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The Probable Role of Tumor Stem Cells for Lymph Node Metastasis in Supraglottic Carcinoma

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Abstract Tumor stem cells (TSC), which are considered as likely candidates for the origin of cancer, are deduced to be responsible for tumor metastasis theoretically. We therefore investigated whether TSC were associated with lymph node metastasis in supraglottic carcinoma. Immunohistochemistry was performed for CD44, CD133, and LYVE-1 to detect TSC and lymphatic vessel density (LVD) in 66 primary supraglottic carcinoma tissue samples from 30 patients with lymph node metastasis (N+) and 36 patients without (N0). Reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot were used to detect the expression of CD44 and CD133 at mRNA and protein levels in N+ and N0 primary tumors. The LVD was 22.4±10.26 in 30N+ and 6.8±4.09 in 36N0 samples subjected to immunohistochemistry, which was associated with their clinical nodal stages. There were 43.33% CD44-positive and 93.33% CD133-positive samples in 30N+, and 13.89% CD44-positive and 44.44% CD133-positive samples in 36N0 ($P<0.05$). However, in each positive slide, there were only 5~10% CD44-positive cells, but 70~85% CD133-possitive cells. The expressions of CD44 and CD133 of N+ obtained through RT-PCR and Western blot were significantly higher than those of N0. These results suggest that TSC identified through CD44-positive cells in N+ were significantly higher than those in N0, indicating that TSC may be responsible for lymph node metastasis. CD133, whose expression is not restricted to TSC, may be unspecific for TSC identification in hypostatic supraglottic carcinoma.

Keywords CD133 · CD44 · Lymph node metastasis · Supraglottic carcinoma · Tumor stem cell

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Introduction

Supraglottic carcinoma, one of the most common malignancies of the head and neck region, results in substantial morbidity and mortality annually. Eighty-five percent of supraglottic carcinoma preferentially spreads through lymphatic vessels and is accompanied by lymph node metastasis [1]. Lymph node metastasis represents the major step of dissemination and serves both as a major prognostic indicator for disease progression and a guide for therapeutic strategies [2]. However, the mechanism associated with lymph node metastasis still remains unclear. Studies on metastasis mechanism will thus be useful for cancer therapy.

The tumor stem cells (TSC) hypothesis was first proposed in 1983 when Mackillop hypothesized that every tumor contains a rare population of functionally distinct TSC [3]. TSC are defined as a rare cell population in cancer which acts like stem cells: they are self-renewable, tumorigenic, and differentiable [4]. The TSC hypothesis opened a new era in understanding the initiation and progression of cancers. Studies showed that TSC are responsible for tumor growth [5, 6]. However, this topic remains largely uninvestigated because of the absence of reliable specific markers for TSC, especially in head and neck squamous cell carcinomas (HNSCC). Recent studies have reported CD44 and CD133 as the putative markers of TSC in HNSCC. Prince et al. identified a subpopulation of cells with cancer stem cell

properties and proved CD44 as a specific marker for TSC in hypostatic HNSCC [7, 8]. Wei et al. reported CD133 as a putative marker of TSC in the HEP-2 cell line in vitro [9]. In the current study, we hypothesized that TSC identified by CD44 and CD133 in hypostatic tumors might be quite important as a risk factor for lymph node metastasis.

The goal of the present study was to determine whether a relationship exists between TSC and lymph node metastasis in primary supraglottic carcinomas. To our knowledge, such comparison has not been carried out in other studies. We showed that (a) TSC, identified as CD44-positive cells, were significantly higher in N+ than in N0; and (b) CD133 expression was not restricted to TSC. Parts of CD133-positive cells were negative for CD44. To be more precise, CD44-positive cells were responsible for lymph node metastasis, while CD133 was unspecific for TSC identification. This will help us as we conduct further research on TSC, and the mechanism of lymph node metastasis will provide support for tumor therapy.

Patients and Methods

Patients

A total of 66 patients with supraglottic carcinoma (30 cases N+ and 36 cases N0) at Provincial Hospital affiliated to Shandong University from 2006 to 2008 were studied. Hibateral neck dissection and primary tumor resection was performed in all selected patients. The mean age of these patients was 59.5 (range 40~78). All specimens were embedded in paraffin for study by the same researcher.

