Int J Clin Exp Pathol 2015;8(6):6589-6595
www.ijcep.com /ISSN:1936-2625/IJCEP0007570

Original Article

Overexpression of PROM1 (CD133) confers poor prognosis in non-small cell lung cancer

Zhi-Xin Qiu[1], Shuang Zhao[1], Xian-Ming Mo[2], Wei-Min Li[1]

[1]*Department of Respiratory Medicine, West China Hospital, Sichuan University, Chengdu 610041, China;* [2]*Laboratory of Stem Cell Biology, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, China*

Received March 4, 2015; Accepted April 21, 2015; Epub June 1, 2015; Published June 15, 2015

Abstract: The surface marker PROM1 is considered one of the most important marker of tumor-initiating cells, and its high expression is believed to be an adverse prognostic factor in gliomas, medulloblastoma and in other malignancies. The aims of our research were to explore the expression profile of the PROM1 in non-small cell lung cancer (NSCLC) and to assess its possible role as a prognostic factor. The protein expression profiles were determined via immunohistochemical staining assay. The clinical prognostic values of protein expression were investigated with univariate and multivariate survival analysis. The quantitative variable PROM1 expression was dichotomized according to the best cutoff value obtained by the receiver operating characteristics (ROC) analysis. The protein level of PROM1 of NSCLC was higher compared with normal tissues, and the survival analysis demonstrated the positive membrane expression and combination of membrane/cytoplasm groups of PROM1 had worse prognosis than those negative expression groups. Also, multivariate Cox regression analysis showed membrane expression of PROM1 and lymph node invasion were the independent prognostic factors. The expression of PROM1 was significantly higher than normal tissue, and high levels of PROM1 membrane expression and combination of membrane/cytoplasm expression were associated with adverse prognosis.

Keywords: PROM1, CD133, non-small cell lung cancer, prognosis

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide and the incidence increased year by year. Non-small cell lung cancer (NSCLC) is the main type of lung cancer. CD133, a member of prominent family, formerly known as PROML-1 or AC133, was first discovered as a pentaspan transmembrane glycoprotein of murine neuroepithelial stem cells located in plasma membrane protrusions [1]. CD133 is found in embryonic stem cells, normal tissue stem cells, stem cell niches, and circulating endothelial progenitors as well as cancer stem cells. Its antigen has been identified as a putative stem cell marker in normal and malignant brain tissues. According to the cancer stem cell hypothesis, PROM1 (CD133) - positive cells determine long-term tumor growth and, therefore, are suspected to influence clinical outcome [2]. Although most researchers had studied PROM1 expression in correlation with prognosis and clinicopathological variables, that indicated high PROM1 expression is associated with decreased survival in a variety of human tumors, including brain, liver, stomach, endometrium, ovary, colorectum, gliomas [2] and medulloblastoma [3], to date, the relationship between PROM1 and NSCLC lack of in-depth study. Thus, in this study, we aimed to evaluate the expression profile of the PROM1 in NSCLC with use of immunohistochemical staining assay and to determine its possible prognostic significance.

Material and methods

Tissue collection

One hundred and eighty-three surgically resected primary NSCLC cases, during the period from 2007 to 2008, were obtained from the archives of the Pathology Department of West China Hospital, Sichuan University, 175 cases of NSCLC patients included in survival analysis at last. A total of 96 normal control tissue sam-

Table 1. Expression of PROM1 protein in NSCLC and normal control group

Location	Total (No.)	Positive expression (No. %)	Negative expression (No. %)	*P* value
Normal tissue	96	14 (14.6%)	82 (85.4%)	Ref.
Tumor membrane	175	81 (46.3%)	94 (53.7%)	*<0.001*[a]
Tumor cytoplasm	183	107 (58.5%)	76 (41.5%)	*<0.001*[a]
Tumor nucleus	28	14 (50%)	14 (50%)	*<0.001*[a]

[a]Statistically significant.

ples were randomly taken from the normal tissues that adjacent to the tumor tissues according to the surgically resected. Data on stage were according to the International Union Against Cancer's tumor-node-metastasis system, and differentiation and histological type were according to the World Health Organization classification for NSCLC [4, 5]. The tissue specimens consisted of 91 adenocarcinomas (ADC), 74 squamous cell carcinomas (SCC), and 10 other types. All the tissues were fixed in 10% formalin immediately and embedded with paraffin within 12 to 24 hours post resection. All patients were adjuvant therapy-free before surgical resection and underwent standard therapeutic procedure after surgical resection according to the Clinical Oncology Information Network guidelines for nonsurgical management of lung cancer [6]. Institutional review board approval for this research was obtained from West China Hospital. All the participants provide their verbal informed consent to participate in this study. Because all the patients came from different parts of China, it is really difficult to let them written consent, but we have their contact information, so we ask them if they agree to participate in our research by telephone. If "yes", we included these patients' tissues. The ethics committees in our hospital (Medical Ethics Committee of Sichuan University) approve this consent procedure.

