E-cadherin and aquaporin 1 co-expression analysis in hepatocellular carcinoma: a pilot study

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Abstract
Hepatocellular carcinoma (HCC) is the main primary liver malignancy, being associated with both health and economic burden worldwide. Recently, novel molecular markers and possible therapeutic targets were identified. Different adhesion molecules, as well as possible angiogenesis-associated targets can be prime candidates when investigating novel therapies. Considering these premises, our goal was to study the co-existence of E-cadherin and aquaporin 1 (AQP1) in a series of HCC diagnosed patients. Utilizing archived tissue fragments from 17 patients diagnosed with well-to-moderate and poorly differentiated HCC, as well as four samples of normal liver tissue and using a highly specific biotin-free tyramide amplification technique, we have assessed here the expression of E-cadherin and AQP1 during HCC carcinogenesis. Moreover, as we have observed that some of the AQP1 expression seems membrane-bound, we have sought to evaluate their co-localization. Our data showed, as expected, that E-cadherin decreases from control tissue to low-grade and respectively, high-grade HCC. AQP1 was expressed, also as already known, at the level of endothelial blood vessels and bile ducts epithelia, however, we have showed here for the first time that this water pore is also expressed in the cytoplasm and membranes of hepatocytes, both in control and HCC tissue. Moreover, AQP1 expression parallels the decrease of E-cadherin expression during carcinogenesis, but together with this downregulation, we have also found a spatial decrease in the colocalization of the two proteins. Altogether, utilizing a biotin-free tyramide signal amplification technique, this study shows for the first time that AQP1 is expressed at the level of liver epithelia, in both control and HCC tissue.

Keywords: hepatocellular carcinoma, E-cadherin, aquaporin 1, histopathology.

Introduction
Hepatocellular carcinoma (HCC) can be considered the most prevalent primary liver cancer worldwide [1]. Digestive cancers generally represent an important public health issue because of the high rate of mortality [2]. According to GLOBOCAN, liver cancer was the sixth most frequently diagnosed cancer worldwide in 2018. Annually, there are approximately 840 000 new cases and 780 000 deaths due to HCC, placing it on the fourth place for cancer death. In most regions of the world males are two to three times more affected than women. Moreover, liver cancer is considered as the second leading cause of cancer-related death for men. Hepatitis B virus (HBV) and hepatitis C virus (HCV) chronic infection are the most important risk factors for HCC, and advanced cirrhosis significantly increases this correlation. By taking into consideration recent developments in HCV treatment regimes, an important number of deaths caused by HCC could be avoided [3]. The use of direct-acting antivirals (DAAs) was an important part of HCV eradication, which led to significantly decrease of the risk of liver cancer [4]. In addition, the success of HCV treatment showed a substantial role on liver cirrhosis prevention and on the incidence of HCC [5]. On the other hand, widespread hepatitis B vaccination brings hope for a diminution of related HCCs [1]. Metabolic syndrome, type II diabetes, obesity, [6, 7], non-alcoholic metabolic syndrome (non-alcoholic fatty liver disease – NAFLD) [1] and alcohol consumption [8] represent important risk factors for HCC [9].

E-cadherin is a characteristic marker of epithelial–mesenchymal transition involved in embryogenesis and adult tissue homeostasis [10]. E-cadherin, α-catenin and β-catenin are strongly expressed in non-cancerous epithelial tissue. In recent studies, this observation was compared with the decreased expression in malignant tissues [11]. Intracellular catenins may determine the prevalence of metastasis and tumor growth [12]. Most of the human malignant tumors originate from epithelial cells. To invade adjacent tissues, carcinoma cells must lose their intercellular adhesion and hence allow cell migration. Moreover, loss of E-cadherin expression was demonstrated at early stages,
The sections were deparaffinized, rehydrated in decreasing alcohol series, processed for antigen retrieval by micro-waving in 0.1 M citrate buffer pH 6 for 20 minutes, incubated in 1% hydrogen peroxide in distilled water for 30 minutes to block the endogenous peroxidase activity, and kept for another 30 minutes in 3% skimmed milk in phosphate-buffered saline (PBS) for blocking unspecific antigen sites. For enzymatic single immunohistochemistry, the primary antibodies were incubated on the slides at 4°C for 18 hours, and the next day the signal was amplified for 60 minutes utilizing a species-specific peroxidase polymer-based system adsorbed for human immunoglobulins (Nichirei Bioscience, Tokyo, Japan). The signal was then detected with 3,3’-Diaminobenzidine (DAB) (Nichirei Bioscience) and the slides were coveredslipped in DPX (Sigma-Aldrich, St. Louis, MO, USA) after a hematoxylin counterstaining. Negative controls were obtained by omitting the primary antibodies, and intrinsic staining patterns in normal liver tissue were utilized as positive controls.

