

RESEARCH ARTICLE

Co-Expression of Putative Cancer Stem Cell Markers, CD133 and Nestin, in Skin Tumors

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Abstract

Background: Cancer stem cells (CSC) are populations of cells responsible for tumor initiation, progression and therapeutic resistance in many cancers. In the present study, we aimed to investigate the expression pattern and clinical significance of two CSC markers, CD133 and Nestin, in a series of skin tumors. **Materials and Methods:** One hundred and thirteen paraffin blocks from skin cancers including 16 (14%) cases of melanoma, 37 (33%) of squamous cell cancer (SCC) and 60 (53%) of basal cell cancer (BCC) were collected and assembled in a tissue microarray (TMA). The samples were immunohistochemically examined for the expression of CD133 and Nestin. Expression of these markers was also correlated with clinicopathological parameters. **Results:** A significant difference was observed in the expression of CD133 and Nestin in melanomas, SCC and BCC (p value=0.001). Furthermore, the level of expression was significantly higher in the melanomas compared to the SCC and BCC tumors. Expression of CD133 in the melanoma was significantly associated with increased tumor invasiveness (p value=0.05), a higher rate of metastasis (p value=0.04) and the presence of ulceration (p value=0.02). Increased expression of Nestin was observed in metastatic melanoma (p value=0.04), while no statistically significant correlation was found with other clinicopathological parameters including Breslow thickness, Clark level and ulceration. **Conclusions:** Elevated expression levels of CD133 and Nestin in the melanomas are associated with advanced disease, with more aggressive and metastatic skin tumors. Therefore, these markers could be potential therapeutic targets for malignant tumors of the skin.

Keywords: Cancer stem cells - melanoma - SCC - BCC - CD133 - Nestin

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Introduction

Malignant skin cancers are an extremely heterogeneous group of diseases that are very common among various populations (Andrade et al., 2012). There are three main types of skin cancer including basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and melanoma (Andrade et al., 2012). BCC and SCC are derived from keratinocytes, while melanomas originate from melanocytes (La Porta and Zappari, 2013). Melanoma is the most invasive form of skin cancer accounting for only about 4% of the cases of skin cancer, while it causes 74% of all skin cancer related deaths (Mueller and Bosserhoff, 2009). The incidence of these malignancies in the world was 54.41% for BCC, 23.77% for SCC and 8.16% for melanoma during 2000 to 2008 (Leiter and Garbe, 2008). It is noteworthy to mention that the incidence of these malignancies was 71.26% for BCC, 21.32% for SCC and 3.28% for melanoma in Iran during the same period (Semnani et al., 2008).

BCC is the most common skin cancer which tends to

grow slowly and does not metastasize to the bloodstream (Schatten et al., 2008), while SCC tumors are metastatic, aggressive and fatal. Malignant melanoma (MM) is an aggressive tumor that its occurrence has increased rapidly during the past two decades (Blackwood et al., 2002; Gyrylova et al., 2014). The incidence rate of melanoma has been reported to be correlated with age, socioeconomic status, tumor localization (Jemal et al., 2011), and gender (Gyrylova et al., 2014).

However, the major risk factor for developing melanoma is exposure to ultraviolet radiation (UVR), especially associated with the occurrence of sunburns. Patients diagnosed with distant metastases have median survival of 6-9 months, while early diagnosis of melanomas seems to be the key to improve the survival rates (Gajda and Kaminska-Winciorek, 2014).

Snail homolog 2 (SNAI2), mesenchymal marker (TWIST1) cooperatively with BMI1 (stemness marker), as the important transcription factors in Epithelial-Mesenchymal Transition (EMT), have been implicated

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in aggressive behavior of SCCs and melanomas, while their expression levels in BCCs has not been elucidated yet. Thus, BCC could be a good model system to elucidate mechanisms which inhibit processes that drive tumor metastasis. Although up regulation of BMI1 and TWIST1 have been reported in aggressive SCC, melanoma and metastatic BCC, a recent study showed decreased levels of BMI1 and TWIST1 mRNA expression in non-metastatic BCCs (Rajabpour et al., 2014).

