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Running title: Low SVEP1 expression correlates with poor prognosis in HCC

Decreased expression of SVEP1 is closely related to a cancer stem cell-like phenotype and poor prognosis in hepatocellular carcinoma

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The objective of this study was to investigate the expression of SVEP1 in hepatocellular carcinoma (HCC) and to evaluate the association among SVEP1, cancer stem cell-like phenotype, and the prognosis of patients to provide new possibilities for the accurate diagnosis and stratification of HCC. Two hundred HCC and paired adjacent tissues were analyzed by immunohistochemistry and scored, and their relationships with clinicopathological parameters and survival rates were analyzed. We found that compared with adjacent tissues, the expression of SVEP1 in HCC was relatively low and was closely related to tumor size, satellite nodule formation, and histological grade ($p < 0.05$). Statistical analysis showed that the survival rate of patients with low expression of SVEP1 decreased significantly ($p < 0.05$). Our results showed that the expression of SVEP1 was negatively correlated with the expression of the cancer stem cell markers CD44 and CD133 ($p < 0.05$). Moreover, multivariate Cox regression analysis showed that SVEP1 was an independent prognostic factor for the survival of HCC patients. In conclusion, our results suggest that decreased SVEP1 expression may promote HCC acquisition of a cancer stem cell-like phenotype, ultimately leading to heterogeneity and poor prognosis of HCC. This work may provide new insight into the development of HCC and suggests a potential marker for predicting the prognosis of patients.

Key words: SVEP1; hepatocellular carcinoma; cancer stem cells; prognosis

Primary liver cancer is currently one of the most prevalent malignancies in the world and the second largest cancer-related cause of death [1]. Hepatocellular carcinoma (HCC) accounts for 90% of primary liver cancers. In recent years, the incidence rate of HCC has shown an unacceptably

increasing trend worldwide [2]. Although some improvements have been made in diagnostic criteria and treatment methods, the current standard management for HCC patients still fails to achieve satisfactory prognosis. Recurrence and metastasis are still the main challenges facing patients [3]. Even in patients with HCC of the same pathological type and clinical stage, some are still more likely to relapse a short time after surgery. Recent studies have shown that the poor prognosis of HCC may be mainly due to the highly complex heterogeneity of tumor cells [4]. Therefore, it is of great significance to further explore instructive markers of poor prognosis of HCC, making more accurate diagnosis and stratification of patients possible.

SVEP1 is also termed polydom, which is a gene located on chromosome 9q32 [5]. SVEP1 serves as an important cell adhesion molecule that can mediate the adhesion between cells and the matrix [6]. Recently, reported findings suggested that SVEP1 deletion affects the development and formation of venous and lymphatic precursors during zebrafish embryonic development [7]. Sprecher et al. reported that knockdown of SVEP1 in keratinocytes can downregulate epithelial marker expression and intercellular adhesion, affecting the phenotypic differentiation of epithelial cells [8]. However, the role of SVEP1 in tumor progression and its prognostic significance still need to be further explored.

Tumor stem cells are a minor subpopulation in tumors, and are considered the main reason for poor tumor prognosis. They have the properties of unlimited proliferation, self-renewal and differentiation into cancer cell allogeneic lines [9]. Similarly, the existence of HCC stem cells was hypothesized to be the main reason for tumor progression and treatment resistance, ultimately leading to tumor recurrence and metastasis [10]. A previous study by our research group showed that the downregulated expression of SVEP1 in HCC was correlated with cancer metastasis and proliferation [11]. In view of the important role of SVEP1 in cell differentiation and development, we explored the expression levels of SVEP1, CD44 and CD133 in 200 cases of hepatocellular carcinoma and analyzed the association between SVEP1 expression, clinicopathological parameters and survival rates. In this study, the objective was to clarify the expression of SVEP1 in HCC and to explore the relationship between SVEP1 and HCC stem cell phenotype and its significance in predicting prognosis.

