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RESEARCH ARTICLE

# INVESTIGATION OF CANCER STEM CELL SURFACE MARKERS IN THE TUMOR TISSUES OF PATIENTS WHO HAD LIVER TRANSPLANTATION DUE TO HEPATOCELLULAR CANCER AND EVALUATION OF THE EFFECT OF THESE MARKERS ON PROGNOSIS

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**ABSTRACT**

**Background and purpose:** To investigate the relevance between cancer stem cell(CSC) markers and tumor progression in hepatocellular carcinoma(HCC).

**Methods:** Data of patients who underwent liver transplantation(LT) for HCC between February 1998 and September 2018 were collected. Patients over 18 years of age were included. Immunohistochemical staining were performed in paraffin blocks of liver explants containing HCC in terms of CSC markers, CD13, CD44, CD47, CD90 and EpCAM. Follow-up period, cancer recurrence, disease-free and overall survival were investigated.

**Results:** There were 71 patients who met the inclusion criteria. Optimal evaluation conditions were not met for CD13 and CD90 staining. Disease recurrence was found to be more frequent in CD 44+ cases (p=0.008). Disease-free survival was significantly longer in CD44- group(160.2 vs 103.0 months, p=0.043). Overall survival was significantly shorter in CD44+ cases(171.7 vs 107.8 months, p=0.018). No statistically difference was found between CD47+/- or EpCAM+/- groups in terms of recurrence (p=0.27, p=0.24). There was no significant difference in disease-free and overall survival in CD47+/- or EpCAM+/- cases, respectively (CD47+/-; p=0.82, p=0.90, EpCAM; p=0.76, p=0.69).

**Conclusion:** Positive CD44markers in HCC is associated with a more aggressive course of disease. Targeted therapies for CD44antigens of CSCs may prevent disease recurrence and increase survival.

**Keywords:** Hepatocellular cancer, cancer stem cell, tumor markers, liver transplantatio

## INTRODUCTION

Hepatocellular cancer (HCC) is the 3rd most common cause of death worldwide (1). The most important reason for this is that it is resistant to treatment and it relapses easily. Local recurrences and metastases that occur in a short period of time lead to the death of patients due to the lack of effective treatment option. Currently, the most effective treatment modalities for HCC are liver resection with tumor-free surgical margins or liver transplantation (LT) in selected cases (2,3). Liver transplantation provides the chance to eliminate both HCC and the underlying chronic liver disease (3,4). Different criteria have been defined for selection of patients with HCC as liver transplant recipients (4). Although Milan and University of California San Francisco (UCSF) criteria are frequently used, some liver transplant centers may also use other criteria. Recurrence rate of HCC after liver transplantation is reported to be around 20-25% worldwide (5). Unfortunately, there is still no effective treatment for prevention and treatment of recurrence. Systemic chemotherapeutic agents are inadequate. Therefore, solutions to prevent and treat HCC recurrence after surgical resection or LT are needed.

In recent years, importance of cancer stem cells (CSCs) in tumor biology has been revealed (6,7). This topic has been widely investigated and studies have discovered many specific CSC surface markers for certain tumors. Determining spesific CSCs markers promises to develop target therapies for tumors (1,6,7). As a result of current studies, the main CSCs surface markers of HCC are: CD 133, CD44, CD47, CD13, CD24, OV6, CD90 and EpCAM (epithelial cell adhesion molecule) (8-10). Experimental and clinical researches for targeted treatment strategies regarding defined markers continue worldwide. However, most of the studies are cell line based and there are few studies using human tissues.

The aim of this study was to investigate the presence of CSCs markers in the liver tissues of patients that underwent LT for HCC and to determine the relationship between these markers and disease prognosis.

## MATERIAL AND METHODS

The study was designed as a cross-sectional study. Institutional ethics committee approval was obtained. Cases who underwent LT for HCC between February 1998 and September 2018 in our Hospital were analysed. Patients over 18 years of age and whose data could be obtained reliably were included.

