

RESEARCH ARTICLE

The High Expressed Serum Soluble Neural Cell Adhesion Molecule, a High Risk Factor Indicating Hepatic Encephalopathy in Hepatocellular Carcinoma Patients

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Abstract

Objective: To investigate whether the expression of serum soluble neural cell adhesion molecule (sNCAM) is associated with hepatic encephalopathy (HE) in hepatocellular carcinoma (HCC) patients. **Materials and Methods:** The Oncomine Cancer Microarray database was used to determine the clinical relevance of NCAM expression in different kinds of human cancers. Sera from 75 HCC cases enrolled in this study were assessed for expression of sNCAM by enzyme linked immunosorbent assay (ELISA). **Results:** Dependent on the Oncomine Cancer Microarray database analysis, NCAM was down regulated in 10 different kinds of cancer, like bladder cancer, brain and central nervous system cancer, while up-regulated in lung cancer, uterine corpus leiomyoma and sarcoma, compared to normal groups. Puzzlingly, NCAM expression demonstrated no significant difference between normal and HCC groups. However, we found by quantitative ELISA that the level of sNCAM in sera from HCC patients with HE (347.4 ± 151.9 ng/ml) was significantly more up-regulated than that in HCC patients without HE (260.3 ± 104.2 ng/ml), the p-value being 0.008. sNCAM may be an important risk factor of HE in HCC patients, the correlation coefficients was 0.278 ($P < 0.05$) on rank correlation analysis. **Conclusions:** This study highlights that up-regulated level of serum sNCAM is associated with HE in HCC patients and suggests that the high expression can be used as an indicator.

Keywords: Soluble neural cell adhesion molecule - hepatic encephalopathy - hepatocellular carcinoma

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignant cancer and ranks the third leading cause of death worldwide (Ferlay et al., 2010; Sangmala et al., 2014). In China, the HCC shows two significant characteristics: the high correlation with obvious liver cirrhosis and the bad therapeutical efficacy (Zhang et al., 2014). HCC is the second leading cause of cancer-related mortality among Chinese male and its five-year postoperative survival rate is only 30%-40% (Zhu, 2012; Guo et al., 2014).

Hepatic encephalopathy (HE) is a syndrome of neuropsychiatric dysfunction seen in patients with liver dysfunction after exclusion of other known brain disease (Ferenci et al., 2002). Patients with HE often go through mental status changes ranging from subtle psychologic abnormalities to profound coma (Munoz, 2008). It has been considered that the brain-blood barrier disturbances, altered neurotransmission,

neuroinflammation, oxidative stress, benzodiazepine pathway abnormalities, manganese neurotoxicity, brain energetic disturbances, and brain blood flow abnormalities are involved in the development of HE. While hyperammonemia may play the most important role in the pathogenesis of HE (Ciecko-Michalska et al., 2012). Although HE is usually a serious complication of acute liver failure and chronic liver diseases, predominantly liver cirrhosis, there are a certain number of HE resulting from HCC (Yoneyama et al., 2004). Over the years, a variety of concepts of pathogenesis have been put forth in an attempt to explain the pathogenesis of HE, but its molecular indicator is still poor understood.

Neural cell adhesion molecule (NCAM, also the cluster of differentiation CD56) as the first identified cell adhesion molecule was originally detected in the study of neurons (Jorgensen and Bock, 1974). It belongs to the immunoglobulin superfamily and always strongly expresses in the nervous system. It also could be detected expressing in heart, and skeletal muscles (Cunningham

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et al., 1987). NCAM is a cell surface glycoprotein involved in cell-cell interactions and can influence junctional communication, the association of axons with pathways and targets, as well as signals that alter levels of neurotransmitter enzymes (Rutishauser et al., 1988). It has been shown that NCAM is expressed in a variety of human tumours, including small cell lung cancer, neuroblastoma, rhabdomyosarcoma, brain tumours, and acute myeloid leukaemia (Jensen and Berthold, 2007). NCAM as a putative marker for the malignant stem/progenitor cell population has been suggested to use in the Wilms' tumour (WT) progenitor cell population (Podshakked et al., 2009). There is also evidence that NCAM can be employed to enrich for hepatic stem/progenitor cells in damaged livers and hepatocellular carcinomas (Tsuchiya et al., 2009).

Until now, researches about the expression of NCAM in HCC are few. A previous research showed that only an approximately 8.3% of the operated HCC tissue samples expressed NCAM (Tsuchiya et al., 2011). Nevertheless, there is still no report describing the expression of serum soluble neural cell adhesion molecule (sNCAM) in HCC patients with HE. In this study, we identified the expression of serum sNCAM was up regulated in HCC patients with HE by ELISA. This report indicated that the positive correlation between high expressed serum sNCAM and HE in HCC patients.

