RESEARCH ARTICLE

The High Expressed Serum Soluble Neural Cell Adhesion Molecule, a High Risk Factor Indicating Hepatic Encephalopathy in Hepatocelular 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 hepatocelular 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, rhabdomyosarkoma, 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 (Pode-Shakked 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 Table2.

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		
		Bladder	48		
		Infiltrating Bladder Urothelial Carcinoma	13	2004	(Dyrskjot et al., 2004)
		Bladder	9		
	Brain and CNS Cancer	Brain Glioblastoma	542	2013	The Cancer Genome Atlas
		Glioblastoma	5		
		Brain	10		
		Glioblastoma	81	2006	(Sun et al., 2006)
		Brain	23		
		Anaplastic Oligoastrocytoma	4	2005	
		Anaplastic Oligodendroglioma	23		(French et al., 2005)
		Brain	6		
	Breast Cancer	Invasive Ductal Breast Carcinoma	35	2004	(Zhao et al., 2004)
		Breast	3		
		Intraductal Cribriform Breast Adenocarcinoma	3	2011	The Cancer s Genome Atla
		Breast	61		
	Cervical Cancer	Cervical Squamous Cell Carcinoma	40	2008	(Biewenga et al., 2008)
		Cervix Uteri	5		
	Colorectal Cancer	Cecum Adenocarcinoma	22	2011	
		Colon Mucinous Adenocarcinoma	22		The Cancer Genome Atlas
		Rectal Mucinous Adenocarcinoma	6		
		Colon	19		
		Colon Adenoma	25	2007	(Sabates-Bellver et al., 2007)
		Colon	25		
	Gastric Cancer	Gastric Cancer	80	2011	(Cui et al., 2011)
		Gastric Tissue	80		
		Gastric Mixed Adenocarcinoma	4	2009	(D'Errico et al., 2009)
		Gastric Intestinal Type Adenocarcinoma	26		
		Gastric Mucosa	31		
	Head and Neck Cancer	Tongue Squamous Cell Carcinoma	31	2009	(Estilo et al., 2009)
		Tongue	26		
		Thyroid Gland Papillary Carcinoma	9	2005	(He et al., 2005)
		Thyroid Gland	9		
	Lung Cancer	Lung Carcinoid Tumor	20	2001	(Bhattacharjee et al., 2001)
		Small Cell Lung Carcinoma	6		
		Lung	17		
		Small Cell Lung Carcinoma	4	2001	(Garber et al., 2001)
		Lung	5		
	Lymphoma	Follicular Lymphoma	5	2008	(Brune et al., 2008)
		Centroblast	5		
		Memory B-Lymphocyte	5		
		Naive Pregerminal Center B-Lymphocyte	5		
		Plasma Cell	5		
		Small Cleaved Follicle Center Cell	5		
	Other Cancer	Uterine Corpus Leiomyoma	50	2009	(Crabtree et al., 2009)
		Myometrium	27		
	Ovarian Cancer	Ovarian Serous Adenocarcinoma	40	2009	(Yoshihara et al., 2009)
		Peritoneum	10		
	Prostate Cancer	Prostate Carcinoma	25	2001	(Welsh et al., 2001)
		Prostate Gland	9		
	Sarcoma	Dedifferentiated Liposarcoma	46	2010	(Barretina et al., 2010)
		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)

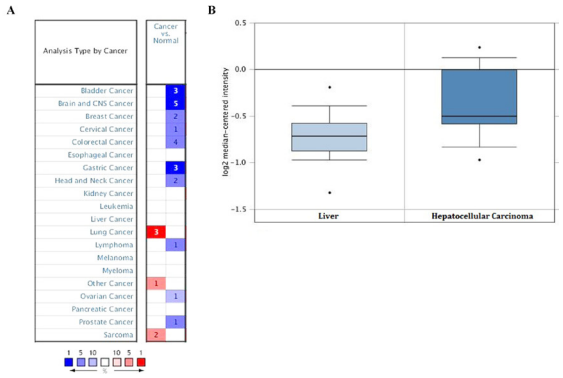

Figure 1. Expression of NCAM in Different Kinds of Cancers in Oncomine Database. (A) The significant unique analysis of the NCAM expression in various cancers tissues versus normal tissues. **(B)** NCAM expression in HCC tissues compared to normal livers in Mas Liver Study