Cancer stem cells (CSCs) are a population of cells responsible for tumor initiation, cancer progression and therapeutic resistance in many cancers (Schatten et al., 2008). They are similar to stem cells and are capable of both self-renewal and differentiation into all of the cells within a tumor (Siclari and Qin, 2010; Rangwala et al., 2011). CSCs have been identified in a number of cancers including acute myeloid leukaemia (AML) (Bonnet and Dick, 1997; Passegue et al., 2003), glioblastoma (Singh et al., 2003), breast (Al-Hajj et al., 2003; Ponti et al., 2005), lung (Kim et al., 2005), prostate (Collins et al., 2005), ovarian (Bapat et al., 2005), gastric (Houghton et al., 2004), esophagous (Li et al., 2013), Head and Neck SCC (Satpute et al., 2013) and skin cancers (Frank et al., 2005; Monzani et al., 2007). Different markers have been identified to be expressed on melanoma stem cells (Quintana et al., 2010; Shakhova and Sommer, 2013) comprising CD20 (Fang et al., 2005) and ABC transporter family members such as MDR1, ABCG2 and ABCB5 (Frank et al., 2003; Frank et al., 2005; Monzani et al., 2007; Keshet et al., 2008; Schatten et al., 2008), CD271 (Boiko et al., 2010; Civenni et al., 2011), CD44 (Fernandez-Figueras et al., 1996), CD133 (Klein et al., 2006) and Nestin (Piras et al., 2010; Fusi et al., 2011). Among these markers, CD133 and Nestin have been described as markers of melanocytic stem cells and two of the most important surface markers with increased expression in the cancer stem cell fraction in different human malignancies, including melanoma (Klein et al., 2006; Monzani et al., 2007; Rappa et al., 2008; Al Dhaybi et al., 2010; Shakhova and Sommer, 2013). CD133 or human prominin-1/AC133 is a transmembrane glycoprotein with a molecular weight of 120 kDa that is expressed on the hematopoietic stem cells, endothelial progenitor cells and dermal-derived stem cells (Belicchi et al., 2004; Shmelkov et al., 2005).

CD133 positive melanoma cells have an enhanced capability to initiate primary tumors in NOD/SCID mice compared to CD133 negative melanoma cells (Monzani et al., 2007). The CD133 positive cells isolated from melanoma specimens have also showed a tumor-initiating property *in vivo* and in melanoma cell line (Monzani et al., 2007).

CD133 has come to the view as a major stem cell marker in different cancers including melanoma due to strong tendency of CD133+ cells to metastasize as a feature that is supposed to be related to cancer stem cell component (Clarke and Fuller, 2006; Sharma et al., 2010).

Nestin (NES) is an intermediate filament that was described as a marker of neural stem cells and expressed in the cytoplasm of neuroepithelial stem cells (Lendahl et al., 1990; Dahlstrand et al., 1992). This marker is

expressed in the repair processes in the muscle, liver and infarcted myocardium (Ishiwata et al., 2011). Nestin has been reported to be correlated with poor prognosis in some tumors including central nervous system tumors, gastrointestinal, pancreatic, prostate, breast cancer (Ishiwata et al., 2011) and metastatic melanoma (Tohyama et al., 1992; Florenes et al., 1994). However, the role of Nestin in malignancies has not been elucidated yet (Ishiwata et al., 2011). The expression of Nestin in melanoma tumor cells was also correlated with melanoma invasiveness (Tanabe et al., 2010). Moreover, Nestin was detected in the blood of the patients with melanoma (Fusi et al., 2011), and its detection was associated with the presence of circulating melanoma tumor cells indicating the metastatic tendency of melanoma. Therefore, Nestin might be an early biomarker for melanoma recurrence (Fusi et al., 2011).

Concerning the lack of any available data in the literature on the topic of the presence of CSC in various types of skin cancers including SCC and BCC compared to malignant melanomas, this study aimed, for the first time, to examine the staining patterns and clinical significance of the two common putative CSC markers, CD133 and Nestin, on the TMAs of various skin tumor specimens including melanomas, SCC and BCC.

Materials and Methods

Patients and tumor characteristics

Samples of 113 skin cancer tissues were collected from Shohada-e-Tajrish Hospital, a referral Medical Centre in Tehran (Iran) during 2003-2011. This series included 16 melanomas, 37 squamous cell carcinoma (SCC) and 60 Basal cell carcinoma (BCC) samples. Pathology reports were reviewed by a pathologist to consider patients' age, sex, tumor size, tumor type and grade. Tumor tissues were fixed in 10% buffered formalin and embedded in paraffin. This study was approved by the Ethics Committee of Iran University of Medical Sciences (IUMS).

Tissue microarray (TMA) preparation

Skin cancer tissue microarrays were constructed as described previously (Kononen et al., 1998; Mehrazma et al., 2013; Mohsenzadegan et al., 2013). All Hematoxylin and Eosin (H and E)-stained slides were reviewed by a pathologist (AR) to determine the best area for preparing the TMA of each specimen. Tissue arrays were then constructed by placing 0.6 mm diameter samples from tumor samples per single block, with 1 mm spacing separating each core. The TMA blocks were made in three copies, each containing one sample from a different region of the tumor using tissue-arraying instrument (Minicore; ALPHELYS, Plaisir, France). The mean scoring of the three cores were calculated as the final score.