Antibody preparation

All the cases of lung cancer tissue samples underwent Envision two-step immunohistochemical staining. Primary antibodies used as follows: Prominin-1 mouse monoclonal antibody (17A6.1, 1:100 dilution, #MAB4399, Millipore). Secondary antibodies of Dako EnVision were purchased from Dako Corporation. All paraffin tissues were made of 4 μm slices. Envision method according to kit instructions, and antigen retrieval was done by heating Tris/ethylenediaminetetraacetic acid retrieval solution (pH 6.0) at 95°C for 45 minutes in water bath. The method of immunohistochemical staining according to the literature [7].

Immunohistochemical scoring

We used dual-rate semi-quantitative method according to the literature [7], and the scores composed of stained area and staining intensity of the tumor cells. Evaluation of sections was carried out by 2 pathologist (Drs. DN Liang and XS Qiu) without the knowledge of clinical information as previous described. The fraction score was defined as the average of 10 randomly selected fields by light microscope: 0, no tumor cell stained; 1, <20% of cells stained; 2, 20% to 50% of cells stained; and 3, >50% of cells stained. The intensity score was defined as follows: 0, no appreciable staining in the tumor cells; 1, barely detectable staining in the tumor cells; 2, readily appreciable brown staining; and 3, dark brown staining in tumor cells. The total score was calculated by multiplying the fraction score and the intensity score, producing a total range from 0 to 9. Then the quantitative variable PROM1 expression was dichotomized according to the best cutoff value obtained by the receiver operating characteristics (ROC) analysis (Supplementary 1: ROC analysis).

Statistical analysis

The association of clinical characteristics with status of protein expression was determined by Pearson chi-square test. The Kaplan-Meier method was used to estimate univariate survival. The log-rank test and univariate Cox regression analysis were used to compare survival distributions between positive and negative staining groups. Independent prognostic factors of survival were identified with a multivariate Cox regression analysis. "PROM1 expression" was dichotomized by the ROC. $P<0.05$ (2-side) was considered to be statistically significant. Data analysis and summarization were conducted using SPSS 17.0 for Windows (SPSS Inc., Chicago, III) [7] and Graphpad. prism. 6. x. C.

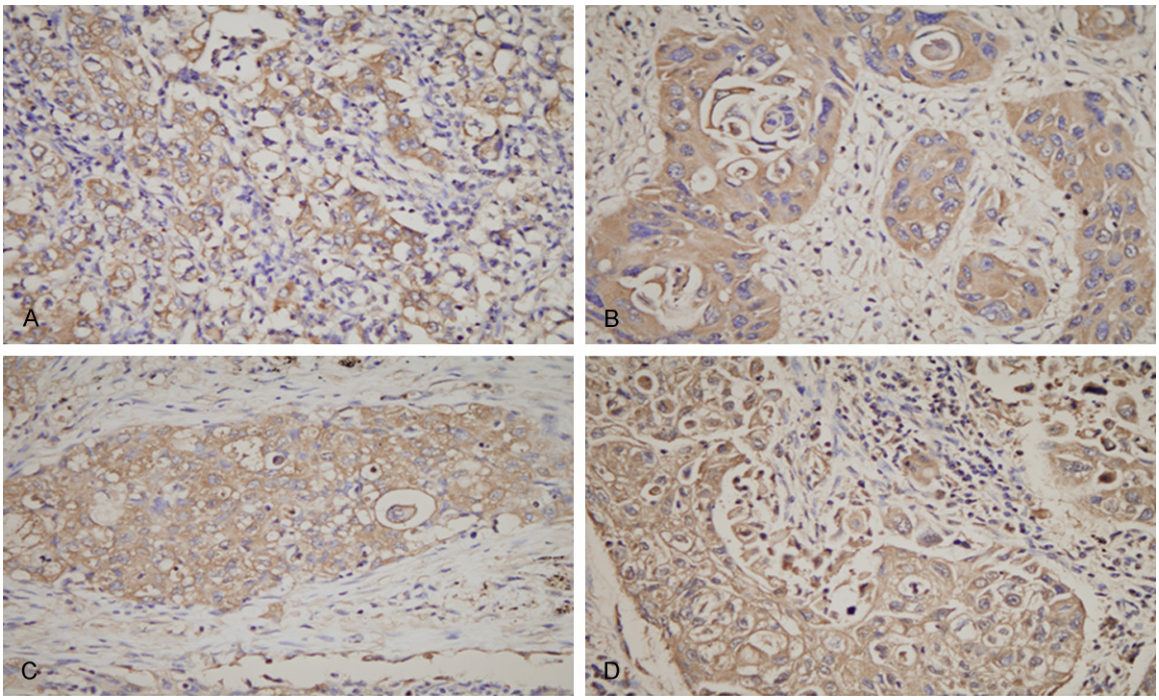


Figure 1. Representative examples of PROM1/CD133 expression at different locations. A. Positive expression of tumor cell membrane; B. Positive expression of tumor cell cytoplasm; C. Positive expression of tumor cell nuclear membrane; D. Positive expression of tumor cell nuclear. Red arrows shown. Original magnification, × 400.