For fluorescence double immunohistochemistry, the slides were processed as above, incubated simultaneously overnight with both primary antibodies, and the second day they were further incubated with a mix of anti-mouse peroxidase polymer-based system (Nichirei Bioscience) and anti-rabbit Alexa Fluor 596 secondary antibodies (Thermo Fisher Scientific, Waltham, MA USA; 1:300, two hours at room temperature). AQPI signal was further amplified and detected with an Alexa 488–tyramide precipitation step (Thermo Scientific, 1:200, 10 minutes). In all cases, the slides were counterstained with 4',6-Diamidino-2-phenyl-indole (DAPI) (Thermo Scientific, 1:200, 10 minutes). In all cases, the slides were counterstained with 4',6-Diamidino-2-phenyl-indole (DAPI) (Thermo Fisher Scientific) for 15 minutes, incubated for 20 seconds in a 0.3% Sudan Black (Sigma-Aldrich), alcoholic solution to reduce autofluorescence, washed in distilled water and coverslipped with a fluorescence anti-fading mounting medium (Vectashield, Vector Laboratories, Burlingame, CA, USA) [23].

Transmitted light microscopy and fluorescence imaging has been performed utilizing a Nikon 90i motorized microscope (Nikon Europe B.V., Amsterdam, The Netherlands) equipped with a high-resolution low noise 16 Mp DS-Ri Nikon complementary metal oxide semiconductor (CMOS) camera and a light-emitting diode (LED) fluorescence source. After confirming the staining on enzymatic immunohistochemistry, for semiquantitative analysis, images have been captured on fluorescence-stained slides, and images have been captured utilizing the 40× objective. Images were obtained by sequential scanning of each channel with specific pairs of highly selective custom-made filters to eliminate the crosstalk of the fluorophores and to ensure a reliable quantification for DAPI, Alexa 488, and Alexa 594 spectra (Chroma Technology Corp., Bellows Falls, VT, USA). All fluorescence image data has been processed for blind deconvolution utilizing Nikon’s deconvolution algorithms, at five iterations.

Image analysis and statistical assessment

As we have targeted only the tumor epithelium through this analysis, prior to any quantification, in all fluorescence image sets stroma, blood vessels and bile ducts have been manually removed and thus not considered any further. All fluorescent signals have been quantified as area, and then reported as percentage of total epithelium in the
E-cadherin and aquaporin 1 co-expression analysis in hepatocellular carcinoma: a pilot study

respective image utilizing the Image-Pro Plus AMS 7 image analysis software (Media Cybernetics, Bethesda, MD, USA). Percentages have been averaged for all images in each slide (patient), and then slides from patients with the same pathological grading.

Moreover, E-cadherin and AQP1 colocalization degrees have been calculated in Image-Pro Plus AMS 7 image analysis software, utilizing the colocalization between their respective fluorescence channels, and have been reported as overlapping coefficients.

Continuous numerical data obtained were exported and plotted in Microsoft Office Excel 2010 and were analyzed using the Statistical Package for the Social Sciences (SPSS) software (IBM SPSS Statistics, version 20.0). To assess statistical differences, we used the one-way analysis of variance (ANOVA) with least significant difference (LSD) post hoc analysis to compare the means of more than two groups. Correlations were assessed using the Pearson’s correlation coefficient. Data were reported as mean ± standard error of the mean (SEM), and in all cases, \( p < 0.05 \) was used to indicate statistical significance.