Immunohistochemistry

Immunohistochemistry was performed on the TMA slides (Superfrost plus, Thermo Scientific, Germany) with a standard chain polymer-conjugated (Envision) technique as described previously (Madjd et al., 2011; Mohsenzadegan et al., 2013) applying specific monoclonal

antibodies. The slides were deparaffinized in 60°C for 20 minutes and cleared in xylene and were then rehydrated in ethanol. The endogenous peroxidase was blocked with 3% H₂O₂ for 20 minutes. After three washes in Tris-buffered saline (TBS), antigen retrieval was achieved by autoclaving slides in citrate buffer (pH 6.0) for 10 minutes. After the elimination of the excess serum, sections were exposed to primary antibodies, Anti-Nestin antibody (ab11306, Abcam, UK) and Anti-CD133 (Gifted by Avicenna Research Institute, Monoclonal Antibody Research Center (MARC) with optimal dilutions which was found to be 1/200 and 1/150, respectively. The sections were then washed in TBS and incubated with anti-rabbit/anti-mouse Envision (Dako, Denmark) as a secondary antibody for 10 minutes. Then, the TMA slides were visualized with 3,3'-diaminobenzidine (DAB, Dako) substrate as chromogen for 15 minutes at the room temperature. The sections were lightly counterstained with haematoxylin, dehydrated in alcohol, cleared with xylene and mounted for examination. Human normal kidney tissues were used as positive controls for CD133 antibody, whereas the brain cortex of a rat was applied as a positive control for the Nestin antibody.

Scoring TMA slides

The stained slides were reviewed by a semi-quantitative scoring system on a multi-headed microscope by two observers (AR and ZM), and scoring was performed in a coded manner, blinded to clinical data until an agreement was achieved.

First, the TMA slides were scanned at a low magnification to find the distribution of the tumor cells. Then, the positive cores were evaluated for localization and semi-quantitatively for the expression level at higher magnifications, and the final scores were given. Both intensity and percentage of the positive cells were evaluated. Intensity was scored as 0 (absent), 1 (weak), 2 (moderate) or 3 (strong). H-scores reflecting the overall staining were calculated by multiplying the intensity in the percentage of the positive cells and a final score of 0 to 300 was given. The pattern of expression of CD133 was

mainly cytoplasmic and partially cell membrane, while Nestin expression was mostly cytoplasmic.

Statistical analysis

Data were analyzed using the SPSS statistical software package version 20 (SPSS, Chicago, IL, USA). Pearson's χ^2 and Pearson's R tests were used to analyze the significance of the correlation between the expressions of CD133 or Nestin and the clinicopathological parameters. Moreover, the comparisons of CD133 and Nestin expression in different skin cancer types were performed using Mann-Whitney U test. A p value of <0.05 was considered as statistically significant.

Results

Study population

One hundred and thirteen paraffin blocks from skin cancer patients including BCC, SCC and melanoma were collected. Of this series, 16 (14%) of the cases were melanoma, 37 (33%) were SCC and 60 (53%) of the cases were BCC. The mean age of the patients was 61±16 (range 4-96) years; of them, 55 cases (49%) were under 61 and 58 (51%) were older than 61 years.

The mean age of the SCC patients was 64±17 years (age range of 9-96), the mean age of the BCC patients was 61±14 (age range of 4-90), and the mean age of the melanoma cases was 55±18 (age range of 7-91).

The range of tumor size was 0.2 to 25 cm (mean=2.2cm), and 73% of the cases (82) had a tumor size of less than 2.2 cm, and 27% of the cases (31) had a tumor size larger than 2.2 cm in the largest diameter.

The study population consisted of 78 (69%) male and 35 (31%) female participants with a male/female (M/F) ratio of 2.2. The M/F ratio was 3.1 (28/9) among the SCC cases, it was 3 (12/4) in the melanoma, and it decreased to 1.72 (38/22) in the BCC cases.

Of the 16 melanomas, 6 samples were metastatic melanoma, 3 were superficial spreading melanoma, 4 were acral lentiginous melanoma and 3 samples were lentigo malignant melanoma.