Table 2. Association between clinical variables and PROM1 (CD133) expression

Variable	Membrane expression		*P* value	Cytoplasm expression		*P* value
	Positive	Negative		Positive	Negative	
Gender						
Male	67	63	*0.018*[a]	76	54	0.391
Female	14	31		23	22	
Age						
≤60	36	55	0.063	46	45	0.094
>60	45	39		53	31	
Histological type						
SCC	34	40	0.665	45	29	0.128
ADC	41	50		46	45	
Others	6	4		8	2	
Differentiation						
Poor	26	28	0.168	32	22	0.850
Moderate	27	21		27	21	
Well	28	44		39	33	
Tumor size						
T1	13	18	0.992	16	15	0.213
T2	40	43		47	36	
T3	14	15		21	8	
T4	14	18		15	17	
Lymph node invasion						
N0	43	53	0.500	53	43	0.190
N1	21	16		25	12	
N2	16	24		19	21	

N3	1	1		2	0	
Distant metastasis						
M0	77	90	0.829	103	72	0.619
M1	4	4		4	4	
Clinical stage						
I	29	35	0.587	37	27	0.319
II	22	18		27	13	
III	26	37		31	32	
IV	4	4		4	4	

ADC, Adenocarcinoma; SCC, Squamous cell carcinoma; [a]Statistically significant.

Table 3. Relation between clinical variables and survival

Variable	Category	Available No. for Survival (Died/Total)	Median survival (Mean/mo.)	Log-rank *P*
Gender	Male	71/130	45/43.856 ± 2.384	0.064
	Female	17/45	-/51.978 ± 3.844	
Age	<60	40/84	46/44.253 ± 2.859	0.451
	≥60	48/91	-/47.786 ± 2.915	
Histological type	ADC	48/91	46/45.253 ± 2.769	0.832
	SCC	34/74	-/46.459 ± 3.287	
	Others	6/10	46/47.300 ± 6.989	
Differentiation	Poor-Moderate	59/121	-/46.121 ± 2.500	0.727
	Well	29/54	49/45.556 ± 3.545	
Tumor size	T1	16/31	47/44.548 ± 4.917	0.967
	T2	40/83	-/46.910 ± 2.978	
	T3	15/29	54/46.000 ± 4.921	
	T4	17/32	46/44.281 ± 4.693	
Lymph node invasion	N0	36/96	-/52.271 ± 2.629	<0.001[a]
	N1	24/37	36/40.378 ± 4.190	
	N2	26/40	35/37.650 ± 4.354	
Distant metastasis	M0	82/167	-/46.608 ± 2.089	0.094
	M1	6/8	15/30.375 ± 7.756	
Clinical stage	I	25/64	-/51.672 ± 3.229	0.034[a]
	II	19/40	-/48.025 ± 4.172	
	III	38/63	37/40.571 ± 3.447	
	IV	6/8	15/30.375 ± 7.756	

ADC, Adenocarcinoma; SCC, Squamous cell carcinoma; No, number; mo, month; [a]Statistically significant.

Results

Comparison of expression levels of proteins in non-small cell lung cancers versus normal control tissue

The positive expression of PROM1 could be detected in the cell membrane, cytoplasm, nuclear membrane (only a few cases) and nucleus. Comparison of PROM1 expression in NSCLC with normal controls is shown in **Table 1**. Protein level of PROM1 was significantly increased (all $P<0.001$) in NSCLC compared with normal controls. Representative examples of PROM1 expression at different locations are shown in **Figure 1**.

Relationship between protein expression profiles and clinical characteristics of NSCLC

The relationship between protein phenotypes and clinical variables was estimated by the univariate analysis (**Table 2**). The membrane expression of PROM1 was associated with gender ($P = 0.018$). As it shown, positive membrane expression of PROM1 was much more in men than in women.