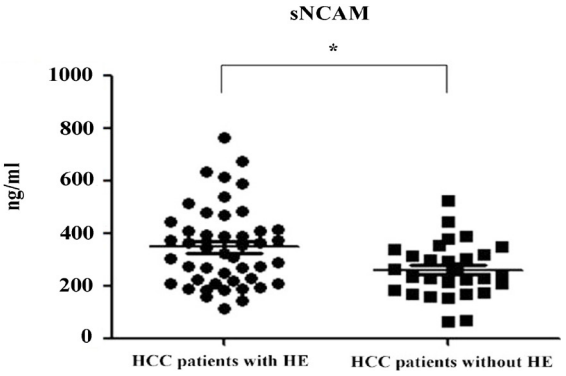

Figure 2. Expression of Serum sNCAM in HCC Patients with or without HE. ELISA was used to measure the expression of sNCAM in sera and the expression of serum sNCAM in HCC patients with HE was significantly up regulated compared to the expression in HCC patients without HE

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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References

Barretina J, Taylor BS, Banerji S, et al (2010). Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy. *Nat Genet*, **42**, 715-21.

Bhattacharjee A, Richards WG, Staunton J, et al (2001). Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. *Proc Natl Acad Sci USA*, **98**, 13790-5.

Biewenga P, Buist MR, Moerland PD, et al (2008). Gene expression in early stage cervical cancer. *Gynecol Oncol*, **108**, 520-6.

Brune V, Tiacci E, Pfeil I, et al (2008). Origin and pathogenesis of nodular lymphocyte-predominant Hodgkin lymphoma as revealed by global gene expression analysis. *J Exp Med*, **205**, 2251-68.

Ciecko-Michalska I, Szczepanek M, Slowik A, et al (2012). Pathogenesis of hepatic encephalopathy. *Gastroenterol Res Pract*, **2012**, 642108.

Crabtree JS, Jelinsky SA, Harris HA, et al (2009). Comparison of human and rat uterine leiomyomata: identification of a dysregulated mammalian target of rapamycin pathway. *Cancer Res*, **69**, 6171-8.

Cui J, Chen Y, Chou WC, et al (2011). An integrated transcriptomic and computational analysis for biomarker identification in gastric cancer. *Nucleic Acids Res*, **39**, 1197-207.

Cunningham BA, Hemperly JJ, Murray BA, et al (1987). Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science*, **236**, 799-806.

D'Errico M, de Rinaldis E, Blasi MF, et al (2009). Genome-wide expression profile of sporadic gastric cancers with microsatellite instability. *Eur J Cancer*, **45**, 461-9.

Dyrskjot L, Kruhoffer M, Thykjaer T, et al (2004). Gene expression in the urinary bladder: a common carcinoma in situ gene expression signature exists disregarding histopathological classification. *Cancer Res*, **64**, 4040-8.

Estilo CL, P Oc, Talbot S, et al (2009). Oral tongue cancer gene expression profiling: Identification of novel potential prognosticators by oligonucleotide microarray analysis. *BMC Cancer*, **9**, 11.

Ferenci P, Lockwood A, Mullen K, et al (2002). Hepatic encephalopathy-definition, nomenclature, diagnosis, and quantification: final report of the working party at the 11th World Congresses of Gastroenterology, Vienna, 1998. *Hepatology*, **35**, 716-21.

Ferlay J, Shin HR, Bray F, et al (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer*, **127**, 2893-917.

French PJ, Swagemakers SM, Nagel JH, et al (2005). Gene expression profiles associated with treatment response in oligodendrogliomas. *Cancer Res*, **65**, 11335-44.

Garber ME, Troyanskaya OG, Schluens K, et al (2001). Diversity of gene expression in adenocarcinoma of the lung. *Proc Natl Acad Sci USA*, **98**, 13784-9.

Guo X, Xiong L, Yu L, et al (2014). Increased level of nucleolin confers to aggressive tumor progression and poor prognosis in patients with hepatocellular carcinoma after hepatectomy. *Diagn Pathol*, **9**, 175.

He H, Jazdzewski K, Li W, et al (2005). The role of microRNA genes in papillary thyroid carcinoma. *Proc Natl Acad Sci USA*, **102**, 19075-80.

Jensen M, Berthold F (2007). Targeting the neural cell adhesion molecule in cancer. *Cancer Lett*, **258**, 9-21.