Table 1. Association between CD133 and Nestin Expression (Intensity, Percentage of Positive Cells and H-score) and Clinicopathological Parameters of Skin Cancer (P value; Pearson χ^2)

Patients and tumor characteristics	No. of cases (%)	Expression of CD133 (P value; Pearson χ^2)			Expression of Nestin (P value; Pearson χ^2)		
		Intensity of staining	Percentage of positive cells	H-score (cut-off=89)	Intensity of staining	Percentage of positive cells	H-score (cut-off=155)
All cases	113						
Age (years)		0.47	0.39	0.2	0.4	0.96	0.63
<61	55 (49)						
>61	58 (51)						
Gender		0.46	0.41	0.23	0.44	0.24	0.52
Male	78 (69)						
Female	35 (31)						
Tumor Type		0.001	0.001	0.001	0.001	0.16	0.002
Melanoma	16(14)						
SCC	37(33)						
BCC	60(53)						
Tumor Grade (SCC)							
Well differentiated	12(32)	0.51	0.08	0.1	0.03	0.13	0.1
Moderately differentiated	22 (60)						
Poorly differentiated	3(8)						

Of the 37 SCC samples, 12 (32%) were well differentiated, 3 (8%) were moderately differentiated and 22 (60%) cases were poorly differentiated.

Among the 55 BCC samples, only 4 cases (7%) showed local recurrence without metastasis, while in the SCC samples 12/37 cases (32%) had local recurrence and 8 cases (22%) showed metastasis.

In melanoma, 5/16 (31%) local recurrence was reported, whereas 11/16 cases (69%) showed metastasis.

Patients' data, tumor characteristics and the relationship between the intensity of expression of CD133 and Nestin and different prognostic factors such as age, gender, grade of tumor (only for SCC), tumor size, tumor type, metastasis and recurrence were examined in 113 skin neoplasm as summarized in Table 1.

Expression of CD133 in melanoma, SCC and BCC tumors

The level of expression of both markers was examined by three scoring methods; namely, the intensity of the staining, the percentage of positive cells and H-score. Normal kidney tissue showed strong and uniform staining of CD133 mainly in cytoplasm and partially in cell membrane of the tumor cells (Figure 1A).

This was a retrospective study on a collection of 113 paraffin embedded skin tumors. Of these tumors, 7 cases were excluded from the study due to technical problems in tissue processing or absence of tumor cells within the cores, leaving a total of 106 for the final evaluation of CD133.

From the 106 tissue cores stained with CD133 antibody, 91% (96) of the tumors showed positive staining with variety of intensities, while only 9% (10) of the cases were negative for CD133 staining. Weak, moderate and strong intensities were detected in 62 (58%), 27 (26%) and 7 (7%) cases, respectively (Table 2).

The analysis of each tumor type showed that negative,

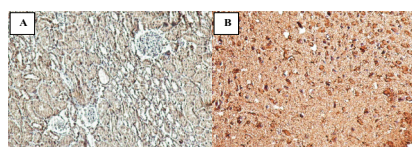


Figure 1. Expression of CD133 and Nestin Proteins. A) Normal kidney for CD133: ×20 and; B) Brain cortex of rat for Nestin ×20

weak and moderate intensities were observed in 10 (19%), 35 (66%) and 8 (15%) tumors among the 53 BCC cases, whereas none of the tumors showed a strong intensity (Table 2, Figure 2A and B).

All SCC tumors expressed CD133 with weak, moderate and strong intensities which was detected in 20/37 (54%), 14/37 (38%) and 3/37(8%) of the SCC samples, respectively (Table 2, Figure 2C and D).

Similarly, all the 16 melanoma cores showed the expression of CD133 with weak, moderate or strong intensities which was detected in 7 (44%), 5 (31%) and 4 (25%) cases (Table 2, Figure 2E and F).

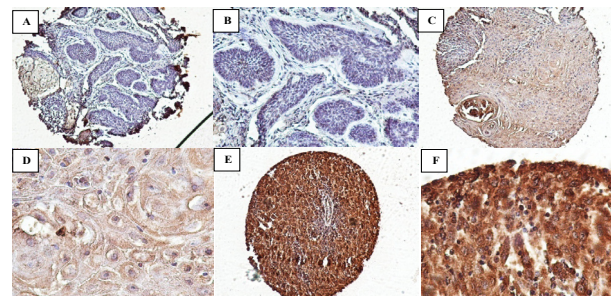


Figure 2. Cytoplasmic Staining of CD133 Observed in BCC Tumors. A) With original magnification ×10 and; B) With original magnification ×20; In SCC tumors C) With original magnification ×10 and; D) With original magnification ×20 and; In melanoma tumors E) With original magnification ×10 and; F) With original magnification ×20

