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Differential expression of anterior gradient protein 3 in intrahepatic cholangiocarcinoma and hepatocellular carcinoma

Veronika Brychtova [a], Vita Zampachova [b], Roman Hrstka [a], Pavel Fabian [c], Jiri Novak [d], Marketa Hermanova [b], Borivoj Vojtesek [a,*]

[a] Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Zluty kopec 7, 65653 Brno, Czech Republic
[b] First Department of Pathological Anatomy, Medical Faculty of Masaryk University and St. Anne's University Hospital, Pekarska 53, 65691 Brno, Czech Republic
[c] Department of Pathology, Masaryk Memorial Cancer Institute, Zluty kopec 7, 65653 Brno, Czech Republic
[d] Department of Comprehensive Oncology Care, Masaryk Memorial Cancer Institute, Zluty kopec 7, 65653 Brno, Czech Republic

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ABSTRACT

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver cancer next to hepatocellular carcinoma (HCC). Despite the significant difference of the therapeutic strategy for both diseases, their histological appearance may be very similar. Thus the correct diagnosis is crucial for treatment choice but is often difficult to achieve. The aim of our study was to evaluate anterior gradient 3 (AGR3) as a new diagnostic marker helping to distinguish between ICC and HCC. AGR3 is a putative transmembrane protein implicated in breast, prostate and ovary tumorigenesis and belongs to the family of protein disulfide isomerases.

Since there is little information on how AGR3 is expressed in normal and diseased tissues and what its exact function is, we analyzed its expression pattern in normal liver and tumor tissue of ICC and HCC. The immunohistochemical analysis in normal tissue revealed specific AGR3 expression in intrahepatic bile duct cholangiocytes which was not present in liver hepatocytes. Consequently we analyzed AGR3 expression in 74 representative samples of puncture biopsies, tissue excisions and resection specimens from which 48 samples were diagnosed as HCC and 26 as ICC. Our results showed AGR3 expression negative and weakly positive respectively in hepatocellular carcinomas compared to stronger AGR3 positivity in cholangiocellular carcinomas. AGR3 expression statistically significantly correlated to acid mucopolysaccharide expression and negatively correlated to glypican-3 expression. We conclude that according to receiver operating characteristics (ROC) analysis AGR3 expression is relatively specific for ICC and is potentially linked to mucosecretion, which may indicate potential implication in treatment resistance.

Introduction

Malignant liver tumors are frequent malignancies worldwide. In Europe and North America, viral hepatitis C and in Asia and Africa viral hepatitis B are major risk factors. Both infectious illnesses result in their chronic stage in cirrhosis which is present in more than half of hepatocellular carcinoma patients (El-Serag and Rudolph, 2007; Jemal et al., 2011). The majority of primary adult liver tumors are classified as either hepatocellular carcinoma (HCC) or intrahepatic cholangiocarcinoma (ICC). There also exists a rare subtype of primary liver cancer designated as combined hepatocellular and cholangiocarcinoma (cHCC-CC) (Bosman et al., 2010).

The therapeutic strategy and the prognosis for HCC and ICC are significantly different and therefore the distinction between these two diagnoses is essential. Common immunohistochemical markers used for differential diagnostics favoring HCC include hepatocyte paraffin antigen-1 (Hep Par 1), carcinoembryogenic antigen (CEA), CD10, alpha-fetoprotein (AFP), β-catenin, Hsp70, glutamine synthetase and glypican-3 (GPC-3) (Fatima et al., 2011; Kakar et al., 2007; Tremosini et al., 2012). However, each of these markers has limitations given by suboptimal sensitivity or difficulty of interpretation at certain conditions. Particularly nonstandard conditions arise in poorly differentiated tissue, atypical cases or in needle core biopsies where available tissue is limited and the positive expression of markers is commonly only focal.

During our work in analyzing the expression of anterior gradient 3 (AGR3), we found that it shows a restricted expression pattern in the liver, being expressed by intrahepatic bile duct cholangiocytes but not hepatocytes. We thus tested AGR3 as a potential new marker of ICC applicable in differential diagnostics.

Abbreviations: ICC, intrahepatic cholangiocarcinoma; HCC, hepatocellular carcinoma; AGR3, anterior gradient 3; AGR2, anterior gradient 2; GPC-3, glypican-3; ROC, receiver operating characteristics; AUC, area under curve.

* Corresponding author. Fax: +420 543211169.
E-mail address: vojtesek@mou.cz (B. Vojtesek).

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AGR3 is also known as HAG-3 or BCMP11 and it is a homologue of anterior gradient 2 (AGR2) protein. AGR2 is an endoplasmic reticulum chaperone that participates in mucin production in intestine and asthmatic lung (Park et al., 2009; Schroeder et al., 2012) and has been associated to different cancers. Elevated AGR2 expression has been detected especially in cancers derived from epithelial cells including breast, prostate, ovarian, lung, esophageal and pancreatic cancer and in some cases was shown to be hormone dependent (Brychtova et al., 2011; Hrstka et al., 2010; Zhang et al., 2005). AGR2 is specifically expressed in tall epithelial cells of normal liver and liver cancer where it potentially promotes secretory functions of mucins (Lepreux et al., 2011). AGR2 overexpression was found in majority of fibrolamellar carcinomas but only exceptionally in conventional hepatocellular carcinomas (Vivekanandan et al., 2009).

There are only limited data on AGR3 expression in normal and cancer tissue. Originally it has been identified as a membrane bound protein in breast cancer cell lines (Adam et al., 2003) and together with AGR2 the expression has been associated with estrogen receptor positive breast cancer (Fletcher et al., 2003). Recent data on ovarian cancer has shown that AGR3 expression can be also independent of estrogen signaling and is coupled to mucinous type of ovarian cancer (Gray et al., 2012).