Jorgensen OS, Bock E (1974). Brain specific synaptosomal membrane proteins demonstrated by crossed immunoelectrophoresis. *J Neurochem*, **23**, 879-80.

Livingston BD, Jacobs JL, Glick MC, et al (1988). Extended polysialic acid chains (n greater than 55) in glycoproteins from human neuroblastoma cells. *J Biol Chem*, **263**, 9443-8.

Mas VR, Maluf DG, Archer KJ, et al (2009). Genes involved in viral carcinogenesis and tumor initiation in hepatitis C virus-induced hepatocellular carcinoma. *Mol Med*, **15**, 85-94.

Munoz SJ (2008). Hepatic encephalopathy. *Med Clin North Am*, **92**, 795-812.

Pode-Shakked N, Metsuyanim S, Rom-Gross E, et al (2009). Developmental tumourigenesis: NCAM as a putative marker for the malignant renal stem/progenitor cell population. *J Cell Mol Med*, **13**, 1792-808.

Rhodes DR, Yu J, Shanker K, et al (2004). ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia*, **6**, 1-6.

Rutishauser U, Acheson A, Hall AK, et al (1988). The neural cell adhesion molecule (NCAM) as a regulator of cell-cell interactions. *Science*, **240**, 53-7.

Sabates-Bellver J, Van der Flier LG, de Palo M, et al (2007). Transcriptome profile of human colorectal adenomas. *Mol Cancer Res*, **5**, 1263-75.

Sanchez-Carbayo M, Socci ND, Lozano J, et al (2006). Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays. *J Clin Oncol*, **24**, 778-89.

Sangmala P, Chaikledkaew U, Tanwandee T, et al (2014). Economic evaluation and budget impact analysis of the surveillance program for hepatocellular carcinoma in Thai chronic hepatitis B patients. *Asian Pac J Cancer Prev*, **15**, 8993-9004.

Shan YS, Hsu HP, Lai MD, et al (2015). Increased expression of argininosuccinate synthetase protein predicts poor prognosis in human gastric cancer. *Oncol Rep*, **33**, 49-57.

Sun L, Hui AM, Su Q, et al (2006). Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain. *Cancer Cell*, **9**, 287-300.

Tsuchiya A, Kamimura H, Takamura M, et al (2009). Clinicopathological analysis of CD133 and NCAM human hepatic stem/progenitor cells in damaged livers and hepatocellular carcinomas. *Hepatol Res*, **39**, 1080-90.

Tsuchiya A, Kamimura H, Tamura Y, et al (2011). Hepatocellular carcinoma with progenitor cell features distinguishable by the hepatic stem/progenitor cell marker NCAM. *Cancer Lett*, **309**, 95-103.

Vilstrup H, Amodio P, Bajaj J, et al (2014). Hepatic encephalopathy in chronic liver disease: 2014 practice guideline by the American association for the study of liver diseases and the european association for the study of the liver. *Hepatology*, **60**, 715-35.

Welsh JB, Sapinoso LM, Su AI, et al (2001). Analysis of gene expression identifies candidate markers and pharmacological targets in prostate cancer. *Cancer Res*, **61**, 5974-8.

Yoneyama K, Nebashi Y, Kiuchi Y, et al (2004). Prognostic index of cirrhotic patients with hepatic encephalopathy with and without hepatocellular carcinoma. *Dig Dis Sci*, **49**, 1174-80.

Yoshihara K, Tajima A, Komata D, et al (2009). Gene expression profiling of advanced-stage serous ovarian cancers distinguishes novel subclasses and implicates ZEB2 in tumor progression and prognosis. *Cancer Sci*, **100**, 1421-8.

Zhang ZM, Zhang YM, Gao S, et al (2014). Treatment efficacy and prognostic factors for huge HCC based on barcelona clinic liver cancer staging. *Asian Pac J Cancer Prev*, **15**, 8823-8.

Zhao H, Langerod A, Ji Y, et al (2004). Different gene expression patterns in invasive lobular and ductal carcinomas of the breast. *Mol Biol Cell*, **15**, 2523-36.

Zhu AX (2012). Molecularly targeted therapy for advanced hepatocellular carcinoma in 2012: current status and future perspectives. *Semin Oncol*, **39**, 493-502.