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IMMUNOHISTOCHEMICAL EXPRESSION OF CD44 IN PATIENTS WITH HEPATOCELLULAR CARCINOMA

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Abstract

Introduction**:** CD44, a transmembrane glycoprotein with a role in cell-cell and cell-matrix interactions and one of the stem cell markers, is considered to participate in progression and prognosis of hepatocellular carcinoma (HCC), which makes it a potential prognostic marker and therapeutic target.

We aimed to evaluate immunoexpression of CD44 in tumor and surrounding non-tumor liver tissue and to correlate it to multiple clinicopathological data in order to determine its prognostic value in patients from the Republic of North Macedonia.

Material and Methods**:** Presence of the immunosignal and the percentage of CD44+ tumor cells at the whole tumor tissue sample and adjacent cirrhotic liver tissue were semi-quantitatively determined. The immunohistochemistry results were correlated to B and C hepatitis, tumor dimensions, enlarged lymph nodes, T status, differentiation (G), microvascular invasion, and survival.

Results**:** We found a significant difference in CD44 expression between tumor and non-tumor liver tissue ($p < 0.000$) and significantly higher CD44 expression was also found in T4 tumors in comparison with T1 tumors ($p < 0.01$).

Conclusion**:** Expression of CD44 was significantly higher in tumor in comparison to non-tumor tissue and was significantly associated to T4 local tumor growth, making it a potential prognostic marker and therapeutic target.

***Keywords*:** CD44, HCC, immunohistochemistry, T status, survival

Introduction

CD44 is a multifunctional transmembrane glycoprotein, a cell surface adhesion molecule and a major hyaluronan receptor, expressed in many epithelial and mesenchymal cells. It has mainly a role in lymphocyte activation, recirculation, homing, adhesion, and it is also involved in cell-cell and cell-matrix interactions [1-3]. CD44 is expressed in various normal tissues, either in standard or variant isoforms, which occur through alternative splicing and it can also be expressed in cancer tissue [3]. It is involved in the adhesion of tumor cells to the host cells and host matrix [4] and has a function in gene transcription [3].

CD44 is considered to be one of the stem cell markers and to participate in proliferation, progression, metastasis and prognosis in several cancer types including hepatocellular carcinoma (HCC) [5-7], which makes it a therapeutic target for treatment of HCC [4].

The aim of this study was to evaluate immunoexpression of CD44 in HCC and surrounding non-tumor liver tissue, to correlate it to multiple clinical data and survival of patients and to correlate it with pathological characteristics of the tumor in order to evaluate the possible prognostic value of CD44 immunoexpression in patients with HCC from the Republic of North Macedonia.

Material and Methods

The investigation of CD44s immunoreactivity in HCC was performed on a well-defined cohort of patients [8,9] comprising 60 patients, 19 female (31.67%) and 41 (68.33%) male, ranging in age from 31 to 85 years, median 61.88±10.5. Patients were diagnosed and treated at the University Clinic for Gastroenterohepatology and the University Clinic for Abdominal Surgery in Skopje, Republic of North Macedonia in a period of 6 years. The biopsy and surgical material of patients, without any other previous therapy, were analyzed at the Institute of Pathology in Skopje.

Immunohistochemical staining with an antibody against CD44 (Monoclonal Mouse, Anti-Human, Clone DF1485, DAKO, dilution 1:50) using Avidin-Biotin immunoperoxidase technique was made in all tumors (60 cases) and adjacent non-neoplastic liver tissue in 35 cases. For the visualization of the antigen-antibody reaction, LSAB and En-Vision kit from DAKO was used.

Presence and distribution of the signal, membranous or cytoplasmic and the percentage of positive tumor cells were evaluated. The percentage of the stained cells (regardless of the signal intensity) was semi-quantitatively determined in the whole tumor tissue sample at the slide and the whole non-tumor tissue at the slide on microscopic fields at x 200 magnification. For obtaining objective results of the CD44 immunoexpression, evaluation of the histological slides was made by two pathologists.

Correlations of the CD44 immunoexpression and B and C hepatitis, tumor dimensions, enlarged lymph nodes, T status, differentiation (G), microvascular invasion, and survival were made.

T status of the disease was determined according to the TNM classification [10].

Statistical software package Statistica 7.1 for Windows and SPSS Statistics 23.0 was used for statistical analysis, applying: descriptive statistics, Mann-Whitney U Test, survival analysis (Kaplan-Meier curves) and multiple linear regression. Statistical significance was accepted for *p* values <0.05.