Immunohistochemistry

Paraffin-embedded laryngeal cancer sections (4 μm thickness) were dewaxed and subjected to antigen retrieval through water bathing in 0.01 M citric buffer (pH 6.0) at 95~98°C for 15 min. To quench endogenous peroxidase, sections were incubated in 3% hydrogen peroxide solution (H_2O_2) for 30 min. Non-specific binding was prevented by incubating the samples in 10% normal goat serum for 30 min in a humid chamber. The slides were incubated overnight at 4°C in antibodies for LYVE-1 (Abcam, Rabbit polycolonal antibody, 5 μg/ml), CD44 (Abcam, Rabbit monocolonal antibody, 1:100), and CD133 (Abcam, Rabbit polycolonal antibody, 1:200). For the negative control, equal PBS was used instead of primary antibodies. After being washed, the primary antibody was detected with an appropriate secondary antibody for 30 min at 37°C. 3, 3′-diamino-benzidine tetrahydrochloride (DAB) solution was used to visualize positive staining, and haematoxylin was used to counterstain the nucleolus.

Interpretation of Immunohistochemical Staining

For CD44 and CD133 expressions, positive cells were stained dark brown and both detected as cytoplasmic immunoreactivity within the tumor mass. The intensity of immunohistochemical staining was randomly evaluated in five areas of the slide sections under 100× objects. The lymphatic vessels were considered positive if the vessels were stained claybank by LYVE-1. The quantification of lymphatic vessels was conducted using the methods of Daisuke Mori [13]. Briefly, we detected two compartments: the peritumoral compartment, a 2 mm-wide area around the tumor mass, and the intratumoral compartment, an area located within the tumor mass. The LVD was defined by counting the number of LYVE-1 positive vessels in the two selected areas and adding the number up for an average vessels count. All slides were validated by two investigators independently. The validation was performed in a blind manner or without knowledge of the eventual clinical parameters. When differences between inter-observers occurred, the respective slides were reinvestigated jointly by both investigators.

RNA and Protein Detection for CD44 and CD133

Total RNA was extracted using TRIzol (Invitrogen) from 12 primary tissues (6 of each for N0, and N+) and frozen quickly into liquid nitrogen after surgical excision. Expressions of CD44 and CD133 mRNA were determined through RT-PCR using M-MuL V reverse transcription (Takara). β-actin was used as standard. The primers were as follows:

CD44: Sense: 5′ AGCGGCTCCTCCAGTGAAA3′; Anti-sense: 5′TCTGTCTGTGC TGTCGGTGATC3′; CD133: Sense: 5′CTGGTGGGGTATTTCTTTTGTATG3′; Anti-sense: 5′ AACGCCTTGTCCTTGGTAGTG3′. β-actin: Sense: 5′CTCCTTAAT GTCACGCACGATTT3′; Anti-sense: 5′GTGGGGCGCCCCAGGCACCA3′. Total protein was extracted from eight primary tissues (N0, N+ equally half) frozen in-80°C freezer using radioimmune precipitation buffer (RIPA) protein lysis buffer under protocols. The Bradford method was used to determine the protein concentration of the supernatant. The samples (40 μg of total protein each) were used for Western blot analysis with the primary antibodies (CD44, 1:5000; CD133, 2 μg/ml; β-actin, 1:2000). The CD44, CD133, and β-actin bands were visualized at apparent molecular weights of 82 kDa, 110 kDa, and 43 kDa, respectively.

Statistical Analysis

Statistical analyses were performed using the SPSS statistical software (version 13.0; SPSS Inc, Chicago, Ill). To test the associations between CD44, CD133, LVD, and clinical