Materials and Methods

Specimen collection

75 HCC sera were collected at First Affiliated Hospital of Dalian Medical University for sNCAM ELISA. Access to these samples complied with both Chinese laws and the guidelines of the Ethics Committee. This study was approved by the Research Ethics Committee of First Affiliated Hospital of Dalian Medical University and Zhongshan Hospital, Fudan University. The informed consent was obtained from each patient and the clinical characteristic of these patients was described in Table 1. The diagnostic criteria of HE was complied with the 2014 practice guideline by the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases (Vilstrup et al., 2014).

Table 1. General Information of HCC Patients

	HCC patients with HE	HCC patients without HE
Number of individuals	46	29
Gender (male/female)	36 (78.3%)/ 10 (21.7%)	23 (79.3%)/ 6 (20.7%)
Age (years)	59±9	59±11
ALT (IU/L)	47.7±36.6	55.2±45.4
AST (IU/L)	70.9±71.2	98.3±101.9
AFP (IU/ml)	303.9±480.2	439.9±548.3
HbsAg(s/co) (1/0)	39 (84.8%)/ 7 (15.2%)	24 (82.8%)/ 5 (17.2%)
PT(s)	14.5±2.1	14.0±1.9

*Mean±standard deviation ALT, Alanine aminotransferase; AST, Aspartate transaminase; AFP, alpha fetoprotein; HbsAg, hepatitis B surface antigen; PT, Prothrombin time

Oncomine analysis

Oncomine Cancer Microarray database (<http://www.oncomine.org/>) was used to systematically assess expression levels of NCAM in various cancers tissues versus normal tissues. (Rhodes et al., 2004; Shan et al., 2015) Threshold by $P \leq 0.001$, fold change ≥ 2 and gene rank was top10%. The corresponding data sources used in this study were summarized in Table 2.

Enzyme linked immunosorbent assay (ELISA)

sNCAM was measured quantitatively in sera from 75 patients with HCC by using the NCAM1 (Human) ELISA Kit (Abnova), according to the manufacturer's protocol. Briefly, the diluted sera and standards were pipetted into the detective plate and incubated at 37°C for 90 min. Then biotinylated antibodies were pipetted. After incubating and washing, Avidin-Biotin-Peroxidase Complex (ABC) working solution was added, and finally, a color development step was performed. The O.D. absorbance values were read at 450 nm using the Infinite M200 (Tecan).

Statistics

The student t-test was used to compare two groups of parametric variants. The correlation of serum sNCAM expression and HE in HCC patients was evaluated with spearman rank correlation analysis. SPSS 17.0 was used to process the statistical analysis and GraphPad prism 5.0 was used to draw the graphs. $P \leq 0.05$ was considered statistically significant.

Results

NCAM expression in cancer tissues

To determine the clinical relevance of NCAM in different kinds of human cancers, NCAM expression in bladder cancer, brain and central nervous system cancer, breast cancer, cervical cancer, colorectal cancer, gastric cancer, head and neck cancer, lung cancer, lymphoma, other cancer (uterine corpus leiomyoma), ovarian cancer, prostate cancer, sarcoma and so on were from Oncomine Cancer Microarray database. We compared NCAM expression levels in cancer tissues to that in normal tissues with the threshold by p-value below 0.001, fold change ≥ 2 , gene rank was top10%. The result was listed in Figure 1 A. It indicated that 29 analyses met all of these conditions. The results demonstrated that NCAM expression was significantly changed in 13 different cancers versus normal tissues, respectively. In bladder cancer, brain and central nervous system cancer, breast cancer, cervical cancer, colorectal cancer, gastric cancer, head and neck cancer, lymphoma, ovarian cancer and prostate cancer, the expressions of NCAM were significantly decreased. While, in lung cancer, other cancer (uterine corpus leiomyoma) and sarcoma there were significantly higher expression levels of NCAM.