Results

Hepatitis infection was detected in 42 (70%) out of 60 patients. Thirty-six of them (60%) had hepatitis B infection and 6 (10%) had hepatitis C infection.

Cirrhosis was detected in 52 (86.67%) patients and it was also histologically confirmed.

Enlarged lymph nodes were found in only 6 (10%) patients. Most of the patients had tumors with dimensions 5 to 10 cm (26 patients - 23.33%), with moderate differentiation (27 patients - 45%) and tumors with microvacular invasion (38 patients - 63.33%).

The distribution of tumor T status and survival associated to T status are shown in Table 1.

Table 1. The distribution of tumor T status and survival associated to T status in patients with HCC

T	n	(%)	Survival months (Mean)	P value
T1	10	16.66	15.80	0.026*
T2	25	41.66	13.50	
T3	20	33.33	8.80	0.026*
T4	5	8.33	4.80	0.004**

p<0.05*; p<0.01**, n = Number of patients

The mean survival time for female patients was 8.86±1.76 months, for male 13.03±1.50 and overall survival was 11.6051±1.19 months.

Cox regression analysis showed that hepatitis B and hepatitis C infection (p<0.001; p<0.001), microvascular invasion (p<0.002) dimensions of the tumor (p< 0.055), T4 tumor status (p<0.01) and poor differentiation of the tumor (p<0.05) significantly influenced on patients' survival, as it has already been published (9).

There was no statistical difference in survival of patients with surgical and non-surgical treatment (p>0.05).

CD44 immunoexpression was found in 22/60 (36.66%) of the tumor tissue samples and in 0/35 (0%) of non-tumor tissue samples.

In the tumor tissue, the percentage of CD44 positive cells was determined in range from 10% to 70%. Expression of CD44 was membranous in 20/22 (90.90%), and cytoplasmic in 2/22 (9.09%) cases. The intensity of the signal was heterogeneous, from weak to strong, in different cases and in different areas in the same tumor. The tumor tissue showed immunoreactivity to CD44 in few patterns. Positive staining was found in smaller or larger clusters of cells, small sheets of cells and there was intensive staining of cells at the border of the tumor lobules in some cases (Fig. 1 a,b,c,d,e). Some non-parenchymal cells in

the tumor stoma and adjacent cirrhotic liver tissue were also stained (Fig. 1 a,b). In both, tumor and non-tumor liver tissue, 100% of Kupffer cells were immunopositive for CD44 antibody (Fig. 1 f).

