Gene Expression of Hepatocellular Carcinoma Reveals Glypican-3 Role as Potential Biomarker

Received for publication, May 8, 2014
Accepted, July 2, 2014

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Abstract
Aim: To assess gene expression of glypican-3 in patients with hepatocellular carcinoma and in HUH7 cell lines treated with doxorubicin and to evaluate its role as early potential biomarker.

Material and methods: Tissue samples from 30 patients (liver transplant, n=18, liver resection, n=12) with hepatocellular carcinoma were evaluated by qPCR along with HUH7 cell lines culture treated with doxorubicin.

Results: Statistically significant correlations were found between GPC3 mRNA level and tumor size (R=0.505, p=0.004). In the liver resection group alone we found a statistically significant correlation between GPC3 mRNA and survival months (R=0.629, p=0.028).

Differences in GPC3 mRNA expression were depicted in patients group related to type of surgical intervention, hepatitis infection, Milan criteria, type of transplant and relapse. The cell lines treated with doxorubicin changed their morphology and after 24h incubation they were damaged or dead. By qPCR GPC3 mRNA level decreases directly proportional with the increased doses of doxorubicin.

Discussions: HCC uniformly over-expressed GPC3 that might demonstrate its role as a diagnostic biomarker. Diminishing GPC3 mRNA level in cell lines treated with doxorubicin could be indicative for the interference of the drug with cancer cell growth, proliferation and apoptosis, and could demonstrate GPC3 role as a prognostic biomarker.

Keywords: hepatocellular carcinoma; liver transplantation; HUH-7; glypic-an-3; doxorubicin

1. Introduction
Hepatocellular carcinoma (HCC) is a primary malignancy of the hepatocyte being the 5th most common cancer and the third cause of cancer-related death worldwide (1). About 80% of HCCs develops on underlying liver cirrhosis (2, 3). HCC is often diagnosed at an advanced stage when most potentially curative therapies are of limited effectiveness. Treatment with chemotherapeutic drugs is one of the most frequent strategies in HCC patients, especially in those with unresectable tumors.

Doxorubicin (adriamycin, DOX) is anthracycline which is known to interact with DNA by intercalation and inhibition of macromolecular biosynthesis is an anti-neoplastic chemotherapeutic drug used in treating advanced HCC (4). It is a cytotoxic agent commonly used
in chemotherapeutic regimens which makes it ideal to estimate the response to systemic therapies to HCC (5) and therefore was selected as a model treatment in our study.

In cells that are exposed to doxorubicin, DNA damage response, epigenome and transcriptome are deregulated. As a consequence inhibition of cell proliferation occurs which leads to cell lysis (cytotoxicity) in cancer cells (6).

Recently, in humans, glypicans received a tremendous attention due to their expression in various amounts and degrees depending on the tissue, and on different stages of development (7). Mutations in glypican genes are correlated with phenotypes that show a deregulation of cellular growth and morphogenesis. In the process of development glypicans are involved either as controllers or effectors (8). From glypicans family, glypican-3 is a cell surface heparan sulfate proteoglycan that may play a role in the control of cell division and growth regulation.

Alteration of glypican-3 (GPC3) expression is associated with several malignancies and has been identified as highly expressed in tissues, serum samples of HCC patients (9, 10, 11, 12) and HCC cell lines like Huh7, Huh6 and HepG2 (13). Several studies investigated glypican-3 protein encoded by the GPC3 gene which is used as a diagnostic marker which distinguishes small hepatocellular carcinomas from cirrhosis, dysplastic nodules, and focal nodular hyperplasia–like nodules (14, 15, 16).

The aim of our study was to explore the potential diagnostic and prognostic value of glypican-3/GPC3 in HCC patients who underwent liver transplantation or liver resection by qPCR and to determine correlation of its gene expression levels with clinicopathological parameters of the patients. Also we investigated variation of GPC3 gene expression in HUH-7 exposed to a range of doses of doxorubicin.