Paraffin blocks of liver explants containing HCC from the patients included in the study were obtained. For immunohistochemical staining of CSCs surface markers, CD13, CD44, CD47, CD90 and EpCAM specific monoclonal antibodies were used. These

antibodies and their brands/models were CD90 (Santa Cruz Biotechnology, SCBT, sc-5316), CD44 (Santa Cruz Biotechnology, SCBT, sc-9960), CD13 (Santa Cruz Biotechnology, SCBT, sc-13536), CD47 (Thermo Fisher Scientific, Invitrogen B6H12), EpCAM (Thermo Fisher Scientific, Invitrogen MA512604). Staining patterns were scored and recorded. Data of age, gender, type of liver transplantation (living/cadaveric), follow-up period, cancer recurrence, disease-free and overall survival were recorded.

The values were entered into a Microsoft Office Excel 2020 (Microsoft Corp., Redmond, Washington, USA) database. The records underwent an extensive data editing process to check for inconsistencies between data fields. After validation, error-free data were entered into the master file. Data were imported into IBM SPSS statistics 23.0 (SPSS, Chicago, Illinois, USA) for analysis. Descriptive statistical methods (mean, median, standard deviation), univariate and multivariate analyses and survival analyses were performed.

In univariate analysis, t-test or Mann-Whitney U test was used for univariate analysis according to the compliance with normal distribution in the comparison of the data obtained by measurement. Chi-square test was used to compare the data obtained by counting. Chi-square test or Fisher's exact test was used in the analysis of census and/or categorical data. According to these results, $p<0.05$ was considered significant. "Kaplan-Meier" method was used for survival analysis and "log-rank" test was used for comparison of groups.

## RESULTS

There were 71 patients who met the inclusion criteria. After exclusion of 5 patients with early mortality (first 90 days), 54 (81%) males/12 (18%) females, total 66 patients were analysed. The mean age of the patients was 55.95±7.4 years (37-68) and the median age was 55 years. Thirty-five (53.7%) of the patients received LT from living and 31 (46.9%) from cadaveric donors. The mean follow-up period was 107.46±65.82 (3.22-231.36) months. HCC recurrence occurred in 14(21%) patients. The sites of recurrence were lung, bone, liver and skin. The median time to recurrence was 25.17±21.48 (2.10-58.35) months. The median disease-free survival was 145.81±11.92 and the median overall survival was 145.81±11.58 months.

Paraffin blocks were evaluated immunohistochemically for the presence of HCC stem cell markers CD13, CD44, CD47, CD90 and EpCAM. Despite many attempts, optimal immunohistochemical evaluation could not be achived for CD13 and CD90. Therefore these two markers could not be evaluated. Assesment of other 3 markers accomplished successfully.