Results

Enzymatic IHC

On simple immunohistochemistry, E-cadherin showed, as expected, a distinct membrane staining of liver cells in both control tissue and HCC cases, with a large staining heterogeneity for different tumors, but with the overall tendency of decreasing signal intensity and area for tumors compared to control liver tissue (Figure 1, A–C). E-cadherin expression was restricted to most liver cells’ membranes, although usually the pattern was not completely continuous, probably due to morpho-functional differences between the vascular and biliary poles of the cells, and sometimes a faint sub-membranous staining pattern could be observed.

AQP1 was strongly expressed adjacent to the blood vessel endothelia, the membrane of red blood cells, biliary ducts in the portal spaces, the membrane of some mononucleate inflammatory cells, but also showed a diffuse, generalized, and mostly homogenous expression in the cytoplasm of control liver cells (Figure 1D). Only on occasion, hepatocytes with more intense staining in their cytoplasm could be identified on control liver tissue. In the tumor tissue, the vascular and biliary expression was conserved, however there was a much higher heterogeneity of staining of the hepatocytes, with patchy intracytoplasmic staining grouped mostly around the nucleus, with a granular staining in the rest of the cytoplasm, and on frequent occasions with a clear-cut membranous staining pattern (Figure 1, E and F). This staining was overall non-homogenous between neighboring cells.

Fluorescence IHC

The heterogeneity of the two markers was next followed more closely on double immunofluorescence staining, compared to control liver tissue. Although AQP1 was most of the times expressed in the cytoplasm, we could also clearly identify a membrane staining pattern that showed a clear-cut colocalization with the E-cadherin staining, in both control and HCC cases (Figures 2 and 3). AQP1 expression did not show a uniform expression for the cytoplasm of all tumor areas, and in some tumors, AQPI seemed to be massively reduced in the cytoplasm and increased at the level of the membranes. Thus, there were tumor regions with scant expression of AQPI, as well as tumors that would express AQPI with both a membrane and cytoplasmic patterns, but without any E-cadherin in these respective tumor areas.

AQPI severe loss in the cytoplasm also seemed to be accompanied by a decrease of E-cadherin signal, and a translocation in the cytoplasm, with a granular pattern (Figures 3, A–C). For most of the tumors, the cell areas with the highest AQPI content in their cytoplasm seemed

Figure 1 – Exemplary images (×400) from control liver and HCC tissue stained for E-cadherin (A–C) and AQP1 (D–F). E-cadherin is expressed at the level of the membranes in control tissue (A), and in HCC its expression is highly variable (B), with areas almost negative (C). AQP1 is expressed in the cytoplasm of the normal hepatocytes, bile ducts, blood vessels, inflammatory cells, and red blood cells (D), while in HCC the expression is patchy in the cytoplasm, mostly surrounding the nucleus and on occasion with a clear-cut membrane staining (E), while in some areas it is more uniform in the cytoplasm and without a clear-cut membrane staining (F). AQPI: Aquaporin 1; HCC: Hepatocellular carcinoma.
to be exhibiting a low co-expression with E-cadherin on their membranes, but interestingly, in the areas with strong membrane E-cadherin and low cytoplasmic AQP1, there seem to be a densification of AQP1 in and under the membrane, an observation suggested by evident membrane colocalization areas (Figures 3, D–F). In undifferentiated tumors, AQP1 was also showing a granular intra-cytoplasmic expression, with increasing heterogeneity as ranging from no expression at all to this granular pattern. Association of granulovacuolar degeneration in the areas of AQP1 cytoplasmic loss increased the granular–vesicular staining for AQP1, as if the water pore would be localized preferentially in the membranes of hydropic organelles.

Altogether, there were areas with almost no E-cadherin expression, and a perfect AQP1 membrane expression, and regions where both markers would share an almost perfect colocalization.