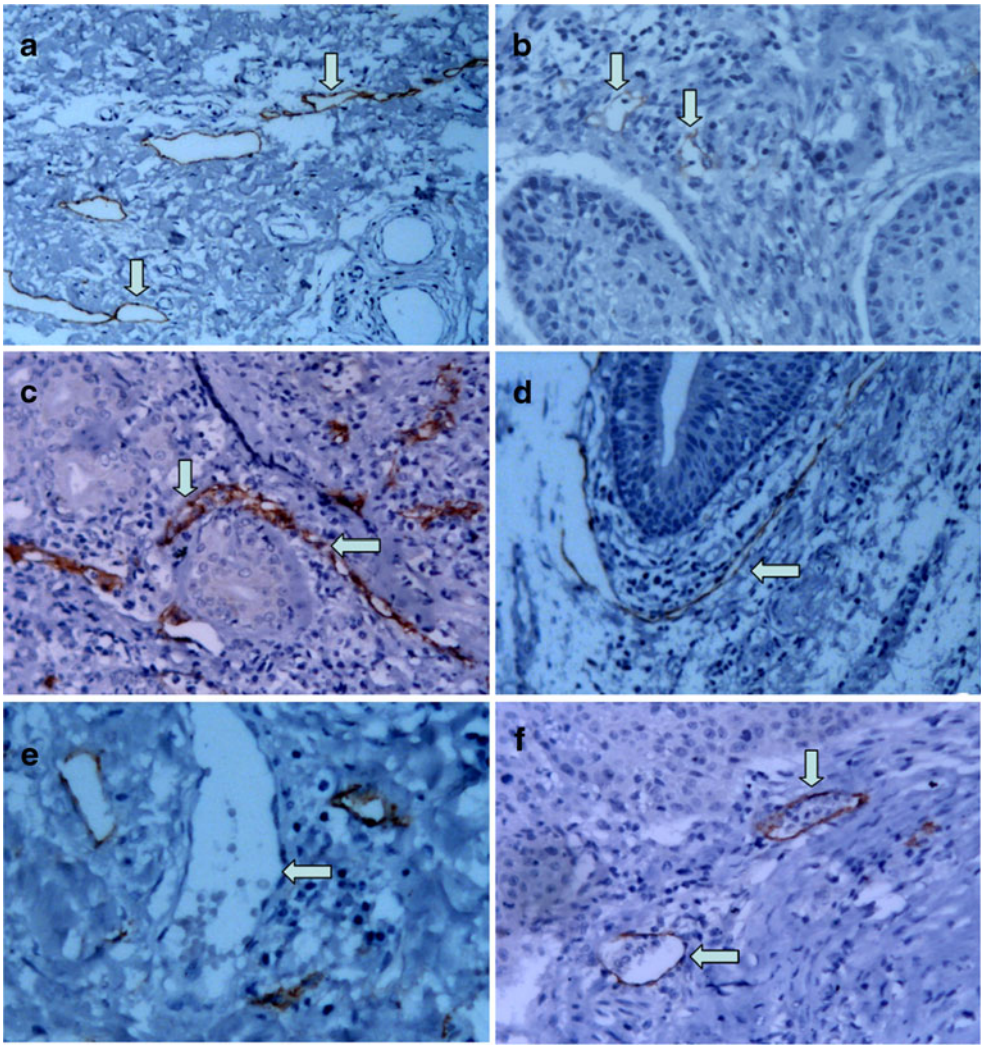

Fig. 1 Immunohistochemistry of LYVE-1 in supraglottic carcinoma. **a** A large patent of LYVE-1 positive lymphatics at the tumor margin. **b** Some small LYVE-1 positive vessels inside carcinoma tissue. **c** LYVE-1 positive vessels stream between different cancer nests. **d** Lymphatic invasion at the periphery of tumors. **e** The adjacent blood vessel surrounded by a smooth muscle which contained red blood cells was negative for LYVE-1 immunostaining. **f** Tumor cells inside some LYVE-1 positive vessels

Table 1 Associations between CD44, CD133 expressions and clinicopathological parameters in supraglottic carcinoma

Variables	No	CD44			CD133		
		(+)	(-)	*P* Value	(+)	(-)	*P* Value
Age, y				NS			NS
>60	30	8	22		18	12	
<60	36	10	26		26	10	
Gender				NS			NS
men	62	17	45		42	22	
women	4	1	3		2	2	
Stage of disease				<0.05			<0.05
I	9	1	8		7	2	
II	22	3	19		8	14	
III	14	5	9		10	4	
IV	21	9	12		19	2	
N stage				<0.05			<0.05
N+	30	13	17		28	2	
N0	36	5	31		16	20	