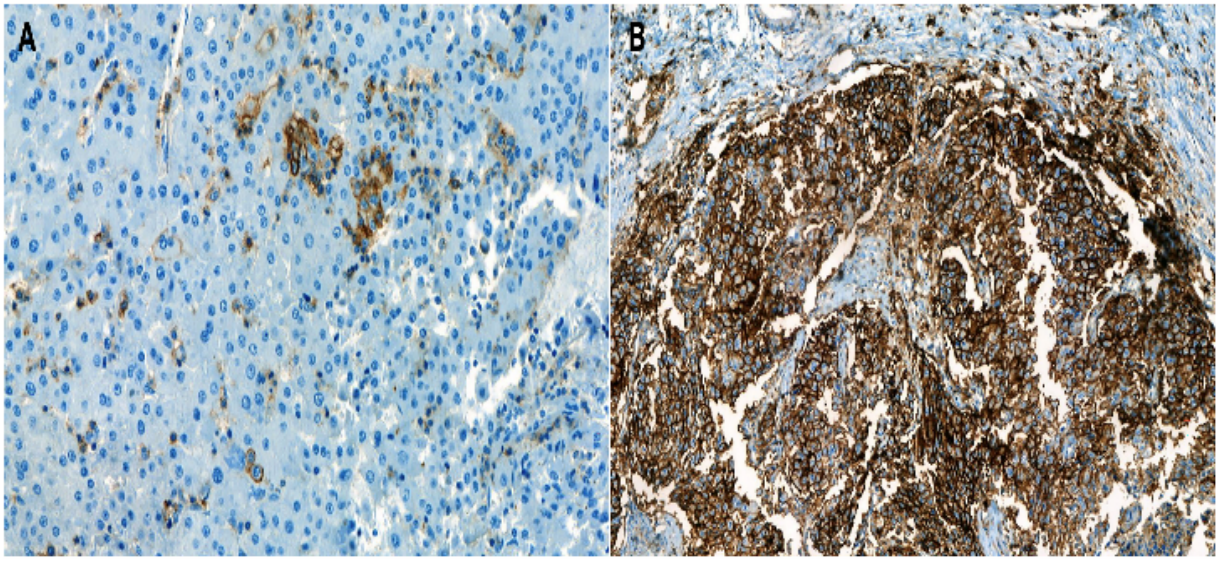


**Figure 1. A.** Hepatocellular cancer case of without recurrence after LT. Low density of CD44+ cells (brown staining) in explanted liver (x20 magnification**. B.** Case with HCC recurrence after LT. Dramatically dense CD44+ cells (brown staining) are seen in (x10 magnification).

CD44 was positive in 20(38%) of 52 patients without recurrence and 11(78%) of 14 patients with recurrence. Disease recurrence was found to be statistically more frequent in CD 44 + cases (p=0.008). A much more intense staining pattern was detected in the 11 CD44+ patients with recurrence compared to CD44+ patients without recurrence (Figure 1A, B). In Kaplan-Meier survival analysis, according to the log-rank test, disease-free survival was significantly longer in CD44- group when compare CD44+ (160.2 vs 103.0 months, p=0.043) (Figure 2). Similarly, overall survival was significantly shorter in CD44+ cases (171.7 vs 107.8 months, p=0.018) (Figure 3).

CD 47 was positive in 5 (9%) of 52 patients without recurrence and 2 (14%) of 14 patients with recurrence. EpCAM was positive in 6 (11%) of patients without recurrence and 2 (14%) of patients with recurrence. No statistically significant difference was found between CD47+/- or EpCAM+/- groups in terms of recurrence (p=0.27, p=0.24). No significant difference in staining pattern was found between either the CD47 +/- or EpCAM +/- groups. There was no significant difference in disease-free survival and overall survival in CD47+/- or EpCAM+/- cases, respectively (CD47+/-; p=0.82, p=0.90, EpCAM; p=0.76, p=0.69).

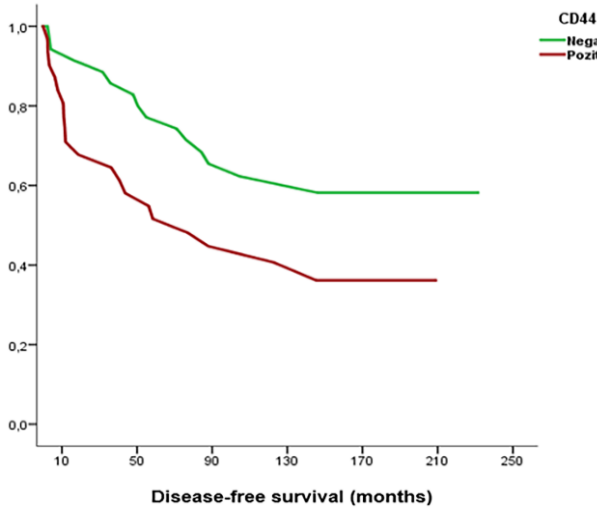


**Figure 2.** Disease-free survival curves in CD44-/+ cases. Disease-free survival is significantly shorter in CD44+ cases (p=0.043)