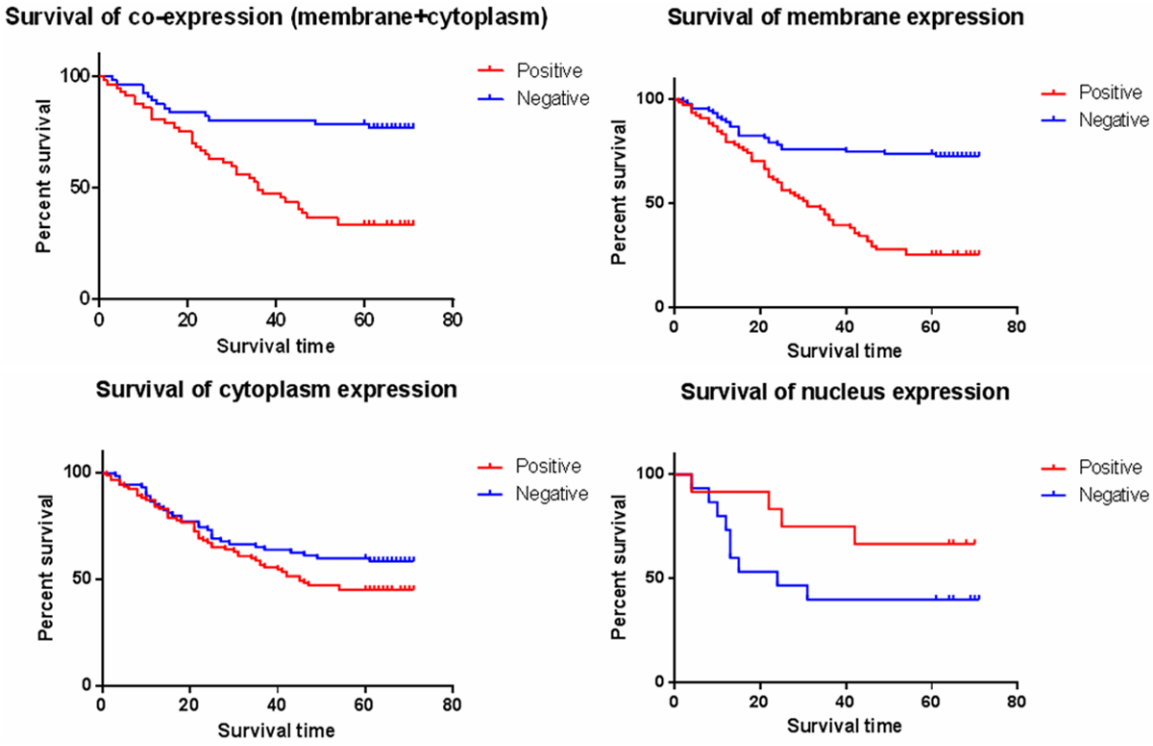


Figure 2. Relation between protein expression and survival are shown. Kaplan-Meier curves of proteins compare positive (red lines) with negative phenotype (blue lines).

Table 4. Relation between protein expression and survival (Univariate analysis)

Protein	P/N	HR	95% CI	*P* value
Membrane expression	P vs. N	3.521	2.232-5.556	*<0.001*[a]
Cytoplasm expression	P vs. N	1.475	0.955-2.278	0.080
Nuclear expression	P vs. N	0.526	0.175-1.580	0.252
Co-expression of membrane and cytoplasm	P vs. N	3.922	2.092-7.407	*<0.001*[a]

P/N indicates positive/negative; HR, hazard ratio; CI, confidence interval; [a]Statistically significant.

Association of clinical characteristics with survival

Clinical pathology data was missing in a small fraction of the cases that were excluded from the survival analysis. The relationship between the survival of patients and clinical pathological features for evaluable cases is shown in **Table 3**. It was indicated that the lymph node invasion and clinical stage were associated with survival (all $P<0.05$).

Comparison of survival among different status of single and combined proteins expression

In the total 175 cases of NSCLC patients, the 5-year survival rates of PROM1 with membrane positive and negative expression were 24.7% and 71.3% respectively, the median survival time were (35.012 ± 2.699) months and (55.367 ± 2.657) months, the difference was statistically significant ($P<0.001$). The 5-year survival rates of PROM1 with cytoplasm positive and negative expression were 43.4% and 57.9% respectively, the median survival time were (43.081 ± 2.713) months and (49.681 ± 3.063) months, there has no statistical difference ($P = 0.1170$). Additionally, The 5-year survival rates of PROM1 with nuclear positive and negative expression were 61.5% and 40.0% respectively, the median survival time were (50.231 ± 7.414) months and (37.067 ± 7.321) months, there has no statistical difference ($P = 0.1288$). Compared the 5-year survival rate between the expression was positive with both membrane and cytoplasm and the expression

Table 5. Multivariate survival analysis (Cox regression model)

Variants	HR	95% CI	*P* value
Lymph Node Invasion	9.709	1.805-52.632	*0.008*[a]
Clinical Stage	1.217	0.399-3.704	0.730
Membrane expression	3.774	2.374-6.061	*<0.001*[a]

HR, hazard ratio; CI, confidence interval; [a]Statistically significant.

was negative with both membrane and cytoplasm, the rates were 32.2% and 76.8% respectively, with the median survival time were (39.119 ± 3.313) months and (59.032 ± 3.096) months, the difference was statistically significant ($P<0.001$) (**Figure 2**). Furthermore, we assessed the prognosis value of expression of proteins (**Table 4**).

Multivariate Cox regression analysis for prognosis

According to the results above, by using multivariate Cox regression model, we evaluated whether membrane expression of PROM1, lymph node invasion and clinical stage could have prognosis value in the assessment of NSCLC (**Table 5**). The analysis revealed that membrane expression of PROM1 and lymph node invasion were independent prognosis factors for NSCLC development (log-rank, all $P<0.05$) (Supplementary 2: Multivariate Survival Analysis (Cox Regression Model)).

Discussion

Our research suggests that there is a significant relationship between high membrane protein levels of PROM1 and the prognosis of NSCLC, finding that PROM1 expression is a potential predictor of survival independent to clinical variables.