**Figure 2** – Exemplary fluorescence co-localization of AQP1 and E-cadherin (×400). On control tissue (A–C), AQP1 is present at the level of the cytoplasm, with evident membrane staining and colocalization with E-cadherin. On HCC, AQP1 is mostly present in the cytoplasm with much reduced colocalization with E-cadherin (D–F). AQP1: Aquaporin 1; HCC: Hepatocellular carcinoma.

**Figure 3** – Exemplary fluorescence variability of AQP1 and E-cadherin on HCC (×400). A high variability was recorded in the tumor areas, e.g., here maintained cytoplasmic AQP1 expression and decreased E-cadherin areas (A–C), and almost exclusively membranous AQP1 with a high degree of colocalization with E-cadherin (D–F). AQP1: Aquaporin 1; HCC: Hepatocellular carcinoma.

Next, we sought to compare the expression levels of E-cadherin and AQP1 in control liver compared to low-grade and high-grade tumors (Figure 4). As expected, E-cadherin expression area decreased from control tissue to low-grade and high-grade HCC. Although the difference was not statistically significant, a gradual decrease pattern was obvious from low-grade to poorly differentiated tumors (Figure 4A). So overall, E-cadherin was downregulated in all tumors, and high-grade tumors showed the lowest values, and importantly, with the lowest heterogeneity.
around the means for this group. AQP1 expression decreased slowly from control tissue to low-grade carcinoma. In fact, due to the heterogeneity of tumor expression, there was no statistically significant difference between control and overall low-grade tumors (Figure 4B). However, there was a drop-in reactivity for high-grade tumors compared to both control and low-grade carcinoma groups ($p<0.05$).

When we analyzed the overlap coefficients between the two signals, there was a high overlap between the two signal areas for control and low-grade cases, most probably due to the fraction of AQP1 that was still expressed on the membrane of the hepatocyte, and the concomitant drop in the expression of both markers (Figure 4C). Moreover, an important decrease of the overlap function was recorded for high-grade tumors, the differences being statistically significant between this group and both control and low-grade HCC ($p<0.05$).

![Figure 4 – Fluorescence signal areas and colocalization for E-cadherin/AQP1: (A) E-cadherin decreases from control liver to low-grade and high-grade carcinoma; (B) AQP1 expression area also decreases towards high-grade tumors, but signal area drop is not so drastically for low-grade tumors; (C) E-cadherin/AQP1 colocalization coefficients decrease drastically from low-grade to high-grade HCC. Significance is showed for a one-way ANOVA followed by a post hoc Fisher’s LSD test. Data are expressed as means ± SEM. ANOVA: Analysis of variance; AQP1: Aquaporin 1; HCC: Hepatocellular carcinoma; LSD: Least significant difference; SEM: Standard error of the mean.](image)

### Discussions

HCC, a primary liver malignancy, develops on a background of chronic liver disease, such as cirrhosis or chronic HBV or HCV infection. Liver cancer along with intrahepatic bile duct cancer is the sixth most commonly diagnosed form of cancer worldwide [24]. Primary liver cancer is the fourth leading cause of death, being the second most lethal tumor after pancreatic cancer [24].