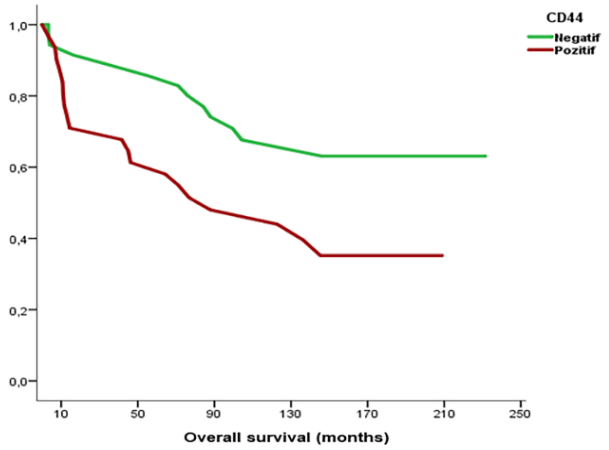


**Figure 3.** Overall survival curves in CD44-/+ cases. Overall survival was significantly decreased in CD44+ cases (p=0.018).

## DISCUSSION

Hepatocellular cancer is the 7th most common cancer worldwide and the 3rd most common disease responsible for cancer-related deaths (8). Surgical resection or LT, combined with chemotherapy are the most effective strategies for cure (2,3). Despite developments in treatment modalities, HCC still has a high recurrence rate and mortality (11-13). Thus, the search for new therapy modalities for better treatment continues.

Since the first successful liver transplant was performed by Starzl in 1963, it became widely practiced worldwide acute or chronic liver failure and HCC in selected cases constitute the most common indications for LT (5). The factors affecting survival after LT are mainly vascular complications graft rejection, infection and disease recurrence in patients with HCC. Egeli et al. reported

that, long-term survival in patients who underwent LT for HCC was significantly lower compared to other etiologies due to early disease recurrence (14). Similarly, studies conducted to determine the prognosis after LT have shown that HCC recurrence has a negative effect on survival (15,16). Many studies have revealed the mechanism of recurrence of HCC or to understand the development of de novo or recurrent malignancy in transplanted patients and important results have been obtained (17,18). However, new findings are needed to prevent recurrence and provide effective treatment.

The CSC theory in cancer development has been known for about 40 years (9). According to this theory, a group of stem cell-like cells with the characteristics of self-renewal, change and proliferation are responsible for the development and progression of cancer (8,9). It has been suggested that CSCs that are resistant to chemotherapy and other treatment

modalities and lead to local recurrence and metastasis. Therefore, it is thought that effective treatment of the disease and prevention of recurrences may be possible by developing therapies targeting CSCs.

In order to target CSCs, important studies have been carried out in recent years to identify cell surface markers specific to these cells and significant progress has been made. The main HCC CSC surface markers identified as a result of current studies are: CD133, CD44, CD47, CD13, CD24, OV6, CD90 and EpCAM (1,9,10). Thanks to these developments, targeted treatment strategies for cells carrying these markers have become more promising. Some new HCC CSC surface markers have also been identified in recent studies (13). Experimental and clinical studies regarding this topic continue intensively (10,11).

In this study, HCC recurrence was found to be statistically significantly higher in CD44+ CSCs after LT. In addition, disease-free and overall survival periods were found to be statistically significantly shorter in CD44+ patients. Consistent with this result, Rozeik et al reported in HCC, increased CD133 and CD44 expression corresponded to higher grade, thus indicating poorer prognosis (1). In accordance with our opinion, they advocated the expression profiles of several CSCs markers may enhance understanding of HCC prognosis, metastasis and relapse. That may facilitate development of novel therapeutic agents targeting and/or preventing HCC. In addition, some novel studies showed higher recurrence rate and shorter disease free survival in CD44+ HCC patients (19,20). On the other hand, no significant difference was found associated with CD47 or EpCAM positivity in terms of recurrence or survival.

There were some limiting factors about this study. COVID-19 pandemic, occurred during the course of the research and it negatively affected our study in many ways. Another important limitation was despite all efforts not being able to study on CD13 and CD90 markers. Thus we could not have information about these markers. Retrospective design of the study and limited number of patients may be considered as other handicaps.

In conclusion, this study demonstrated that HCC with high CD44+ CSCs, is associated with a more aggressive course of the disease. This result suggest that targeted therapies for CD44 surface marker in the treatment of HCC may prevent disease recurrence and increase survival. We consider in near future , comprehensive studies including novel HCC CSC markers will provide the opportunity to development of efficacious target therapies for HCC.

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