During the past years, great advances have been made in developing therapeutic approaches for NSCLC, but the best method to stratify patients with NSCLC into prognostic risk groups and stratify by the optimal treatment is unknown. Cancer stem cell (CSC), also called tumor-initiating cells (TICs), have self-renewal capacity and can produce heterogeneity of tumor cells, it may cause tumors aggressive too [8, 9]. The expression of the PROM1 (CD133) gene, which encoding a 5 transmembrane domain protein, has the feature that identifies brain TICs [10, 11], it also has been used as a marker for purifying CSC in many other solid tumors, including liver, colon, pancreas, prostate and melanoma [12-16]. In addition, high PROM1 expression is associated with poor survival in various human solid tumors, including colon, prostate, etc. However, these studies are limited by relatively small sample size and retrospective study design and thus limit definitive conclusions. Thus, targeting and monitoring PROM1 may lead to significant advances in outcome prediction and cancer therapy.

This study via immunohistochemical analysis found that expression level of PROM1 was significantly increased in NSCLC compared with normal controls. The membrane expression of PROM1 was related to the gender, and the positive expression in men was significantly higher than women (all $P<0.05$). To define a cutoff value that could dichotomize the range of quantitative variables of PROM1 expression, a ROC curve was calculated. In this research, combined with the results of immunohistochemical staining and the 5-year median survival time, found that the 5-year median survival time of membrane positive group of PROM1 and combination of membrane/cytoplasm positive were obviously shorter than the negative groups ($P<0.05$). In addition, based on the results before, we analyzed whether the membrane expression of PROM1, lymph node invasion and clinical stage were as the independent prognostic factors in NSCLS. The results indicated that only PROM1 membrane expression and lymph node invasion indeed as the independent prognostic factors in NSCLS (log-rank, all $P<0.05$).

As we all known that the mechanisms regulating tumorigenesis and progression is multifactorial, so it's hard and insufficiently to use only one biomarker to optimally stratify patients with NSCLC. But, we consider that PROM1 may play a crucial role in determining the patient's outcomes and may be helpful in making treatment strategies.

In summary, the expression of PROM1 was significantly higher in tissues of patients with NSCLC than in normal tissues, and high levels of PROM1 membrane expression and combination of membrane/cytoplasm expression were associated with adverse prognosis. However, in order to incorporate PROM1 expression into risk classification system to be used in the clini-

cal setting, the results of our study need to be confirmed in larger prospective researches.

Acknowledgements

We thank the patients and their families for participating in this study with patience and cooperation, and technicians in the Department of Pathology, West China Hospital, Sichuan University, who assisted us in collecting the tissue samples. This work was supported by the National Natural Science Foundation of China (No. 81372504); the Science and Technology Support Program of Science and Technology Department of Sichuan Province (2014SZ-0148); the International Cooperation Program of Science and Technology Department of Sichuan Province (2014AA022202-2).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Wei-Min Li, Department of Respiratory Medicine, West China Hospital, Sichuan University, Chengdu 610041, P. R. China. Tel: 86-2885423998; E-mail: weimi003@yahoo.com

References

[1] Fargeas CA, Corbeil D and Huttner WB. AC133 antigen, CD133, prominin-1, prominin-2, etc.: prominin family gene products in needof a rational nomenclature. Stem Cells 2003; 21: 506-508.

[2] Zeppernick F, Ahmadi R, Campos B, Dictus C, Helmke BM, Becker N, Lichter P, Unterberg A, Radlwimmer B and Herold-Mende CC. Stem cell marker CD133 affects clinical outcome in glioma patients. Clin Cancer Res 2008; 14: 123-129.

[3] Raso A, Mascelli S, Biassoni R, Nozza P, Kool M, Pistorio A, Ugolotti E, Milanaccio C, Pignatelli S, Ferraro M, Pavanello M, Ravegnani M, Cama A, Garrè ML and Capra V. High levels of PROM1 (CD133) transcript are a potential predictor of poor prognosis in medulloblastoma. Neuro Oncol 2011; 13: 500-508.

[4] Travis WD, Brambilla E, Muller-Hermelink HK and Harris CC. Pathology & Genetics: Tumours of the Lung, Pleura, Thymus and Heart. Lyon, France: IARC Press; 2004.

[5] International Union Against Cancer. TNM Classification of Malignant Tumors. 6th edition. New York, NY: Wiley & Sons; 2002.

[6] The Royal College of Radiologists Clinical Oncology Information Network. Guidelines on the non-surgical management of lung cancer. Clin Oncol (R Coll Radiol) 1999; 11: S1-S53.

[7] Liu D, Huang Y, Chen B, Zeng J, Guo N, Zhang S, Liu L, Xu H, Mo X and Li W. Activation of mammalian target of rapamycin pathway confers adverse outcome in nonsmall cell lung carcinoma. Cancer 2011; 117: 3763-3773.

[8] Jordan CT, Guzman ML and Noble M. Cancer stem cells. N Engl J Med 2006; 355: 1253-1261.

[9] Neuzil J, Stantic M, Zobalova R, Chladova J, Wang X, Prochazka L, Dong L, Andera L and Ralph SJ. Tumour-initiating cells vs. cancer 'stem' cells and CD133: what's in the name? Biochem Biophys Res Commun 2007; 355: 855-859.