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Patients and methods

Patients and tissue samples. This study evaluated 200 patients with hepatocellular carcinoma who underwent hepatectomy at Tianjin Medical University Cancer Institute and Hospital from January 2010 to January 2015. This study excluded patients who received only palliative resection, transarterial embolization or radiotherapy. All patients had complete follow-up data and tumor characteristics, including age, sex, survival time, AFP level, microsatellite lesions, vascular invasion, histological grade and Barcelona clinical liver cancer (BCLC) stage. All HCC cases were reviewed by two certified pathology doctors according to the WHO diagnostic criteria. The ethics committee of Tianjin Medical University Cancer Institute and Hospital approved the study, and all patients signed written informed consent. The tissue microarray (TMA) was constructed by viewing the corresponding HE section and using a hollow needle to drill tissue samples 2 mm in diameter for each HCC paraffin specimen to prepare a single tissue core. This core was transferred to a predetermined position on a paraffin block to make a tissue microarray (TMA). The TMA was cut into 3 μm-thick serial sections, followed by immunohistochemical staining and analysis.

Immunohistochemistry. The tissue chip sections were dewaxed in xylene and hydrated in gradient alcohol, followed by antigen repair at 95 °C for 10 min in EDTA repair solution. To block endogenous peroxidase activity, 3% hydrogen peroxide was added and incubated at room temperature for 10 minutes. After blocking the antigen with serum, the slides were incubated with primary antibody at 4 °C for 12 h. The expression of SVEP1 (R&D Systems Company, mab9774), CD44 (Zhongshan Chemical Co., Beijing, China, zm0051) and CD133 (Abcam Company, ab19898) was detected by pictures PV6001 and PV6002 (Zhongshan Chemical Co., Beijing, China). After washing 3 times, the sections were developed with DAB for 5-10 minutes and counterstained with hematoxylin. Negative controls were incubated with PBS without adding the primary antibody.

Immunohistochemical analyses. Two pathologists who were blinded to the clinicopathological parameters of the cases performed a semiquantitative evaluation of the immunohistochemical staining. Staining was evaluated using the staining index (SI) with the following criteria: 10 visual fields were randomly selected at 400× magnification to analyze 100 tumor cells which were divided into four grades: insignificant staining (score 0), weak staining (score 1), moderate staining (score 2), and strong positive staining (score 3). The average percentage of staining area in tumor tissue

can be divided into four categories: no significant positive area (score 0), positive area ≤ 25% (score 1), positive area 25-50% (score 2), or positive area ≥ 50% (score 3). The results were evaluated using sums of intensity and percentage (SI) scores. An SI score ≥ 2 indicates high expression, while an SI score < 2 indicates low expression.

Statistical analysis. Statistical analysis was performed using SPSS 26.0 software. An independent sample t test was used to compare the differences between groups. The IHC score of paraffin tissues and its relationship with clinicopathological features were analyzed by Pearson's chi-square test or Fisher's exact test. Kaplan-Meier survival analysis was performed to analyze the relationship between SVEP1 expression and survival time in hepatocellular carcinoma, and the log-rank test was used to detect the difference between curves. An analysis of risk factors was conducted using a multivariate Cox regression model. Statistical significance was defined as a p-value < 0.05.

Results

The expression of SVEP1 in hepatocellular carcinoma. To determine the expression of SVEP1 in HCC tissues, we performed immunohistochemical staining on a tissue microarray of 200 HCC tissues and 200 adjacent tissues. The results showed that positive expression of SVEP1 was mainly located in the cytoplasm. SVEP1 was expressed at low or negative levels in 110 cases (55%) of HCC and positive in 90 cases (45%) (Figure 1). In the adjacent tissues, 14 cases (7%) were weakly positive for SVEP1, and 186 cases (93%) showed high expression levels. The expression of SVEP1 was significantly decreased in HCC tissues compared with adjacent tissues (p=0.00).

The relationship between SVEP1 expression and clinicopathological features in hepatocellular carcinoma. We found that the expression level of SVEP1 was closely related to tumor size, satellite nodule formation and histological grade (Table 1). Among 172 cases with a tumor diameter ≥ 3 cm, 100 cases (58.1%) had a low expression level of SVEP1. In contrast, 10 (35.7%) of the 28 cases with a tumor diameter < 3 cm had low SVEP1 expression (p=0.027). Among 88 HCC patients with satellite nodules, 58 (65.9%) had low expression of SVEP1, while among HCC patients without satellite nodules, the proportion of low expression of SVEP1 was 46.4% (52/112; p=0.006). In addition, the results of statistical analysis showed that the low expression of SVEP1 was significantly correlated with the histological grade of HCC. The expression level of SVEP1 was low

in 73/117 cases (62.4%) of moderately and poorly differentiated HCC, while the low expression rate of SVEP1 in highly differentiated HCC was 44.6% (p=0.013).