Intercellular adhesion is an essential feature of epithelia, and loss of adhesion molecules has been documented as an important physiopathological event during tumorigenesis, less intercellular adhesion being linked with a more invasive tumor pattern. Cadherins are a large family of transmembrane or membrane-associated glycoproteins that regulate intercellular adhesion in epithelial tissues in a Ca²⁺-dependent manner [25]. When synthesized, the E-cadherin protein precursor contains a signal polypeptide of approximately 130 amino acid residues (AA) that acts as a signal sequence for uptaking in the endoplasmatic reticulum (ER), and the mature polypeptide of about 728 AA. The cleavage of the signal peptide occurs during processing and maturation of the protein in the ER and Golgi vesicles, before the mature protein is exposed to the cell surface, and cleavage is required in order for the mature protein to exhibit an adhesive function [26]. The mature E-cadherin contains a cytoplasmic domain and an extracellular tail comprising five tandemly repeated domains [25]. Four of these domains are so-called extracellular cadherin repeats, while the fifth is characterized by four conserved cysteine residues. In the cytoplasmic side, E-cadherin is bound to the actin cytoskeleton via the catenins, including α-catenin, β-catenin, γ-catenin and p120 [27]. Besides anchoring the cytoskeleton, E-cadherin/catenin complex has been described with an inhibitory effect on tumor growth and metastasis [28, 29]. Thus, the expression levels of E-cadherin and catenin have been showed to correlate with histopathology features, such as tumor size, grade, stage, invasion, metastasis, and prognosis in several cancers [30–34]. For HCC, many studies found a link between the expression of E-cadherin and histopathological and clinical features of HCC, but the results are not uniform. Thus, Endo et al. [35] revealed that E-cadherin expression was down-regulated and exhibited a significant positive correlation with increasing HCC grade. It has also been showed that a decrease of E-cadherin expression correlates with poor 1-, 3- and 5-year overall survival, increased metastasizing, vascular invasion, advanced differentiation grade and advanced tumor, node, metastasis (TNM) stage [36]. However, Ihara et al. [37] showed an overexpression of E-cadherin in their series of 66 HCCs, which was inversely correlated with tumor histological grade. They concluded that most of thin trabecular- and pseudoglandular-type of tumors preserved or overexpressed E-cadherin, while thick trabecular (diffuse) tumor cell pattern exhibited a significant reduction of the E-cadherin expression levels. E-cadherin expression therefore reflecting in a complex way the histopathology of the tumor.

AQPs, a group of membrane proteins, that serve as channels for water transfer, being preserved in bacteria, plants and animals [38]. In mammals, more than 10 isoforms (AQP0–AQP10) have been isolated, being expressed in different types of cells and tissues of the body. AQP0 type is found in the crystallin lens; AQP1 in the eyes, ear, blood vessels, proximal renal tubules and red blood cells; AQP2 is expressed in the collecting ducts of the kidneys; AQP3...
is found in the epidermis, urinary tract, respiratory tract and digestive tract; AQP4 is present in the ears, astrocytes, eyes, skeletal muscles, gastric parietal cells and renal collecting ducts; AQP5 in the salivary, lacrimal and sweat glands; AQP6 in intracellular vesicles of the renal collecting ducts; AQP7 is expressed in adipocytes, testis and kidneys; AQP8 is expressed in the liver, kidneys and testis; AQP9 in the liver and leukocytes; AQP10 is present in the gut [39]. Although some studies have initially described that water metabolism and secretion across the hepatocyte membrane is AQP independent [40], following studies have documented expression of AQP1, AQP3, AQP7, AQP8, AQP9 and AQP0 in the liver parenchyma [18, 41]. Thus, the exocrine secretion of hepatocytes implies an intense water exchange at the level of basolateral and canicular membranes, a process regulated by the presence of these channels. AQP1 has been initially described only in endothelial cells, AQP3 in Kupffer cells, AQP7 in hepatocytes and endothelial cells, AQP8 in liver cells, and AQP9 in cholangiocytes. Moreover, AQP4 has also been documented in regulating cell–cell adhesion by modulating the level of tight junction proteins [42]. When overexpressed on cell culture, the C-terminal tail of AQP5 interacted with and reduced the levels of plakoglobin, β-catenin, zonula occludens-1 (ZO-1) and desmoglein-2 tight junction associated proteins, suggesting a mechanism independent from the water-buffering pathway. Mice lacking AQP1 present with impaired angiogenesis and endothelial cell migration, apparently by reduced water influx into expanding cellular protrusions which would normally lead cell migration, supporting the fact that cellular migration is strongly influenced by a balanced expression of AQP4 [43]. There is evidence that AQP1 acts in fact as both a water and ions channel, mediating osmotic equilibrium through individual subunit water pores, and cation permeability through the central pore of the tetramer [44–46]. The idea that the AQP1 cation pore is in fact separate from the individual water channels for each monomer is supported by different effects of specific mutations [47, 48]. Combined administration of AQP1 water and ion channel blockers lead to reduced colon cancer cells migration in cell culture, suggesting thus a less invasive profile [49].