In this study we aimed to examine AGR3 expression in healthy liver and hepatic cancer. We focused on the analysis of AGR3 expression pattern in ICC and HCC as well as mucosecretion and verified the possibility to use AGR3 as a diagnostic marker for distinction between liver tumor types compared to GPC-3.

Materials and methods

Study group and tissue specimens

The study group consisted of 74 patients: 26 were diagnosed with ICC and 48 patients with HCC. All patients underwent surgical procedure at the Faculty Hospital Brno, at the Masaryk Memorial Cancer Institute, or at St. Anne's University Hospital from 2005 to 2011. The cohort consisted of 52 males (70.3%) and 22 females (29.7%). Patient age at the time of diagnosis was within the range of 17–81 years (median 65 years). Tissue material included both diagnostic biopsies and surgical specimens. Tissues were fixed in 10% neutral buffered formalin for 24 h, routinely processed and then embedded in paraffin wax. Informed consent has been obtained from all patients involved in this study. The data used were anonymized and they were handled according to Czech Republic existing legislation.

Immunohistochemistry

Immunohistochemical staining was performed on 4 μm thick freshly cut tissue sections and the optimal antibody concentration and retrieval were set separately for each antibody used. Sections were deparaffinized in xylene and rehydrated into PBS through a graded ethanol series. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide in PBS for 15 min. Antigen retrieval was performed in citrate buffer pH 6 at 94 °C for 20 min. For AGR3 immunodetection, the sections were incubated overnight at 4 °C with mouse monoclonal antibody to AGR3 (clone 1.2, in house; Gray et al., 2012) and for GPC-3 immunodetection, the sections were incubated overnight at 4 °C with mouse monoclonal antibody to GPC-3 (clone 1G12, Cell Marque, CA, USA). A streptavidin-biotin peroxidase detection system was used according to the manufacturers' instructions (Vectastain Ellite ABC Kit, Vector Laboratories, Burlingame, CA, USA). Signal was visualized by 3,3′-diaminobenzidine (Liquid DAB + Substrate Chromogen System, Dako, Glostrup, Denmark). Nuclear counterstaining was performed with Gill's hematoxylin.

Histochemistry

Neutral and acid polysaccharides were detected by PAS-Alcian Blue. Briefly, histochemical staining was performed on 4 μm thick freshly cut tissue sections that were deparaffinized in xylene and rehydrated into distilled water through a graded ethanol series. The sections were then stained with Alcian blue in acetic acid at pH 2.5, washed with distilled water, treated with 0.6% periodic acid, washed with distilled water and stained with Shiff's reagent. Nuclear counterstaining was performed with Gill's hematoxylin.

Evaluation of immunostaining and PAS-Alcian Blue staining

For immunohistochemical evaluation, 5 conventional categories according to the number of positive cells were assessed: 0 — negative (less than 1% of positive cells); 1 — border (1–5% of positive cells); 2 — weakly positive (5–25% of positive cells); 3 — moderately positive (25–50% of positive cells) and 4 — strongly positive (more than 50% of positive cells). For PAS-Alcian Blue staining evaluation, two categories were considered: 0 — negative (less than 1% of positive cells) and 1 — positive (more than 1% of positive cells).

Statistical analysis

All statistical analyses were performed using STATISTICA Version 10 (StatSoft, Inc., Tulsa, OK, USA). The associations of AGR3 and acid mucopolysaccharides expression with selected clinico-pathological features were analyzed using Pearson's chi-squared test. Differences at $p \leq 0.05$ were considered to be statistically significant.

Results

The study group comprised 26 patients diagnosed with ICC and 48 with HCC. Regarding histologic grade, the cohort encompassed 5 (6.7%) G1 tumors, 39 (52.0%) G2 tumors, 15 (20.0%) G3 tumors and 16 cases (21.3%) with unknown grade (mostly comprising the needle biopsies due to their limited size). According to the tumor size, the study group included 7 (9.3%) cases falling into T1 group, 13 (17.3%) cases of T2 group, 21 (28.0%) cases of T3 group, 4 (5.4%) cases of T4 group and the rest 30 (40.0%) cases were of unknown T status (encompassing some of the diagnostic biopsies without further available pathological or clinical data). Considering nodal involvement, 31 (41.3%) patients did not have any affected lymph nodes, 11 (14.7%) had lymph node metastases and in 33 (44.0%) patients the data were absent. Distant metastases were not detected in 37 (49.3%) patients while 16 (21.4%) patients presented distant metastases and in 22 (29.3%) patients the data describing distant metastasis involvement were unavailable.

From demographical factors, only sex showed significant role in HCC incidence ($p = 0.00152$; Pearson's chi-squared test). The distribution of hepatocellular carcinoma between males and females was uneven with males suffering more often from hepatocellular carcinoma than females. There was no similar association in ICC. Neither tumor extent nor nodal and distant metastasis involvement showed any significant association with clinicopathological characteristics.

Immunohistochemical analysis showed the expression of AGR3 protein in tissue samples of healthy liver and liver neoplasm. In normal healthy liver tissue AGR3 was expressed in cholangiocytes, while hepatocytes were AGR3 negative (Fig. 1).

During evaluation of AGR3 expression in ICC and HCC distinct disparity between the two diagnoses was found. Immunohistochemical analysis showed that high AGR3 expression is a characteristic feature of ICC ($p < 0.00001$; Pearson's chi-squared test) (Fig. 2; Table 1). AGR3 expression was more homogenous in cholangiocellular rather than hepatocellular carcinoma, but rarely there were observed regions with different intensity. There were two negative samples which constituted