Decreased SVEP1 expression is significantly correlated with the HCC stem phenotype. The results of the correlation analysis between the expression of SVEP1 and cancer stem cell-related markers are shown in Table 2. The low expression of SVEP1 in HCC was significantly correlated with the phenotypic markers CD44 (Figures 2A, 2B) and CD133 (Figures 3A, 3B) (p-values were 0.014 and 0.02, respectively). The results showed that 53/110 cases had high expression of CD44, and 48/110 cases had high expression of CD133 in the low expression group of SVEP1. In the SVEP1 high expression group, the numbers of cases with high expression of CD44 and CD133 were 28/90 and 25/90, respectively (Table 2). In addition, we further analyzed the significance of CD44 and CD133 in the prognosis of HCC patients, and survival analyses demonstrated that the overall survival rate of HCC patients in the high CD44 and CD133 expression group was significantly lower than those in the low expression group (p=0.021 and 0.048) (Figures 2C, 3C).

The relationship between decreased SVEP1 expression and the prognosis of HCC patients. Among the 200 HCC patients, the median survival time of 110 patients with low SVEP1 expression was 28.78 months, and the 5-year survival rate was 20.90%, while the median survival time of patients with high SVEP1 expression was 47.01 months, and the 5-year survival rate was 46.67%. Survival analysis showed that the overall survival rate (OS) and disease-free survival rate (DFS) of patients in the SVEP1 low expression group were significantly lower than those in the SVEP1 high expression group (p=0.029 and 0.004) (Figure 4). A stepwise forward multivariate Cox regression analysis for OS (including sex, age, cirrhosis, HBV, tumor size, histological grade, satellite nodule, macrovascular invasion, microvascular invasion, BCLC stage, AFP $\geq$ 20 ng/ml, SVEP1, CD44 and CD133) were performed (Supplementary Table S1), and results showed that BCLC stage, histological grade, AFP level and low expression of SVEP1 were independent prognostic factors of HCC ($p < 0.05$) (Table 3). Subsequently, we further investigated the prognostic differences between HCC with high SVEP1 expression and low SVEP1 expression in two high-risk recurrence subgroups (the histological medium/low-differentiation group and the AFP $\geq$ 20 ng/ml group). The results showed that the OS time and DFS time of patients with low expression of SVEP1 decreased significantly in the medium/low-differentiated HCC group (p=0.014 and 0.002). In patients with

AFP higher than 20 ng/ml, patients with higher SVEP1 expression had longer DFS times than patients with lower expression (p=0.022) (Figure 5), even though there was no significant difference in OS time (p=0.187). These results suggest that low levels of SVEP1 expression are a key feature of HCC and may suggest a poor prognosis for HCC patients.

Discussion

HCC, as a highly heterogeneous tumor, is one of the most lethal malignant digestive system cancers worldwide [12]. With the rising incidence of HCC in recent decades, more than 600,000 deaths have occurred each year [13], and surgical resection is still the mainstay of curative treatment options. However, the overall prognosis of HCC varies considerably from patient to patient, and some tumors recurred and metastasized within a short period of time after the operation. Therefore, it is of great significance to further explore the markers of poor prognosis of HCC. SVEP1 is a cell adhesion factor and extracellular matrix protein involved in remodeling of lymphatics and differentiation of the epidermis [7]. Studies have shown that SVEP1 deletion affects the development and formation of venous and lymphatic precursors during zebrafish embryonic development [7]. In addition, studies have demonstrated that the spliceosome of SVEP1 and its regulation might contribute to the invasion of bone niches by tumor cells [14]. Previous studies by our research group showed that the downregulated expression of SVEP1 in HCC was correlated with HCC metastasis and proliferation. This study evaluated the expression level of SVEP1 in tumor tissues and corresponding adjacent tissues of 200 patients with HCC. The results revealed that the decreased expression level of SVEP1 in tumor tissues was significantly higher than that in adjacent tissues (p=0.00), and the low expression of SVEP1 was closely related to tumor size, satellite nodule formation and histological grade ($p < 0.05$). Multivariate Cox regression analysis showed that low expression of SVEP1, BCLC stage, histological grade and AFP level were independent prognostic factors for HCC ($p < 0.05$).