On HCC tissue, overall levels of AQP3, 7 and 9 have been evaluated by reverse transcription–quantitative polymerase chain reaction, western blotting and immunohistochemistry [50]. Compared with control liver tissue, HCC showed a significant increase in the expression of AQP3, but with a concomitant reduction in the expression levels of AQP7 and AQP9, at both the messenger ribonucleic acid (mRNA) and protein levels. Immunohistochemistry showed that while AQP9 was mainly found on the plasma membrane of hepatocytes, AQP3 and AQP7 were mostly detected in the cytoplasm and the nuclei. What was interesting, was that a high expression of AQP3 was associated with low expression levels of AQP7 and AQP9, but with high tumor grade and lymphatic metastasis.

We have showed here for the first time that AQP1 is also expressed in normal hepatocytes liver tissue, and to a lesser extent, in liver carcinoma cells, by utilizing a biotin-free tyramide signal amplification technique that offered more amplification compared to avidin–biotin and polymer techniques routinely utilized for diagnostic purposes. Thus, besides the well described pattern of expression in the membranes of red blood cells, endothelial cells, and cuboidal canalicular cells, we have also identified a diffuse signal in the cytoplasm of the normal and tumoral liver cells, that most probably represents maturing protein in the vesicular bodies of the ER and Golgi system. On the tumor tissue, on occasion, the AQP1 signal was also membranous, and probably this is the fraction that has been described as enhancing tumor aggressivity at the advancing edges and metastasizing. Our results showed, however, a significant decrease in overall AQP1 expression for high-grade tumors. Compared to previous studies on histopathology, we have utilized an automated densitometric measurement system that counted both cytoplasmic and membrane signal, and did not score the results utilizing subjective investigator appreciation of signal area and intensity [21]. Also, essentially different from other studies quantifying total AQP1 mRNA or protein levels by Western blotting, we excluded blood vessels from our analysis, and we considered only hepatocyte epithelia. AQP expression, as detected by immunohistochemistry, represents most probably maturing proteins expressed at the level of ER and Golgi vesicles, as it is well documented that AQPs are subjected to multiple post-translational modifications, especially phosphorylation, a process linked to the regulation of the mature protein form on the cellular membranes [51].

**Study limitations**

The main limitation of this study is represented by the small number of patients tissue available, without prior chemotherapeutic regimens that would have altered the metabolism of tumor cells, and from whom pathological material was available. Also, our analysis evaluated only total AQP1 and E-cadherin levels and could not assess the maturing proteins in their pathway towards the membrane compartment, that is to what extent mature proteins are represented in the cytoplasmic regions.

**Conclusions**

Altogether, utilizing a biotin-free tyramide signal amplification method, this study shows for the first time that AQP1 is expressed at the level of liver epithelia, besides the well documented expression in the endothelia of blood vessels and biliary duct system. AQP1 expression parallels the decrease of E-cadherin expression during carcinogenesis, but together with this downregulation, there is a spatial decrease in the colocalization of the two proteins. The myriad of complex pathways that govern cell mobility, edema and adhesiveness might be addressed to efficiently by analyzing the relationship between adhesion molecules and water buffering pores, opening new avenues in controlling cancer extension pathways.

**Conflict of interests**

The authors declare that they have no conflict of interests.

**Acknowledgments**

This work was supported by a grant of the Romanian Ministry of Education and Research, CNCS – UEFISCDI, project number PN-III-P1-1.1-TE-2019-1474, within PNCDI III.

**Authors’ contribution**

Ana-Maria Ciurea and Cristin Constantin Vere equally contributed to the manuscript and share first authorship.
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Received: December 12, 2021
Accepted: December 27, 2021