[10] Miraglia S, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, Bray RA, Waller EK and Buck DW. A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. Blood 1997; 90: 5013-5021.

[11] Corbeil D, Röper K, Hellwig A, Tavian M, Miraglia S, Watt SM, Simmons PJ, Peault B, Buck DW and Huttner WB. The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions. J Biol Chem 2000; 275: 5512-5520.

[12] Yin S, Li J, Hu C, Chen X, Yao M, Yan M, Jiang G, Ge C, Xie H, Wan D, Yang S, Zheng S and Gu J. CD133 positive hepatocellular carcinoma cells possess high capacity for tumorigenicity. Int J Cancer 2007; 120: 1444-1450.

[13] O'Brien CA, Pollett A, Gallinger S and Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007; 445: 106-110.

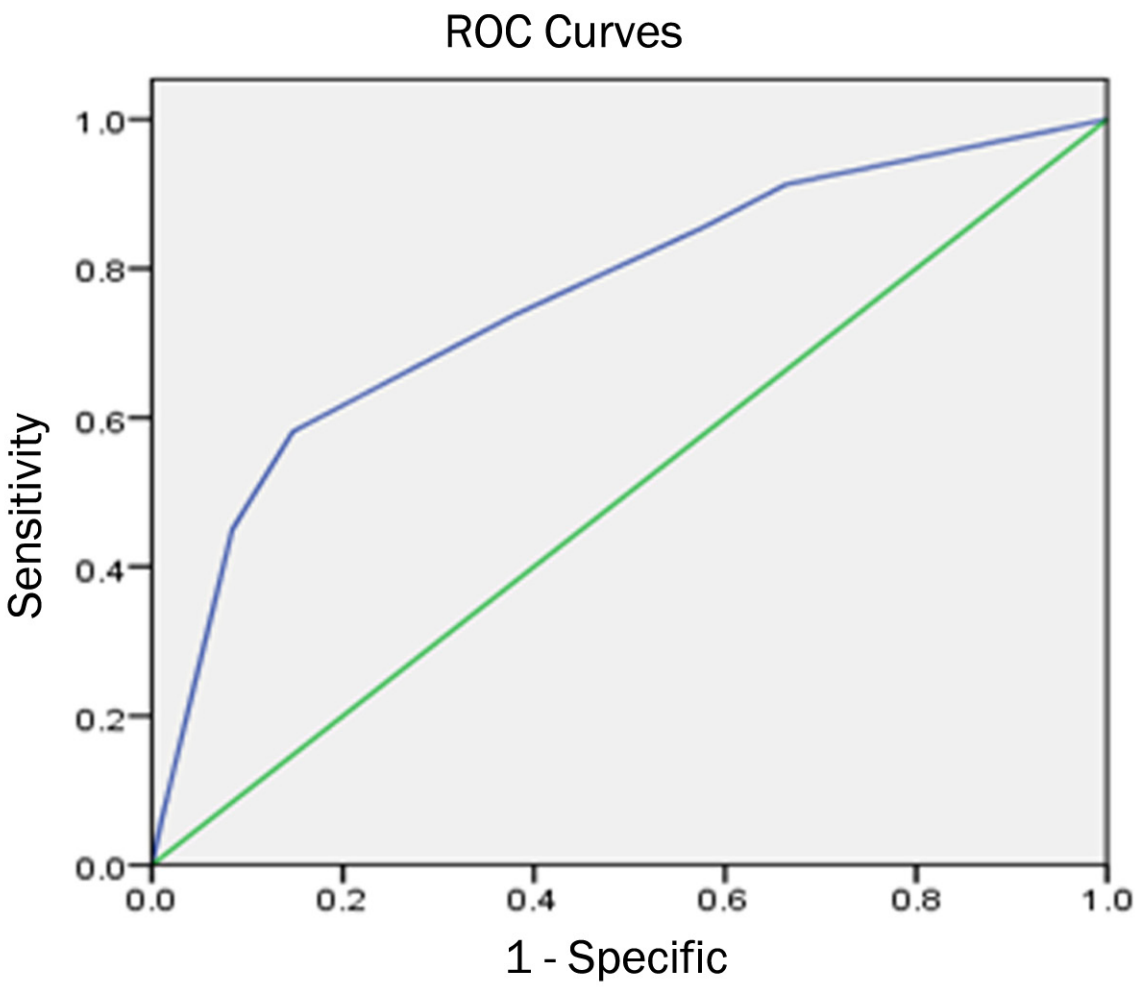
[14] Olempska M, Eisenach PA, Ammerpohl O, Ungefroren H, Fandrich F and Kalthoff H. Detection of tumor stem cell markers in pancreatic carcinoma cell lines. Hepatobiliary Pancreat Dis Int 2007; 6: 92-97.

[15] Collins AT, Berry PA, Hyde C, Stower MJ and Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 2005; 65: 10946-10951.