Cancer stem cells (CSCs) are a group of cells that exist in malignant tumors and have many similarities with normal stem cells or progenitor cells. The common characteristics of these cells are the ability of self-renewal and differentiation into multiple lineages, resulting in the activation of tumor growth and heterogeneity [15]. The commonly used treatment methods for HCC can

eradicate most tumor cells but cause limited damage to liver cancer stem cells. Therefore, the existence of CSCs has always been considered the direct cause of tumor recurrence and metastasis and ultimately leads to poor prognosis of patients [10]. The key to CSC plasticity and metastatic potential is the process of epithelial mesenchymal transformation (EMT) [16, 17], which leads to cytoskeleton remodeling, loss of intercellular adhesion and acquisition of mesenchymal phenotype [18, 19]. As an important cell adhesion molecule, SVEP1 can mediate the adhesion between cells and the matrix. Therefore, its loss of expression may play an important role in promoting the phenotype of HCC stem cells. Our results showed that the low expression of SVEP1 was significantly correlated with the expression of the cancer stem cell markers CD133 and CD44, with *P* values of 0.014 and 0.002, respectively. CD133 and CD44 are the most commonly used surface markers of a variety of cancer stem cells, and studies have shown that CD133- and CD44-positive cells highly express stem cell-related genes in liver cancer [20-22]. In present study, Kaplan-Meier survival analyses showed that the high CD44 and CD133 expression are associated with the poor prognosis of patients with HCC ($p < 0.05$). Therefore, we speculate that the low expression of SVEP1 in HCC is closely related to the phenotype of HCC stem cells. Recent studies have shown that SVEP1 plays an important role in maintaining the microenvironment of hematopoietic stem cell development [23]. Consistent with this, studies have shown that SVEP1 plays a key role in epidermal development, and the downregulation of SVEP1 expression can inhibit epidermal cell differentiation [8].

In the present study, our results showed that the overall survival rate and disease-free survival rate of HCC patients in the low SVEP1 expression group were significantly lower than those in patients with high SVEP1 expression levels (p=0.029 and 0.004). Additionally, in the risk subgroups with medium/low-differentiated HCC and AFP higher than 20 ng/ml, the disease-free survival time was significantly shorter in patients with low SVEP1 expression than in those with higher expression (p=0.002 and 0.022). These results suggested that decreased SVEP1 expression may serve as a potential marker to identify high-risk populations and predict prognosis in HCC patients. Activation of the PI3K/Akt signaling pathway is an important part of maintaining the stem phenotype in mouse and human pluripotent stem cells. Studies have shown that PI3K pathway activation is associated with increased stemness in breast cancer, lung cancer and colorectal cancer [24]. Previous studies

by our research group have shown that the loss of SVEP1 expression can activate the PI3K/Akt signaling pathway and promote HCC metastasis [11]. Therefore, we speculate that the decreased expression of SVEP1 may induce an HCC stem cell phenotype by activating the PI3K/Akt pathway, ultimately leading to poor prognosis in patients. In a follow-up study, we will further clarify the role and mechanism of SVEP1 in regulating the HCC stem cell phenotype in vitro.

In conclusion, this study demonstrated that the decreased expression of SVEP1 in hepatocellular carcinoma was closely related to tumor size, satellite nodule formation and histological grade. We also found that low SVEP1 expression was associated with an HCC stem cell-like phenotype. Survival analysis showed that low SVEP1 expression was an independent prognostic factor for HCC. In conclusion, the results of this study may provide a basis for further clarifying the mechanism of the development and high heterogeneity of hepatocellular carcinoma and are expected to provide new possibilities for more accurate stratification of HCC and predicting the prognosis of patients.

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Supplementary data are available in the online version of the paper.

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Figure Legends

Figure 1. SVEP1 immunohistochemistry in HCC tissues and paraneoplastic tissues. SVEP1 expression was primarily localized in the cytoplasm. Adjacent tissues (A1 200×, A2 400×). Positive stain in HCC (B1 200×, B2 400×). Negative stain in HCC (C1 200×, C2 400×).