Abbreviation: NS, not significant

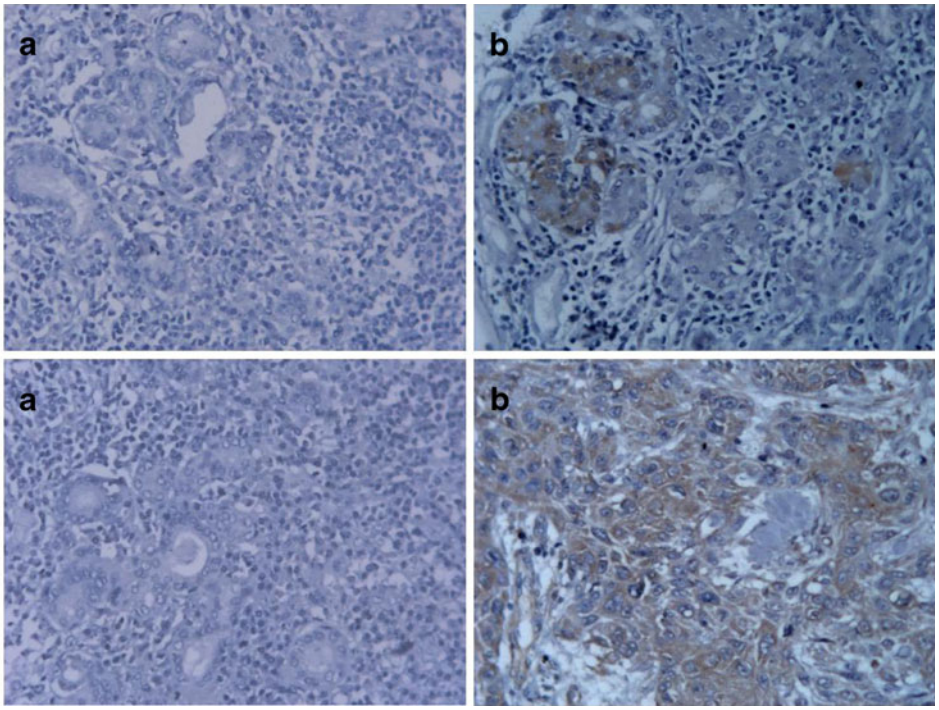

Fig. 2 Immunohistochemistry of CD44 and CD133 in supraglottic carcinoma. PBS was added to slide instead of primary antibody as negative control, and no stained was detected (Fig. 2**A**-*a*: CD44 negative control, Fig. 2**B**-*a*: CD133 negative control). In CD44-positive samples, there were about 5~10% cells stained by CD44 (Fig. 2**A**-*b*). Most CD133-positive slides were in sheets highlighted under DAB (Fig.2**B**-*b*)

nodal stages, we used the X^2 test for trends or the Fisher exact test for categorical variables. P values of less than 0.05 were considered significant.

Results

Quantification of LVD

The numbers of lymphatic vessels in N+ and N0 samples were counted under the microscope using 100× objectives. Any discrete LYVE-1 positive structure, regardless of the presence of lumens, was counted as one lymphatic vessel. The LVD detected through immunohistochemistry was 22.4±10.26 in 30N and 6.8±4.09 in 36N0 samples. A large patent of LYVE-1 positive vessels was detected mainly at the tumor margin (Fig. 1a). Some small LYVE-1 positive vessels could also be seen inside the carcinoma tissue (Fig. 1b). Many LYVE-1 positive vessels were detected between different cancer nests like stream (Fig. 1c). The presence of lymphatic invasion was usually located at the periphery of the tumors (Fig. 1d). The adjacent blood vessel surrounded by a smooth muscle which contained red blood cells was negative for LYVE-1 immunostaining (Fig. 1e). Tumor cells were detected inside some LYVE-1 positive vessels (Fig. 1f).