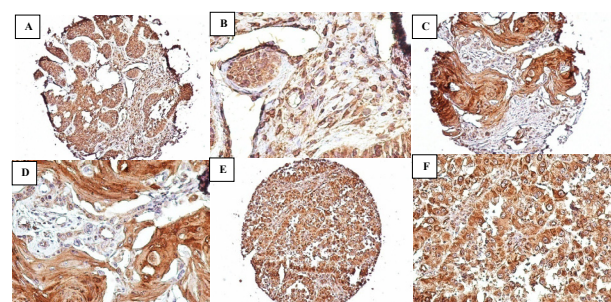


Figure 3. Cytoplasmic Staining of Nestin Observed in BCC Tumors. A) With original magnification ×10 and; B) With original magnification ×20; In SCC tumors; C) With original magnification ×10 and; D) With original magnification ×20 and; In melanoma tumors E) With original magnification ×10 and; F) With original magnification ×20

Table 2. Expression of CD133 and Nestin (Intensity, Percentage of Positive Cells and H-score) in BCC, SCC, Melanoma (p value; Pearson χ^2)

Scoring	BCC No (%)		SCC No (%)		Melanoma No (%)		P value	
	CD133	Nestin	CD133	Nestin	CD133	Nestin	CD133	Nestin
Intensity of staining								
Weak	35(66)	30(55)	20(54)	12(39)	7(44)	2(17)		
Moderate	8(15)	24(44)	14(38)	16(52)	5(31)	6(50)	<0.001	<0.001
Strong	0	1(1)	3(8)	3(9)	4(25)	4(33)		
Percentage of positive cells								
<25	20(38)	1(2)	0(0)	0(0)	1(6)	0(0)	<0.001	0.16
26-50	13(25)	0(0)	9(24)	0(0)	3(19)	0(0)		
51-75	7(13)	3(5)	6(16)	0(0)	1(6)	0(0)		
>75	13(24)	51(93)	22(60)	31(100)	11(69)	12(100)		
H-score								
Low (<89 for CD133 and <155 for Nestin)	40(76)	33(60)	13(35)	12(39)	6(38)	2(17)	<0.001	0.002
High (>89 for CD133 and >155 for Nestin)	13(24)	22(40)	24(65)	19(61)	10(62)	10(83)		
No. total	53	55	37	31	16	12	106	98

Table 3. Expression of CD133 and Nestin in Melanoma (P value ; Pearson χ^2)

Scoring	Age (cut off=56) (cut off=59)		Gender		Size of tumors (cut off=3.5) (cut off=4.1)		Metastasis		Insitu or invasive		ulceration		Clark level		Breslow		Type of tumors	
	CD133	Nestin	CD133	Nestin	CD133	Nestin	CD133	Nestin	CD133	Nestin	CD133	Nestin	CD133	Nestin	CD133	Nestin	CD133	Nestin
Intensity of staining	0.77	0.39	0.05	0.64	0.18	0.59	0.04	0.58	0.58	0.58	0.6	0.84	0.29	0.21	0.76	0.49	0.12	0.04
Percentage of positive cells	0.48	0.17	0.16	0.78	0.43	0.31	0.13	0.05	0.05	0.02	0.27	0.36	0.32	0.21	0.21	0.65	0.02	.
H-score (cut of=156) in CD133 (cut of=212) in Nestin	0.69	0.59	0.01	0.76	0.5	0.59	0.08	0.5	0.56	0.2	0.57	0.52	.	0.58	.	.	0.01	0.22

For the H-score, the evaluation of the cut-off value for the total tumors was calculated based on the mean of the H-score (cut off=89) to define groups showing low and high expression of CD133 (Table 2).

The average intensity of CD133 expression was significantly higher in melanoma cores (mean=1.8) compared to SCC (mean=1.5) and BCC (mean=0.96) cores (p-value<0.001). A significant difference was also observed in the level of expression of CD133 (in terms of H-score) between melanoma (mean H-score=156), SCC (mean H-score=114) and BCC (mean H score=50) cases (Pearson's χ^2 , p-value<0.001, Table 3, 4, 5).

Moreover, the Mann-Whitney U Test was used to compare the differences between the expression of CD133 in various groups indicating a significant difference in CD133 expression (both intensity and H-score) between the BCC cases with SCC cases (p-value<0.001) and melanomas (p-value<0.001), whereas the difference of expression between SCC with melanoma cases was not significant (Table 6).

Analysis of CD133 expression

In univariate analysis, we found a significant association between the expression of CD133 (in terms of H-score) and melanoma subtypes (acral lentiginous, lentigo malignant, super facial spreading melanoma, and metastatic melanoma) (H-score; p value=0.01). The expression of CD133 was also significantly higher in male compared to female cases (H-score; p value=0.01). There was a significant positive correlation between the expression of CD133 and metastasis (p value=0.04), ulceration (p value=0.02) and invasive tumors (p value=0.05).