Figure 2. CD44 immunohistochemistry in HCC tissues. Positive staining (A1×200, A2×400). Negative staining (B1 200×, B2 400×). C) Kaplan-Meier analysis for OS of HCC patients based on CD44 expression.

Figure 3. CD133 immunohistochemistry in HCC tissues. Positive staining (A1 200×, A2 400×). Negative staining (B1, 200×, B2 400×). C) Kaplan–Meier analysis for OS of HCC patients based on CD133 expression.

Figure 4. Kaplan-Meier analysis of the OS (A) and DFS (B) of HCC patients based on SVEP1

328 expression.

329

330 **Figure 5.** Kaplan-Meier analysis of the OS (A, B) and DFS (C, D) of HCC patients in risk
331 subgroups (histological medium/low differentiation or AFP ≥ 20 ng/ml) based on SVEP1
332 expression.

333 **Table 1.** Relationship between SVEP1 expression and clinicopathological features of HCCs.

Clinical parameters	Cases	SVEP1		χ^2	p-value
		Lower, n	Higher, n		
Sex				0.001	0.98
Male	162	89	73		
Female	38	21	17		
Age (years)				2.469	0.116
< 55	90	55	35		
≥ 55	110	55	55		
Cirrhosis				0.281	0.596
negative	87	46	41		
positive	113	64	49		
HBV				2.093	0.148
Absent	45	29	16		
Present	155	81	74		
Tumor size (cm)				4.893	**0.027***
< 3	28	10	18		
≥ 3	172	100	72		
Histological grade				6.226	**0.013***
High	83	37	46		
Median/Low	117	73	44		
Satellite nodule				7.556	**0.006***
Absent	112	52	60		
Present	88	58	30		
Macrovascular invasion				0.910	0.340
Absent	178	100	78		
Present	22	10	12		
Microvascular invasion				1.416	0.234
Absent	87	52	35		
Present	113	58	55		
BCLC stage				0.013	0.91
0/A	166	91	75		
B/C	34	19	15		
AFP (ng/ml)				0.311	0.577
< 20	89	47	42		
≥ 20	111	63	48		

334 Note: *p-value < 0.05 is statistically significant

335

336 **Table 2.** Relationship between SVEP1 expression and cancer stem cell markers expression of
337 HCCs.

Characteristics	Cases	SVEP1		$\chi 2$	p-value
		Lower, n	Higher, n		
CD44				5.986	**0.014***
Negative	119	57	62		
Positive	81	53	28		
C133				5.371	**0.02***
Negative	127	62	65		
Positive	73	48	25		

338 Note: *p-value < 0.05 is statistically significant
339

340 **Table 3.** Multivariate Cox regression analysis for overall survival in HCCs.

Characteristics	B	SE	Wald	p-value	HR (95% CI)
BCLC stage	0.642	0.202	10.087	**0.001***	1.278-2.822
Histological grade	0.445	0.164	7.370	**0.007***	1.132-2.151
AFP ≥ 20 ng/ml	0.379	0.160	5.630	**0.018***	1.068-1.999
SVEP1	-0.099	0.046	4.615	**0.032***	0.828-0.991