However, any studies of the NCAM expression in HCC versus normal tissues were outside the scope mentioned above. Oncomine Cancer Microarray database collected 8 analyses of the NCAM expression in HCC tissues versus normal tissues. While only one of them had a p-value

Table 2. Oncomine Microarray Data Were Used to Analyze the NCAM Expression in Human Cancers

Analysis type	Study	Sample type	Samples (n)	Year of the study	References
Normal vs. Cancer	Bladder Cancer	Infiltrating Bladder Urothelial Carcinoma	81	2006	(Sanchez-Carbayo et al., 2006)
		Superficial Bladder Cancer	28		
Brain and CNS Cancer	Bladder	Infiltrating Bladder Urothelial Carcinoma	13	2004	(Dyrskjot et al., 2004)
		Bladder	9		
	Brain Glioblastoma	Brain Glioblastoma	542	2013	The Cancer Genome Atlas
		Glioblastoma	5		
	Brain	Brain	10	2006	(Sun et al., 2006)
		Glioblastoma	81		
	Anaplastic Oligoastrocytoma	Brain	23	2005	(French et al., 2005)
		Anaplastic Oligodendroglioma	4		
	Anaplastic Oligodendroglioma	Brain	23	2005	(French et al., 2005)
		Brain	6		
Breast Cancer	Invasive Ductal Breast Carcinoma	Breast	35	2004	(Zhao et al., 2004)
		Breast	3		
		Intraductal Cribriform Breast Adenocarcinoma	3		
Breast	Breast	Intraductal Cribriform Breast Adenocarcinoma	61	2011	The Cancer s Genome Atla
		Breast	3		
Cervical Cancer	Cervical Squamous Cell Carcinoma	Cervix Uteri	40	2008	(Biewenga et al., 2008)
		Cervix Uteri	5		
Colorectal Cancer	Cecum Adenocarcinoma	Cecum Adenocarcinoma	22	2011	The Cancer Genome Atlas
		Colon Mucinous Adenocarcinoma	22		
		Rectal Mucinous Adenocarcinoma	6		
		Colon	19		
		Colon Adenoma	25		
Gastric Cancer	Gastric Cancer	Colon	25	2007	(Sabates-Bellver et al., 2007)
		Gastric Cancer	80		
		Gastric Tissue	80		
		Gastric Mixed Adenocarcinoma	4		
		Gastric Intestinal Type Adenocarcinoma	26		
Head and Neck Cancer	Tongue Squamous Cell Carcinoma	Gastric Mucosa	31	2009	(Estilo et al., 2009)
		Tongue	26		
		Thyroid Gland Papillary Carcinoma	9		
Lung Cancer	Thyroid Gland	Thyroid Gland	9	2005	(He et al., 2005)
		Lung Carcinoid Tumor	20		
		Small Cell Lung Carcinoma	6		
Lymphoma	Lung	Lung	17	2001	(Bhattacharjee et al., 2001)
		Small Cell Lung Carcinoma	4		
		Lung	5		
		Lung	5		
Other Cancer	Follicular Lymphoma	Follicular Lymphoma	5	2008	(Brune et al., 2008)
		Centroblast	5		
		Memory B-Lymphocyte	5		
		Naïve Pregerminal Center B-Lymphocyte	5		
		Plasma Cell	5		
Other Cancer	Small Cleaved Follicle Center Cell	Small Cleaved Follicle Center Cell	5	2009	(Crabtree et al., 2009)
		Uterine Corpus Leiomyoma	50		
Ovarian Cancer	Myometrium	Myometrium	27	2009	(Yoshihara et al., 2009)
		Ovarian Serous Adenocarcinoma	40		
Prostate Cancer	Peritoneum	Peritoneum	10	2001	(Welsh et al., 2001)
		Prostate Carcinoma	25		
Sarcoma	Prostate Gland	Prostate Gland	9	2010	(Barretina et al., 2010)
		Dedifferentiated Liposarcoma	46		
		Leiomyosarcoma	26		
		Adipose Tissue	9		

below 0.001 (Mas et al., 2009) and the fold change was only 1.3 (Figure 1 B). By contrast, there was no significant change of NCAM expression in HCC versus normal tissues. This result implied that NCAM may be not a good biomarker for HCC diagnosis.

The quantitative analysis of serum sNCAM expression in HCC patients with or without HE

We studied the expression levels of serum sNCAM by ELISA. 75 sera from HCC patients including 46 sera from HCC patients with HE and 29 sera without HE. The mean level of sNCAM in the sera of HCC patients with HE and without HE was 347.4 ± 151.9 ng/ml and 260.3 ± 104.2 ng/ml, respectively. The expression of serum sNCAM in HCC with HE was significantly up regulated compared to the

Table 3. Spearman Rank Correlation Coefficients and Probabilities between Serum sNCAM Expression and HE in HCC Patients

Spearman's rho		HE	sNCAM
HE	correlation coefficients	1.000	0.278*
	P value (2-tailed)	0.000	0.016
	N	75	75
sNCAM	correlation coefficients	0.278*	1.000
	P value (2-tailed)	0.016	0.000
	N	75	75