[16] Monzani E, Facchetti F, Galmozzi E, Corsini E, Benetti A, Cavazzin C, Gritti A, Piccinini A, Porro D, Santinami M, Invernici G, Parati E, Alessandri G and La Porta CA. Melanoma contains CD133 and ABCG2 positive cells with enhanced tumourigenic potential. Eur J Cancer 2007; 43: 935-946.

Supplementary 1: ROC analysis

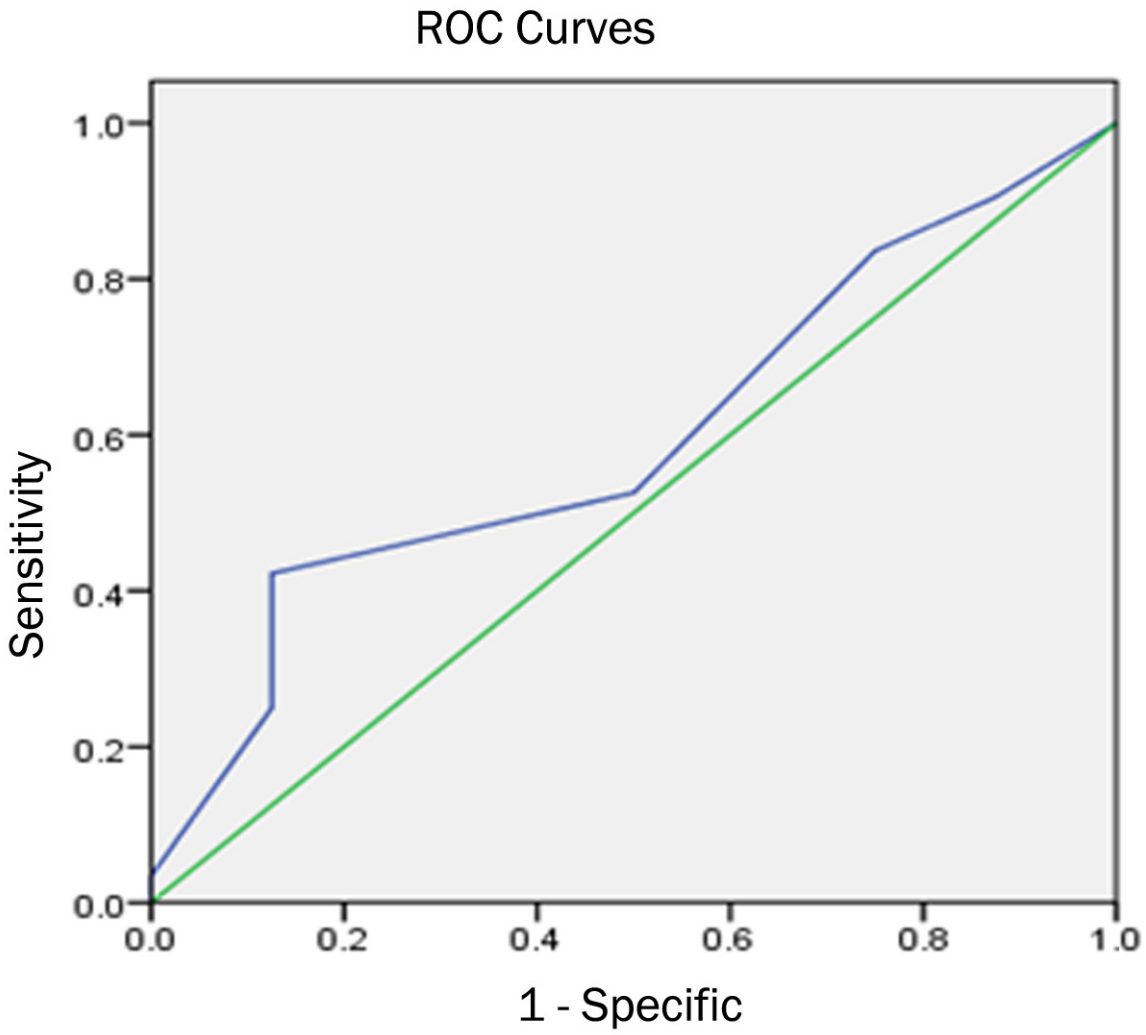
(1) Cutoff-value of cytoplasmic staining:



Cut-off point	Cut-off value	Sensitivity	Specificity	Youden's Index
1	0.5	0.913	0.663	0.250
2	1.5	0.856	0.579	0.277
3	2.5	0.738	0.379	0.359
4	3.5*	0.581	0.147	0.434
5	5	0.450	0.084	0.366
6	7.5	0.063	0.011	0.052

Youden's Index = true positive/(true positives + false negatives) - false positive/(false positive + true negative); *Indicates that the cut-off point corresponding the largest value of Youden's index.