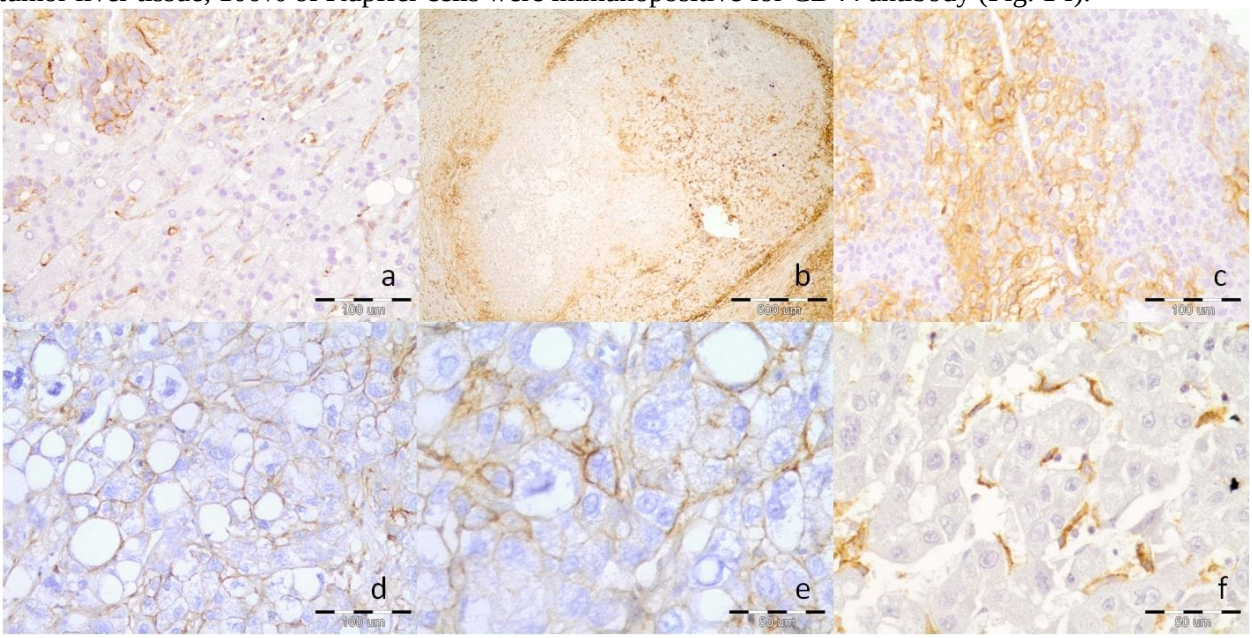

Figure 1. Microphotographs of CD44 immunoexpression in hepatocellular carcinoma and surrounding non-tumor liver tissue. a) Positive staining for CD44 (brown color) in clusters of tumor cells (upper left corner) and negative for parenchymal cells in cirrhotic liver (lower left corner). Some non-parenchymal cells are positively stained in both areas. In non-tumor tissue Kupffer cells are stained (CD44 x 40) b) Positive CD44 cells at the border of the tumor nodule and some within it. The positive cells outside the node are non-parenchymal cells. A second nodule with CD44+ cells is visible in the lower right corner (CD44 x 40) c) Membranous staining of the tumor cells. Approximately 50% of the tumor cells are negative (CD44 x 200) d) Different signal intensity of CD44 immunostaining in tumor tissue (CD44 x 200) e) Higher magnification of the same case in which membranous, somewhat granular positivity is visible (CD44 x 400). f) CD44 negative liver cells and CD44 positive Kupffer cells (CD44 x 200)

A significant difference was detected in the expression of CD44 in tumor tissue and non-tumor surrounding liver tissue (**p<0.00**) (Table 2).

Table 2. Difference between CD44 expression in tumor and surrounding non-tumor cirrhotic liver tissue

Parameter	**Rank Sum Tumor tissue**	**Rank Sum Cirrhotic tissue**	**U**	**Z**	**p-level**	**Valid N Tumor tissue**	**Valid N Cirrhotic tissue**
CD44%	3265.00	1295.00	665.00	4.02*	**0.000***	60	35

* adjusted

No significant correlation was detected between CD44 expression and clinical characteristics of patients.

Regarding pathological parameters, CD44 expression significantly influenced T status of the patients and multiple linear regression showed that patients with T4 tumor status had 22.38% (B=22.38) / (95%CI7.67 to 37.09) **/ p<0.01/** higher expression of CD44 in comparison to patients with T1 status.

Since we did not find a statistically significant difference between the patients with surgical and patients with non-surgical treatment, we included survival in the multiple linear regression analysis.

The multiple linear regression analysis with CD44 as a dependant variable showed that the greatest influence on the CD44 expression, in descending order, had: T4 tumor status (Beta = 0.42), hepatitis C (Beta = 0.27) and hepatitis B infection (Beta = 0.24), G3 (Beta = 0.24), enlarged lymph nodes (Beta = 0.20), patient's survival (Beta = -0.19), tumor dimension (Beta = 0.04) and microvascular invasion (Beta = 0.03).

A smaller percentage of tumor CD44 positive cells was found in patients with better survival, but it was not significant. With each increase in patient survival by one month, the expression of CD44 decreased by 0.32% (B = -0.32) / (95% CI: -0.91 to 0.27) / p>0.05/, with unchanged values for other parameters. With R = 0.49 (F = 1.10; p = 0.38) a moderately strong, but not significant correlation was determined.

Discussion

Hepatocellular carcinoma is a cancer with a rising incidence in many countries in the past few decades. Patients with HCC have a poor prognosis despite the recent achievements in surgical treatment and other therapeutic procedures. HCC is one of the most common cause of cancer-related mortality worldwide [11,12]. The applied therapies still do not give satisfactory results and are followed by post-treatment relapses, drug resistance and tumor progression [4,13,14]. Novel therapeutic strategies are of great importance and urgently needed. In recent years many molecular markers involved in HCC progression have been explored in order to enable basis for new therapeutic strategies and possibilities [15].