341 Note: *p-value < 0.05 is statistically significant

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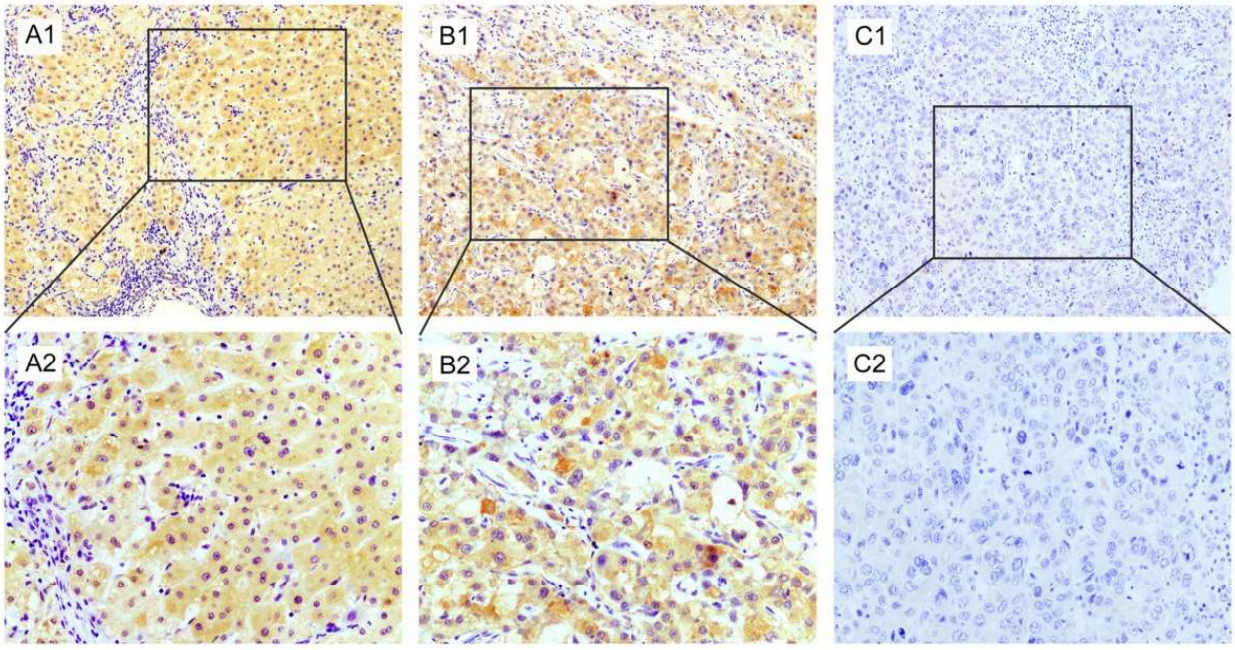

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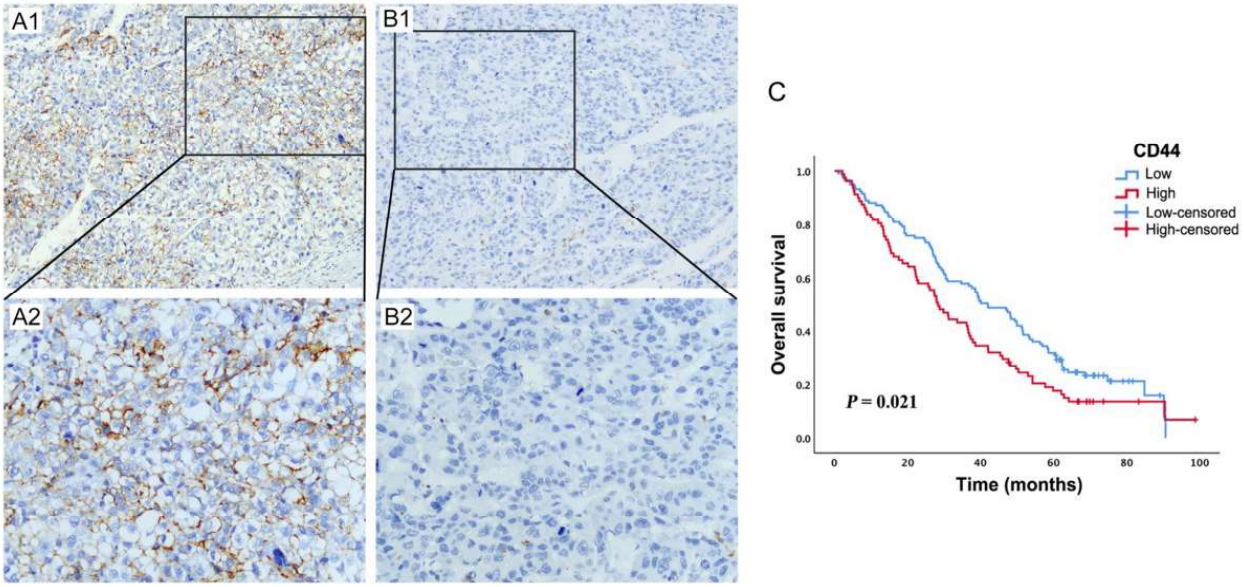