2. Material and methods

Patients and samples collection: In our study we included 30 patients surgically treated for HCC between 2004 and 2012 at the Center of Digestive Diseases and Liver Transplantation in Fundeni Clinical Institute. 12 patients underwent liver resection (LR) and 18 underwent liver transplantation (LT). Patients were followed up for a median period of 18.35 months (range 0.57, 38.46 months). At the enrollment in the study, which was approved by the Ethics Committee of Fundeni Clinical Institute, all patients signed a written informed consent for the use of their samples. Tumoral and non-tumoral liver tissue samples were collected from each patient during surgery for the transcriptomic analysis. All samples used within this study were considered operatory waste, collected in RNA Later (Sigma, St. Louis, MO) and processed according to manufacturer protocol.

Cell culture: The human differentiated hepatocyte derived cellular carcinoma cell line HUH7 was cultivated in Dulbeco modified Eagle medium (Gibco, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cells were seeded at a concentration of 3.5 x 10^5 cells/ml in a 6 wells plate and incubated at 37°C under a humidified atmosphere with 5% CO2. After 24h when cells reached 90% confluence these were treated with 0.0025 µg/µl, 0.005 µg/µl, 0.01 µg/µl, 0.02 µg/µl and 0.03 µg/µl doses of doxorubicin (Actavis, UK).

RNA isolation: Total RNA was prepared from frozen tumoral and non-tumoral tissue samples and from cell lines after 24h of incubation with doxorubicin using Trireagent (Sigma, St. Louis, MO) according to manufacturer’s instructions. The quantity and quality of the total RNA were assessed by spectrophotometry with Nano Drop 1000 (Thermo Scientific, Arlington, TX) and by using lab-on-a-chip Agilent 2100 technology (Agilent Technology,
cDNA synthesis and Quantitative PCR (qPCR): First cDNA was obtained from 2 μg of total RNA using High Capacity cDNA Archive Kit (ABI, Foster City, CA) in a total volume of 20μl. The final dilution of the samples was 2 ng/μl. Two-step relative quantification was performed on 7300 Real time PCR (ABI, Foster City, CA) using hydrolysis probes. Then qPCR amplification was carried out in triplicate for each sample in a total volume of 25 μl at the following conditions: 95°C for 10 min, 95°C for 15 sec and 1 min at 60°C for 40 cycles. The level of each mRNA was normalized to reference gene hu18S (20x). We determined fold changes within tumoral tissues compared with paired non-tumoral tissue. Data were analyzed with SDS 1.4 software using comparative Ct method \[2^{(-\delta\delta Ct)}\]. The tested gene was glypican-3 (GPC3, Hs00170471_m1).

Apoptosis: Apoptotic cells were assessed with Tali® Apoptosis kit (Life Technologies) using the Tali® Image Cytometer (Life Technologies). Briefly apoptotic cells were stained with green annexin V – Alexa Fluor® 488 while dead cells were stained with propidium iodide. 25 μl of cells were loaded into specific slides and run on the Tali cytometer.

Statistical analysis: All statistical analyses were performed using SPSS (Statistical Packages for Social Sciences, Chicago, IL, USA) software version 20.0 and Graph Pad Prism 5.0 (GraphPad Software Inc, San Diego, CA). The comparisons between groups were performed using nonparametric tests (Mann–Whitney U-test). Differences were considered significant if p-value was <0.05.