Genetic, epigenetic and signalling pathways play a role in development of HCC. By stochastic theory (clonal evolution model of cancer) of HCC carcinogenesis, a randomly damaged cell, with injurious changed DNA sequence, can result in cancer growth and all the cells in the tumor are responsible for tumor progression. Another hypothesis supports the existence of few cancer cells with stem cell properties (CSCs), i.e. with characteristics of adult progenitor stem cells, which have capacity of autonomous proliferation, self-renewal and multi-directional differentiation [16-19]. These small amounts of cells are capable of tumor progression and have an important role in cancer development, post-therapy relapses, metastasis, chemotherapy drug resistance and radiotherapy failure [17, 20-22].

Cancer stem cells express surface receptors and using these specific markers CSCs were demonstrated in various tumors including HCC [4]. CSCs specific markers, i.e. their overexpressed receptors may take an important part in developing target therapies that act directly and specifically on the components which strongly participate in tumor progression [4, 21]. CD44, cell surface adhesion molecule is one of these receptors, as are EpCAM, CD133, CD326, CD13, ALDH1, CD24 and CD90 (4).

It has been reported that liver cancer stem cells (LCSCs) dominantly express CD44 standard isoform (CD44s) and CD44 expression in HCC assessed by an antibody against total CD44 was significantly associated with a poor prognosis [22]. It has also been reported that expression of CD44s correlated with high histological grade, and expression of CD44v6 correlated with presence of vascular invasion, p53 overexpression and both with reduced survival rate [23]. Other authors have reported that the expression levels of CD44v6 are correlated with the invasiveness of hepatocellular carcinoma in vitro, but they did not find any significant correlations with clinicopathological parameters of the patients [24].

Zhu Z *et al*. [25] demonstrated in an animal model higher colony formation efficiency in CD133+/CD44+ in comparison to CD133+/CD44− cells. Mima K *et al*.[24] reported that in vitro CD44v6+ cells were associated with the invasive capacity of the tumor. Iacob R *et al*. [26] in a human study of 31 patients with HCC found that CD44 expression had a negative prognostic significance in patients after potentially curative treatment.

Luo Y *et al*. [5] in a meta-analysis on prognostic value of CD44 expression in patients with HCC showed that CD44 expression was not significantly associated with tumor differentiation, AFP level in HCC patients or disease-free survival, but was highly correlated with tumor TNM classification and decreased overall survival.

According to the above-mentioned, the prognostic value of CD44 in HCC still remains debatable and additional studies from different regions and different laboratories are necessary for analyzing CD44 expression in patients with HCC.

In our study CD44 expression was found in 36.66% of patients with HCC, a finding that was consistent with other published reports [23,24].

We found CD44 expression only in a tumor tissue and we did not find any CD44 expression in non-tumor liver tissue. The difference between CD44 expression in tumor and non-tumor tissue was significant, suggesting CD44 role in HCC carcinogenesis. In our study we did not find a statistically significant correlation between CD44 expression and the majority of analyzed clinicopathological parameters, although we found a tendency for greater CD44 expression to be in correlation with larger tumor mass and enlarged lymph nodes; lower CD44 expression was found in patients with better survival, but it was not significant. CD44 expression was significantly higher only in patients with T4 tumor status compared to patients with T1 tumor status, which indicated that CD44 may have a role in tumor growth and in tumor progression according to the tendency shown in relation to other clinicopathological parameters.

Poor prognosis and unsatisfactory therapy in patients with HCC are disappointing facts that require new therapeutic modalities and fast action in more research fields. Targeted molecular therapy has given some results [27], giving courage and hope for patients with HCC and research in this field. It has been reported that in HCC, LCSCs expressing molecular markers as are EpCAM, CD133, CD90, CD44 and CD13 exhibited resistance to radiotherapy and chemotherapy in vitro and in vivo (4). Researchers have made efforts in blocking CD44 activity in vitro and in animal models in different tumors [6,7,28-32], and in blocking CSCs surface markers including CD44 in HCC (4), with promising results. The elimination of CSCs would prevent tumor progression, recurrences and metastasis and therapy resistance [33], which would increase patient's survival.

In conclusion, CD44 in our study was significantly associated with local tumor growth in patients with HCC, making it a potential prognostic marker and therapeutic target.

Compliance with Ethical Standards**:** Authors declare that they have no conflict of interest.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Research Ethics Committee approval number 03-554/2 from 09.02.2018 year, Republic of North Macedonia.

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