However, no significant association was found between the expression of CD133 and age (p value=0.77), size of tumors (p value=0.18), breslow thickness (p value=0.58) and Clark level (p value=0.29) (Table 3).

In the SCC samples, a relative association was observed between the CD133 expression and the grade of tumors (p value=0.06), indicating higher expression of CD133 in poorly differentiated tumors. However, no correlation was found between the expression of CD133 and the size of tumors (p value=0.26), age (p value=0.17), gender (p value=0.57), tumor metastasis (p value=0.35) and recurrence (p value=0.29) (Table 4).

Statistical analysis showed a significant association between the expression of CD133 and the size of tumors (p value=0.05) and the patient's age (p value=0.05) in the BCC samples. While a trend was observed between CD133 expression and recurrence (p value=0.09) (Table 5).

Expression of Nestin in Melanoma, SCC and BCC Tumors

We also performed an immunohistochemical analysis to investigate another putative CSC marker, Nestin on the same series of skin tumors. Embryonic brain tissues which were used as positive controls showed a strong staining (Figure 1B). During the staining of Nestin, 15 out of 113 tumors were disregarded from the study due to technical problems in the tissue processing or absence of tumor cells within the core, leaving a total of 98 cases for the final scoring.

All the remaining tumors (98) including 55 (56%) BCC, 31 (32%) SCC and 12 (12%) melanoma samples stained positively for Nestin with variety of intensities. Weak, moderate and strong intensities were observed in 44 (45%), 46 (47%) and 8 (8%) of the cases, respectively (Table 1).

Considering the staining of each tumor type, we observed weak, moderate and strong staining in 30 (55%), 24 (44%) and 1 (1%) of the BCC cases (Figure 3A and B), while in the SCC cores, weak, moderate and strong staining were detected in 12 (39%), 16 (52%) and 3 (9%) cores (Figure 3C and D).

In the melanoma cases, weak, moderate and strong staining were found in 2 (17%), 6 (50%) and 4 (33%) tumors (Table 2, Figure 3E and F).

The expression of Nestin in terms of H-score was evaluated based on the cut off value of 155 (mean H-score) to define the groups showing low and high expression, demonstrating that 47 (48%) of the cases displayed low expression (H-score \leq 155), while 51 (52%) tumors expressed higher level of Nestin (H-score >155) (Table 2).

The average intensity of Nestin in melanoma was 2.1, which was significantly higher compared to the average intensities of SCC (1.7) and BCC (1.4) (p-value<0.001).

Similarly, the mean of H-score in melanomas (212) was significantly higher than the mean H-score of SCC (166) and BCC (136) cases, indicating stronger expression of this marker in melanoma (Pearson's χ^2 , p-value<0.001, Table 3, 4, 5).

Mann-Whitney U Test indicated a statistically significant difference between the expression of Nestin in SCC cases with the melanoma group (p-value=0.01), and also between the BCC and the melanoma group (p-value=0.001). Moreover, a significant difference was evident between the expression of Nestin in the SCC and BCC groups in terms of H-score (p-value=0.05) (Table 6).

Analysis of Nestin expression

In melanoma, we found a significant positive correlation between the expression of Nestin and the subtypes of melanoma (p value=0.04) and Breslow thickness (p value=0.03). No correlation was found between the expression of Nestin and patient's age (p value=0.39), gender (p value=0.64), tumor size (p value=0.59), tumor metastasis (p value=0.58), Clark level (p value=0.3) and ulceration (p value=0.84) (Table 3).

Similarly, a significant association was observed (p value=0.03) between the expression of Nestin and grade of the tumor in the SCC samples, indicating a higher level of expression of Nestin in the poorly differentiated SCC tumors. The expression of Nestin was also positively correlated with patient's age (p value=0.02), while no correlation was found between the expression of Nestin and gender (p value= 0.29), tumor size (p value=0.84), tumor metastasis (p value=0.58) and local recurrence (p value=0.85) (Table 4).

However, the expression of Nestin was not correlated with any clinicopathological parameters in BCC cases (Table 5).

Combined analysis of CD133 and Nestin

Comparing the results of CD133 and Nestin, we found a significant reciprocal pattern of expression in our series of skin tumors (p value<0.001). Due to the loss of some tissue cores during the preparation of TMAs, a subset of cases from the original series including 91 cases were ultimately analyzed.

The combined analysis of the expression of CD133 and Nestin in all the tumor types showed that 91% (83/91) of the skin tumors displayed the CD133+/Nestin+ phenotype, 9% (8/91) of the tumors expressed CD133-/Nestin+ phenotype, while none of the cases showed either CD133+/Nestin- or CD133-/Nestin- phenotypes.

