ncHCC). Although ductular reaction in cirrhosis contributed to a notable proportion of immunostaining, the cirrhosis-related increase in claudin-1 expression was found to be significant even with the omission of the measurement of bile ducts, therefore, reflecting hepatocyte involvement.

4.2.2 - Claudin-7 – Elevated expression in cirrhosis and HCC

Claudin-7 immunohistochemistry resulted in weak membrane staining on hepatocytes and very strong membrane positivity on bile duct cells in non-cirrhotic livers. Hepatocytes in cirrhotic nodules showed moderate to strong claudin-7 expression, with more pronounced staining in dysplastic foci. HCC cells were also moderately stained with a clearly apical pattern in tumor cell pseudoacini.

By morphometry, hepatocytic claudin-7 immunostaining was found significantly elevated in cSL when compared with NL and ncSL. cHCC, in its turn, was also significantly superior to NL and ncSL, but stained weaker than cSL.

4.2.3 - Claudin-2 – Decreased expression in HCC

Claudin-2 reaction gave granular membranous and cytoplasmic staining in both non-tumorous and tumorous cells. No significant quantitative alteration was measured in non-tumorous tissues. As a contrast, claudin-2 expression was found decreased in all HCCs, with no difference between cHCC and ncHCC.

4.2.4 - Claudin-3 – Unaltered expression in cirrhosis and HCC

Claudin-3 resulted in very weak membrane staining on hepatocytes, while bile ductular cells gave stronger reaction. Weak membranous staining was detected on HCC cells. Morphometry failed to reveal any significant differences between the investigated groups.

4.2.5 - Claudin-4 – Expression Confined to Bile Ducts and HCC Pseudoglandules

Normal or cirrhotic hepatocytes were not stained for claudin-4, while bile ducts revealed strong membranous expression. The majority of HCCs did not show any staining, whereas in 4/30 cases, tumor cells forming alveolar, glandule-like structures exhibited weak membrane staining mainly localized to the apical pole.

4.2.6 - The Effect of HCV Infection on Claudin-1 and -7

Claudin morphometry results were analyzed according to HCV infection status both in tumorous and non-tumorous groups. Mostly non-significant tendencies in immunopositive area percentages were revealed by morphometry. HCV positive non-tumorous samples showed slightly increased claudin-1 expression compared to HCV negative cases, but the differences did not reach statistical significance. Claudin-7 was significantly elevated in HCV infected non-cirrhotic livers compared to HCV-negative cases. Since there is no evidence of

HCV mediated modulation of claudin-7, so far, other factors, such as fibrosis – which was present in HCV-positive, but not in HCV-negative non-cirrhotic cases – might be associated with the observed elevation.

4.2.7 - Western Blot Analysis of Claudin Expression

Western blot analysis on representative samples confirmed the staining specificity and data obtained by immunohistochemistry. The size of the detected claudins were accurate, 21, 23, 24, and 23 kDa for claudin-1, -2, -3, and -7, respectively, while claudin-4 was practically not detectable. Claudin-1 and claudin-7 expressions were elevated in cirrhosis compared with normal liver, and strong expression of claudin-1 was also detected in HCC. Claudin-2 signal was the weakest in the cHCC sample. Bands corresponding to claudin-3 were uniform across samples.

4.2.8 - Relationship between claudin expression and HCC grade and patient survival

When studying the possible association between claudin expression and HCC grade, no significant correlation was found. Nevertheless, claudin-1 showed a not significant, yet increasing expression with higher tumor grades.

From the thirty patients with resected HCC, 25 died by the end of the study (83%). The median overall survival (OS) for all patients was 33 months (95% CI 18 – 48 months). Patients were divided into “claudin high” and “claudin low” subgroups based on the median claudin expression values. Survival analysis failed to reveal any significant association between HCC claudin expression and patient survival.

5 - CONCLUSIONS

1. Our study was the first to evaluate miRNA profiles of pretreatment, diagnostic fine needle aspiration samples of sorafenib-treated HCC patients.
2. Pretreatment high miR-214 levels associate with smaller tumor size, while pretreatment high miR-17-5p levels associate with better ECOG performance status of HCC patients.
3. Our study was the first to identify miR-224 as a putative predictive factor for sorafenib responsiveness of advanced stage HCC patients: high pretreatment miR-224 expression associates with significantly longer overall and progression-free survival.
4. Liver cirrhosis associates with elevated claudin-1 and claudin-7 expressions when compared with non-cirrhotic livers. This enhancing effect of cirrhosis also appears in HCCs developed on cirrhotic background.
5. HCCs show decreased claudin-2 expression compared to non-tumorous liver parenchyma, while claudin-4 expression is confined to bile ducts and HCC pseudoglandules.
6. We found no significant correlation between the entry co-factor of HCV, claudin-1, and the HCV status of the investigated samples. On the other hand, the enhanced claudin-1 expression observed in cirrhosis might contribute to a more effective HCV entry, thus boosting re-infection and chronicity and therefore, it might also contribute to malignant transformation.
7. We did not observe significant correlation between claudin expression and HCC grade or patient survival, while claudin-1 levels show an increasing tendency with higher tumor grades.

6 - LIST OF PUBLICATIONS

Publications related to the Dissertation:

Gyöngyösi B, Végh É, Járay B, Székely E, Fassan M, Bodoky G, Schaff Z, Kiss A. (2014) Pretreatment microRNA level and outcome of sorafenib-treated hepatocellular carcinoma. *Journal of Histochemistry & Cytochemistry* 62:(8) pp. 547-555.

Holczbauer Á*, **Gyöngyösi B***, Lotz G, Törzsök P, Kaposi-Novák P, Szijártó A, Tátrai P, Kupcsulik P, Schaff Z, Kiss A. (2014) Increased expression of claudin-1 and claudin-7 in liver cirrhosis and hepatocellular carcinoma. *Pathology & Oncology Research* 20:(3) pp. 493-502. *equally contributed

Publications not related to the Dissertation:

Bukong TN, Iracheta-Vellve A, Saha B, Ambade A, Satishchandran A, **Gyongyosi B**, Lowe P, Catalano D, Kodys K, Szabo G. (2016) Inhibition of Spleen Tyrosine Kinase activation ameliorates inflammation, cell death, and steatosis in alcoholic liver disease. *Hepatology* 64:(4) pp. 1057-1071.

Bukong TN, Iracheta-Vellve A, **Gyongyosi B**, Ambade A, Catalano D, Kodys K, Szabo G. (2016) Therapeutic benefits of Spleen Tyrosine Kinase inhibitor administration on binge drinking-induced alcoholic liver injury, steatosis, and inflammation in mice. *Alcoholism Clinical and Experimental Research* 40:(7) pp. 1524-1530.

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