3. Results and Discussions

Expression of GPC3 mRNA and correlation with clinicopathological parameters. Tissue (matched non-tumoral and tumoral tissue pair) from a total of 30 patients were included in the study. In table 1 are shown the clinicopathological data of the patients in resection (LR) and liver transplant (LT) groups.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>(LT+LR) group N=30</th>
<th>LT group N=18</th>
<th>LR group N=12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>55.5 (27-75)</td>
<td>55 (27-63)</td>
<td>61.5 (48-75)</td>
</tr>
<tr>
<td>≤60 years</td>
<td>21 (70)</td>
<td>17 (94.4)</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td>&gt;60 years</td>
<td>9 (30)</td>
<td>1 (5.6)</td>
<td>8 (66.7)</td>
</tr>
<tr>
<td>Gender, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>23 (76.7)</td>
<td>15 (83.3)</td>
<td>8 (66.7)</td>
</tr>
<tr>
<td>Female</td>
<td>7 (23.3)</td>
<td>3 (16.7)</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td>Tumor size, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤2 cm</td>
<td>3 (10)</td>
<td>2 (11.1)</td>
<td>1 (8.3)</td>
</tr>
<tr>
<td>&gt;2, ≤5 cm</td>
<td>17 (56.7)</td>
<td>11 (61.1)</td>
<td>6 (50)</td>
</tr>
<tr>
<td>≥5 cm</td>
<td>10 (33.3)</td>
<td>5 (27.8)</td>
<td>5 (41.7)</td>
</tr>
<tr>
<td>Tumor grading, n (%)</td>
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<td></td>
</tr>
<tr>
<td>E/S = I / II</td>
<td>3 (10)</td>
<td>1 (5.6)</td>
<td>2 (16.7)</td>
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<tr>
<td>E/S = II / III</td>
<td>27 (90)</td>
<td>17 (94.4)</td>
<td>10 (83.3)</td>
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<td>Viral infection, n (%)</td>
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<tr>
<td>HVB</td>
<td>16 (53.3)</td>
<td>12 (66.7)</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td>HCV</td>
<td>13 (43.3)</td>
<td>6 (33.3)</td>
<td>7 (58.3)</td>
</tr>
<tr>
<td>No infection</td>
<td>1 (3.4)</td>
<td>0 (0)</td>
<td>1 (8.4)</td>
</tr>
</tbody>
</table>

HCV-hepatitis C virus; HBV-hepatitis B virus

In the group of 30 patients surgically treated for HCC GPC3 mRNA expression was statistically significant correlated with tumor size (R=0.505, p=0.004). A statistical correlation...
was found in LT group alone between GPC3 mRNA expression and tumor size ($R=0.493$, $p=0.038$).

We observed that the median relative quantification (RQ) of GPC3 is bigger in LR group compared to LT group (22.7 vs 2.3) (Fig. 1A). Also GPC3 median RQ was higher in HCV related HCC than in HBV related HCC (37.4 vs 2.0) (Fig. 1B).

Figure 1. Box plots of GPC3 mRNA level in A- transplant versus resection group; B- HBV versus HCV related group; C- Milan versus non-Milan group; D- living transplant versus whole transplant group; E- no relapse versus relapse group

Although there was no significant correlation between GPC3 mRNA expression and Milan criteria in LT group we did observed that in Milan group the median level of GPC3
mRNA level was 2.3 compared with mRNA level in non-Milan group of 65.0 (Fig. 1C). Furthermore there was a difference between the group of patients receiving whole transplant (median mRNA GPC3 level of 7.1) compared with the group of patients with living transplant (median mRNA GPC3 level of 0.7) (Fig. 1D). Also there was a difference between the group of patients with relapse (median mRNA GPC3 level of 65.0) and the group of patients without relapse (median mRNA GPC3 level of 2.3) (Fig. 1E).

In the LR group alone we found a statistically significant correlation between GPC3 mRNA and survival months (R=0.629, p=0.028). No statistically significant correlation was found with Alpha-fetoprotein (AFP) serum level.

**Assessment of GPC3 in HUH7 cell lines after doxorubicin treatment.** After an incubation period of 24h with doxorubicin, cells were evaluated by an inverted microscope (Fig. 2). We observed that the cells markedly changed their morphology along with the increase dose of doxorubicin. Compared with control (without drug) after 24h of incubation the treated cells were damaged and dead. These cells contained multiple nucleoli, an increased number of vacuoles and cytoplasmatic granules. At higher doses of doxorubicin the cells formed packed multilayer islands. Also there was a directly proportional ratio between dead cells and doxorubicin concentration and an inversely proportional ratio between apoptotic cells and doxorubicin dose. We observed that the cell diameter registered by Tali Image cytometer diminished from 12 µm for the un-treated cells to 3 µm for the treated cells.