The combined analysis showed that 100% of the melanoma (12/12) and SCC (31/31) cases possessed CD133+/Nestin+ phenotype, while 83% (39/48) of the BCC tumors co-expressed both CD133 and Nestin markers and 17% (9/48) showed CD133-/Nestin+ phenotype.

Discussion

Cancer stem cells (CSCs) are a small population of the whole tumor cells and are the only cells that possess the ability to initiate and maintain tumor development. The main reason of metastasis, relapse of tumors and resistance to general chemotherapy are related to these populations of cells. Identification and characterization of these cells would be useful in cancer therapy (Hamburger and Salmon, 1977; Reya et al., 2001; Porta, 2009). Among several markers which have been identified for the characterization of cancer stem cells, CD133 and Nestin

Table 4. Expression of CD133 and Nestin in SCC (P value; Pearson χ^2)

Scoring	Age (cut off=64)		Gender		Size (cut off=4.3)		Metastasis		Grade of SCC tumors	
	CD133	Nestin	CD133	Nestin	CD133	Nestin	CD133	Nestin	CD133	Nestin
	Age (cut of=63)	Age (cut of=64)	Size (cut of=3.8)		Size (cut of=4.1)					
Intensity of staining	0.17	0.02	0.57	0.29	0.26	0.84	0.35	0.58	0.43	0.03
Percentage of positive cells	0.81	.	0.71	.	0.37	.	0.23	.	0.09	.
H-score (cut off=114) in CD133 (cut off=166) in Nestin	0.46	0.01	0.61	0.36	0.31	0.63	0.56	0.66	0.06	0.1

Table 5. Expression of CD133 and Nestin in BCC (P value; Pearson χ^2)

Scoring	Age (cut off=64)		Gender		Size(cut off=0.95)		Recurrence	
	CD133	Nestin	CD133	Nestin	CD133	Nestin	CD133	Nestin
	Age (cut off=61)	Age (cut off=60)	Size (cut off=0.8)		Size (cut off=0.98)			
Intensity of staining	0.05	0.5	0.53	0.51	0.18	0.5	0.18	0.91
Percentage of positive cells	0.59	0.8	0.27	0.2	0.11	0.41	0.09	0.62
H-score (cut off=51) in CD133 (cut off=136) in Nestin	0.23	0.41	0.41	0.24	0.05	0.5	0.66	0.59

Table 6. CD133 and Nestin expression (Intensity, Percentage of Positive Cells and H-score) in Melanoma Compared to BCC and SCC (Mann-Whitney U test) CD133 Expression

		Intensity of staining	Percentage of positive cells	H-score (cut off=155)
CD133 expression	BCC and SCC	<0.001	<0.001	<0.001
	BCC and Melanoma	<0.001	<0.001	<0.001
	SCC and Melanoma	0.27	0.72	0.87
Nestin expression	BCC and SCC	0.09	0.12	0.059
	BCC and Melanoma	<0.001	<0.001	<0.001
	SCC and Melanoma	0.086	0.017	0.277

are the most widely reported and have been described as markers of melanocytic stem cells (Bongiorno et al., 2008; Piras et al., 2010).

CD133 is a transmembrane glycoprotein which is expressed as a major stem cell marker in different types of tumors (Hemmati et al., 2003; Singh et al., 2003; Clarke et al., 2006; Wang et al., 2009; Sharma et al., 2010). Previous studies have showed that invasiveness of melanoma can be related to this marker (Clarke and Fuller, 2006; Sharma et al., 2010). Nestin is an intermediate filament that was originally described as a marker of neural stem cells. Increased expression of Nestin has been reported in various tumor cells which was correlated with poor prognosis in some tumors (Ishiwata et al., 2011). The expression of Nestin by melanoma tumor cells was also reported to be associated with melanoma invasiveness (Tanabe et al., 2010). Recent findings suggest that CD133 and Nestin can be used as putative cancer stem cell markers in malignant melanoma (Klein et al., 2006; Monzani et al., 2007; Fusi et al., 2011; Thill et al., 2011).

Because the existence of CSCs in melanomas has been illustrated in the previous studies, the aim of the present study was to investigate the expression of the two common putative CSC markers, CD133 and Nestin, in paraffin-embedded tissue of different skin tumors including malignant melanomas, SCC and BCC which were assembled in TMA in order to compare the level of expression of these markers and their association with pathological features for the first time among Iranian patients. To our knowledge, this is the first study to compare the expression of these CSC markers in various skin tumors. Our staining showed that CD133 was mainly expressed in cytoplasm and partially in cell membrane, while Nestin was mainly expressed in cytoplasm. The pattern of expression of Nestin was reported to be cytoplasmic in a previous study (Laga et al., 2011).