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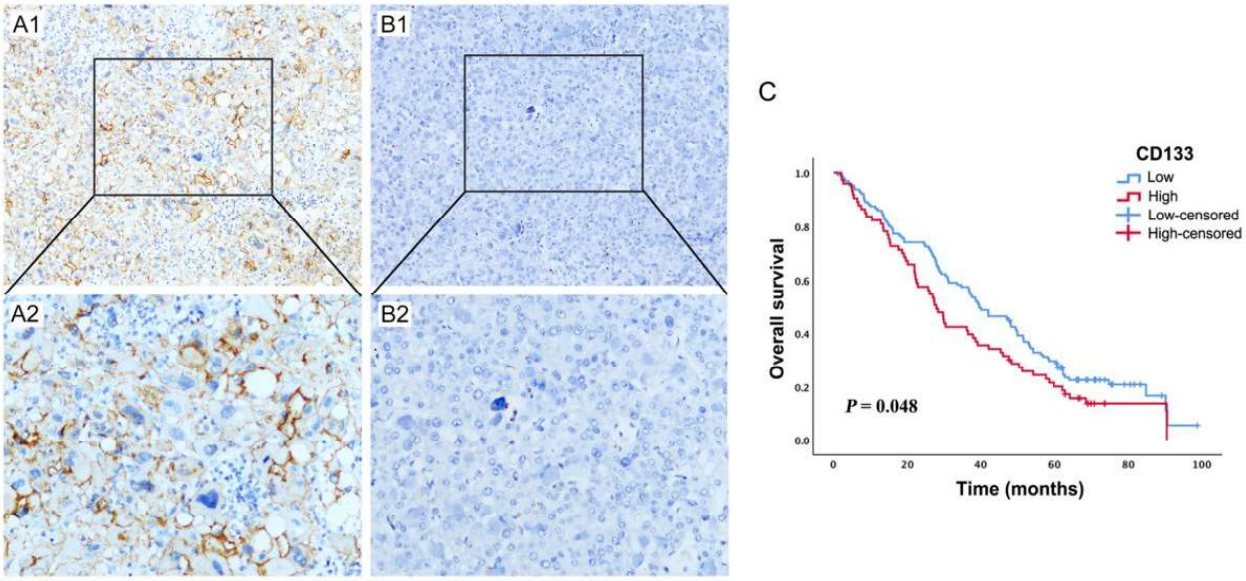

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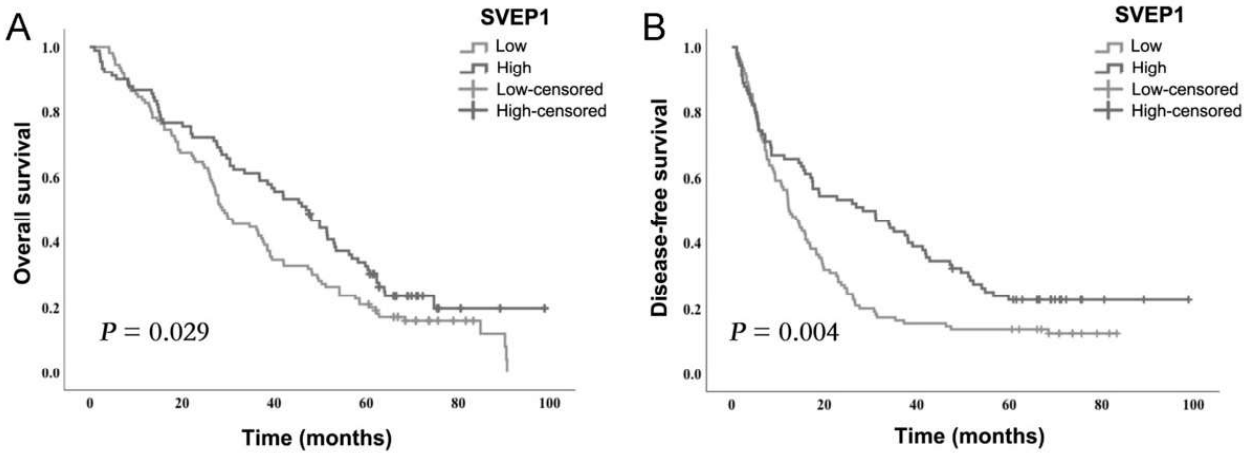

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