*N, number; *Correlation is significant at the 0.05 level (2-tailed)

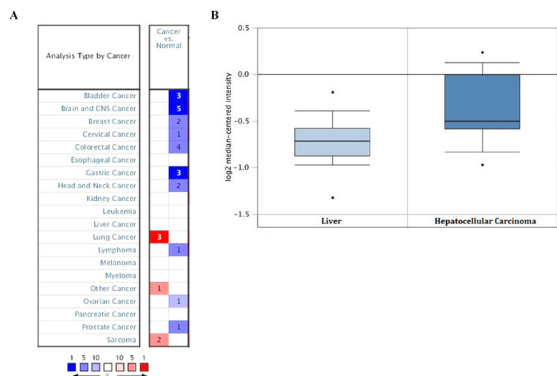


Figure 1. Expression of NCAM in Different Kinds of Cancers in Oncomine Database. (A) The significant unique analysis of the NCAM expression in various cancers tissues versus normal tissues. **(B)** NCAM expression in HCC tissues compared to normal livers in Mas Liver Study

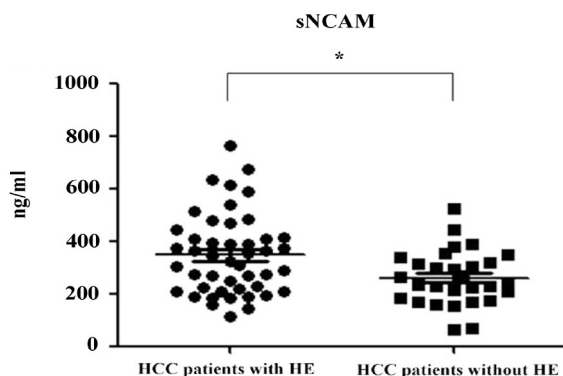


Figure 2. Expression of Serum sNCAM in HCC Patients with or without HE. ELISA was used to measure the expression of sNCAM in sera and the expression of serum sNCAM in HCC patients with HE was significantly up regulated compared to the expression in HCC patients without HE

expression in HCC patients without HE and the p-value was 0.008. Rank correlation analysis demonstrated that positive rank correlation existed between sNCAM and HE in HCC patients, the correlation coefficients was 0.278 ($P < 0.05$).

Discussion

Generally, HE was divided into three types due to its etiology and pathogenesis: type A associated with acute liver failure; type B related to portal-systemic bypass and no intrinsic hepatocellular disease; type C involved in cirrhosis and portal hyper-tension/or portal-systemic

shunts (Ferenci et al., 2002). In China, most HE patients were type C, type A and type B just occupied a relatively small minority. Recent advances have fostered a further understanding of the pathogenesis of HE, but the more detailed investigation about mechanism of HE is needed and it would be crucial to improve the therapeutic effect. If HE can be detected timely and prevented properly, it may be able to reduce the incidence and mortality of HE by taking active therapeutic measures.

In this study, we found expression of serum sNCAM in HCC patients with HE was significantly upregulated than that in HCC patients without HE. There are 3 major isoforms of NCAM as follows: NCAM-180, NCAM-140, and NCAM-120, with molecular masses of 180, 140, and 120 kDa, respectively. The NCAM-120 with no intracellular residues is linked to the membrane via a glycosyl-phosphatidylinositol (GPI) anchor, while NCAM-140 and NCAM-180, have intracellular parts of different lengths (Cunningham et al., 1987). Since NCAM could be released to serum by shedding with or without transmembrane domains as a detectable soluble form of NCAM (Tsuchiya et al., 2011). It implied that using a blood test to assess the sNCAM in the serum may be a convenient method for diagnosis and monitor of HE.

However, the diagnostic value of serum sNCAM in HCC patients with HE needs to be further validated in a large scale investigation and that how sNCAM participates in HE progression also needs to be evaluated. What is more, NCAM is an important glycoprotein with six possible N-linked glycosylation sites. It can carry high levels of the negatively charged polysialic acid (PSA) which consists of a 2-8 linked N-acetylneuraminic acid residues (Livingston et al., 1988). Whether the expression of PSA-NCAM is related to HE progression also requires to be further elucidated.

In conclusion, we report the expression level of NCAM in cancer tissues versus normal tissues according to Oncomine Cancer Microarray database and expression of serum sNCAM in HCC patients with HE was significantly up regulated compared with that in HCC patients without HE by ELISA. This is the first study to illustrate the elevated serum sNCAM expression is correlated with HE in HCC patients and which suggests that the HCC patients with high serum sNCAM expression may be at a high risk to encounter HE and should be paid more attentions to prevent and treat HE.

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