![Figure 2. HUH7 cells treated with various concentrations of doxorubicin as follows: A – Control; B – 0.0025 µg/µl; C – 0.005 µg/µl; D – 0.01 µg/µl; E – 0.02 µg/µl; F – 0.03 µg/µl (20x objective)](image)

GPC3 mRNA was assessed in HUH7 cell line after 24h incubation with doxorubicin. Compared with control (no drug treatment) there was a decrease of GPC3 gene expression along with the increased doxorubicin dose except for 0.0025 µg/µl concentration where we observed a slightly higher GPC3 expression compared with the control (Fig. 3). We can presume that the diminishing GPC3 mRNA level in cell lines treated with doxorubicin could be indicative for the interference of the drug with cancer cell growth and proliferation and could demonstrate GPC3 role as a prognostic biomarker.
4. Discussions

Hepatocellular carcinoma (HCC) is a multi-factorial disease characterized by a poor prognostic and outcome due to late detection. Currently the standard follow up of the patients includes abdominal ultrasounds and serum alpha fetoprotein (AFP) detection, the only serum marker associated with HCC. Unfortunately this strategy is not reliable enough to detect the disease in its early stages (17).

Taking into account that AFP has a low sensitivity and specificity the research orientated towards novel biomarkers identification related to HCC is important.

GPC3 a member of the glypican family of glycosylphosphatidylinositol-anchored cell-surface heparan sulfate proteoglycans has been found to be significantly involved in the onset and development of HCC (18) by regulating cell growth (19) through stimulation of the canonical Wnt signaling pathway (20) and thus making it suitable for use as a novel molecular marker in routine practice for improving the early diagnosis of HCC (21).

Moreover in view of its involvement in multiple signaling pathways it can be considered for targeted therapy in hepatocarcinogenesis (22).

In a previous study we showed that from a group of 30 LT patients, 80% showed positive IHC staining for GPC3 and there was a positive correlation with malignancy grade (23). Moreover in the same study a strong GPC3 protein expression was correlated with poorer prognosis.

Some of the studies regarding the potential value of serum GPC3 show the added value of GPC3 evaluation complementary to AFP in HCC patients (24, 25, 26) while soluble GPC3 (sGPC3), the NH2-terminal portion of GPC3 has higher sensitivity than AFP in the of detecting well or moderately differentiated HCC (27).

In the present study evaluation of GPC3 by qPCR in HCC tissues and in a human differentiated hepatocyte derived cellular carcinoma cell line (HUH-7) revealed that GPC3 could have a diagnostic and prognostic value in investigated groups of HCC.
In all analyzed tumor samples mRNA GPC3 was over expressed compared with matched normal tissues. We found statistically significant correlation between tumor size and GPC3 mRNA level assessed by qPCR.

In LR group we found a statistically significant correlation with survival months. Further a marked increase of median GPC3 mRNA was found in liver resection, HCV related, non-Milan and whole transplant patients. In our study and in accordance with Y.L. Wang et al. (28) patients with GPC3 overexpression had a shorter recurrence-free survival than those with lower GPC3 expression.

Recently both immunostaining and/or detection of gene expression profile of GPC3 are recommended by the International Consensus Panel for differentiating high grade dysplastic nodules from early HCC (29) and endorse the use of GPC3 as early biomarker in HCC.

Further in HUH7 cell lines after doxorubicin monotherapy we observed a change in cell normal morphology and we found a progressively decrease of mRNA GPC3 level with the increased dose of doxorubicin. Compared with control there was a 25.8% decrease of GPC3 gene expression at a doxorubicin concentration of 0.005 µg/µl, a 52.1% at a concentration of 0.01 µg/µl, 58.9% at a concentration of 0.02 µg/µl and a 60.1% at a concentration of 0.03 µg/µl.

Doxorubicin is the most frequently used single chemotherapeutic agent in transcatheter arterial chemoembolization (TACE) usually given either as palliative treatment in end stage un-operable HCC patients or as a “bridging” modality before liver transplantation (30).

5. Conclusions
Taking into account our present results that showed a statistically significant correlation between GPC3 mRNA level and tumor size, we could speculate that a relevant downsizing of tumors size achieved by TACE with doxorubicin could be directly proportional to the decrease of GPC3 gene expression.

In view of our results and with further validation we can conclude that GPC3 could be used to follow up HCC patients after loco-regional therapy advancing it as a potential diagnostic and prognostic marker for HCC.

Acknowledgements: This study was financially supported from project PNII-PT-PCCA 90/2012, 125/2011 (PN-II-ID-PCE- 2011-3-0605) and project PD 23/ 2011 (PN-II-RU-PD-2011-3-0137).

References
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**Conflict of interest:**
The authors report no conflict of interest.