Previously, Klein et al. evaluated the pattern of expression of CD133 and Nestin in 226 melanocytic lesions including banal nevi, in situ and invasive melanomas and metastatic melanomas. They observed that only Nestin showed a statistically significant difference in malignant melanoma in comparison with CD133 (Klein et al., 2006).

In another study, Circulating Melanoma Cells (CMCs) were evaluated for the expression of CD133 and Nestin suggesting that CMCs expressed both markers, while higher expression of Nestin on CMCs have been found to be an index of poor prognosis (Fusi et al., 2011).

The comparison of the CD133 and Nestin expressions in our skin tumor groups showed a significant difference between the levels of expression of these markers. Malignant melanoma expressed a significantly higher level of CD133 and Nestin compared to SCC and BCC samples. SCC showed a statistically significant higher level of CD133 and Nestin in the non-melanoma group compared to BCC cases. The analysis of the intensity of staining in each tumor type demonstrated that the expression patterns of CD133 had a heterogeneous pattern; a variety of staining patterns (weak, moderate and strong) were observed in melanoma and SCC samples; however, BCC cases either did not stain or showed only weak and

moderate staining patterns. All the three types of skin tumors stained with Nestin with a variety of intensities. The averages of intensity for both Nestin and CD133 were significantly higher in malignant melanoma compared to SCC and BCC. Moreover, the intensity of Nestin in melanoma cases was significantly higher compared to CD133.

Our findings were in agreement with those of previous studies indicating that melanoma samples expressed higher level of CD133 and Nestin than non-melanoma skin cancers (Monzani et al., 2007; Sharma et al., 2010; Fusi et al., 2011). Our study demonstrated that the majority of BCC samples expressed low levels of Nestin and CD133, while some previous reports indicated no expression of Nestin in BCC cases (Florenes et al., 1994; Abbas O and Bhawan, 2011). This discrepancy could be due to the selection of different cut-off points and different experimental conditions. Furthermore, the exploration of the relationship between expression of CD133 and Nestin and the subtype of melanomas showed that metastatic melanoma expressed a significantly higher level of CD133 and Nestin.

In a very recent study on a large series of melanomas, Ladstein et al. showed that Nestin expression was significantly associated with increased tumor thickness, high mitotic count and the presence of ulceration and tumor necrosis (Ladstein et al., 2014), but we could not illustrate these correlations in our study probably due to the small sample size.

Our analysis showed a significant correlation between the expression of CD133 and Nestin and histological grade in SCC, indicating that increased levels of CD133 and Nestin were more often found in poorly differentiated SCC. This finding was inconsistent with Patel's study which recommended CD133 as a stem cell marker (Patel et al., 2008).

Our study demonstrated that the majority of BCC samples expressed low levels of CD133 and Nestin which is in line with previous reports that indicated low or no expression of CD133 and Nestin in BCC tumors (Abbas O and Bhawan, 2011).

We also established a phenotype with a combination of positive CD133 and Nestin cells in skin cancer and found that the CD133⁺/Nestin⁺ phenotype occurs in all cases of melanomas and SCC, but not in all cases of BCCs.

Our data showed that CD133 expression in melanoma was significantly associated with increased tumor invasiveness, high metastasis and the presence of ulceration. Also, we found that CD133 expression was higher in the metastatic group rather than primary malignant melanomas, but no significant association was found in mitotic count, Breslow thickness and Clark level. Similarly, strong expression of Nestin was significantly associated with metastatic melanoma, while it was not associated with tumor invasiveness, mitotic count, Breslow thickness, Clark level and ulceration. These findings suggest that Nestin may participate in the step of tumor initiation and carcinogenesis in the skin cancers, particularly melanomas.

The mechanism that causes this condition remains unknown, but it may be associated with the increase in

stem/progenitor-like cells caused by the dedifferentiation of tumor cells or the accumulation of immature phenotype cells observed in patients with an advanced disease.

In conclusion, taken together, our findings revealed that CSC markers, CD133 and Nestin⁺, were highly expressed in melanoma compared to SCC and BCC. These two markers, especially Nestin could be a valuable tool to study skin cancer. Nestin was expressed in metastatic melanoma tissues; therefore, it could be a potentially important marker of melanocytic neoplasms. However, further studies are required to clarify the molecular processes that regulate Nestin expression and to evaluate the potential of Nestin-targeted therapy for malignant melanomas. These results are applicable to the development of new strategies of treatment for skin cancers, suggesting that metastatic melanoma and poorly differentiated SCC patients whose tumors contain higher frequency of CD133⁺ and Nestin⁺ could be appropriate candidates for the targeted therapy of CSCs in combination with conventional therapy.

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