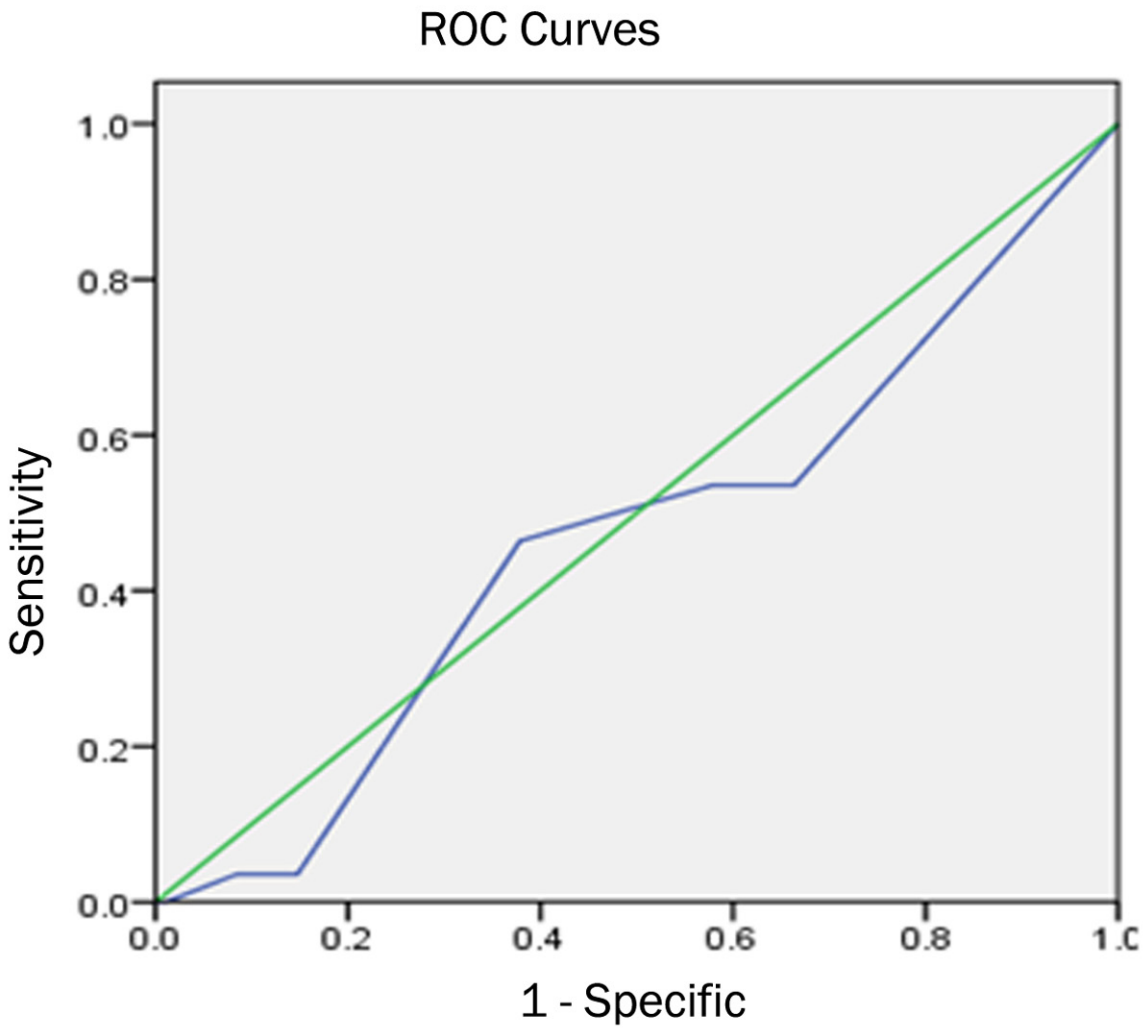
(2) Cutoff-value of membrane staining:



Cut-off point	Cut-off value	Sensitivity	Specificity	Youden's Index
1	0.5	0.905	0.875	0.030
2	1.5	0.836	0.750	0.086
3	2.5	0.526	0.500	0.026
4	3.5*	0.422	0.125	0.297
5	5	0.250	0.000	0.250
6	7.5	0.034	0.011	0.023

Youden's Index = true positive/(true positives + false negatives) - false positive/(false positive + true negative); *Indicates that the cut-off point corresponding the largest value of Youden's index.

(3) Cutoff-value of nuclear staining:



Cut-off point	Cut-off value	Sensitivity	Specificity	Youden's Index
1	0.5	0.536	0.663	-0.127
2	1.5	0.536	0.579	-0.043
3	**2.5***	0.464	0.379	0.085
4	3.5	0.036	0.147	-0.111
5	5	0.036	0.084	-0.048
6	7.5	0.000	0.011	-0.011

Youden's Index = true positive/(true positives + false negatives) - false positive/(false positive + true negative); *Indicates that the cut-off point corresponding the largest value of Youden's index.

Supplementary 2: Multivariate Survival Analysis (Cox Regression Model)

Variants	HR	95% CI	*P* value
Lymph Node Invasion	9.709	1.805-52.632	***0.008***[a]
Clinical Stage	1.217	0.399-3.704	0.730
Membrane expression	3.774	2.374-6.061	***< 0.001***[a]

HR, hazard ratio; CI, confidence interval; [a]Statistically significant.