CD44 and CD133 Expressions by Immunohistochemistry

CD44-positive cells were detected in 43.33% of the 30 case samples of N+ (13/30) and 13.89% of the 36 case samples of N0 (5/36). For CD133, more positive cells were detected both in N+ and N0 samples than for CD44 (28/30 in N+, 16/36 in N0). Associations between CD44, CD133 expressions and their clinicopathological parameters in supraglottic carcinoma were studied as shown in Table.1. Statistical significance could be found between clinical nodal stages both for the CD44 and CD133 expressions (P<0.05). However, for slides that were CD44-positive, there were about 5~10% high-

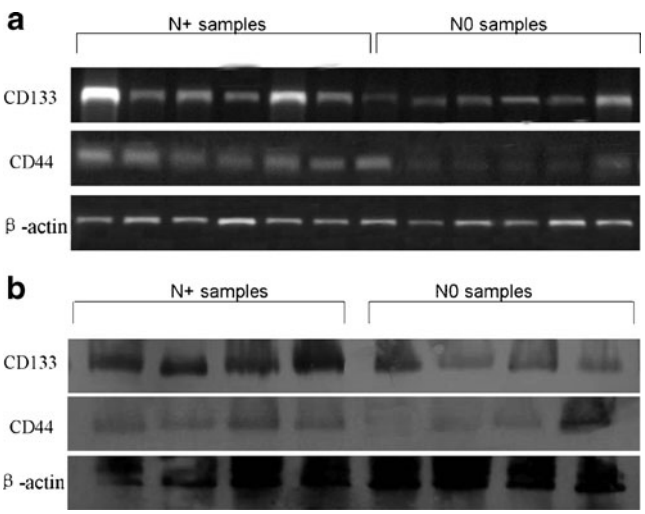

Fig. 3 mRNA and protein levels of CD44 and CD133 in N+ and N0 tissues obtained through RT-PCR and western blot. **a** CD44 and CD133 mRNA in N+ and N0 samples, with β-actin as standard. Lanes 1~6 presents six different N+ tissues, while lanes 7~12 present six different N0 tissues. **b** Expressions of CD44 and CD133 by Western blot. Lanes 1~4 presents four different tissues with N+, while lanes 5~8 present four different N0 tissues

lighted cells by CD44 antibody (Fig. 2A-*b*), with negative control group no stained (Fig. 2A-*a*). For CD133, most positive slides were in sheets highlighted under DAB or about 70~85% cells stained in each slide (Fig. 2B-*b*), with negative control group no stained (Fig. 2B-*a*). Results showed that there were 26 cases with high expression for CD133 but no expression for CD44 in 66 studied tumors in total, indicating that CD133 expression maybe not restricted to TSC.

RT-PCR and Western Blot Analysis for CD44 and CD133

Twelve case samples were selected randomly for RNA extraction, including six N+ and six N0 samples. The RT-PCR results showed that the CD44 and CD133 mRNA in N+ experimental samples were about five times higher than those of N0, with β-actin as standard (Fig. 3a). Eight case samples (four N+ and four N0) were randomly extracted for proteins. The results showed that the expressions of CD44 and CD133 in the N+ group were up-regulated by Western blot compared with N0 samples (Fig. 3b).

Discussion

Lymph node metastasis is a frequent reason for adverse clinical outcome in many carcinomas, including HNSCC [9]. Recently, a large number of reports on lymphangiogenesis have affirmed the existence and significance of lymphatic vessels, which are associated with tumor growth and metastasis in HNSCC [10]. LVD is defined as the number of lymphatic vessels in each slide or average area [11, 12]. Two compartments were defined to distinguish lymphatic vessels in different regions of tumors: the peritumoral (a 2 mm-area around the tumor mass) and intratumoral compartments (the area located within the tumor mass). Peritumoral lymphatic vessel density (P-LVD) refers to the LVD of the peritumoral compartment, while intratumoral lymphatic vessel density (I-LVD) refers to the LVD of the intratumoral compartment [13]. Several studies have reported the clinical significance of P-LVD and I-LVD respectively for tumor growth and progression [14, 15]. In the current study, lymphatic endothelial marker LYVE-1 was used to identify lymph vessels. LVD was quantified by counting the total number of LYVE-1 positive vessels in the peritumoral and intratumoral compartments. Our results showed that the LVD in N+ tumors was significantly higher than that in N0 in 66 primary supraglottic carcinomas, which was consistent with their clinical nodal stages.

TSC are also known as tumor initiating cells (TIC). Four key characteristics are used to define the TSC subpopulation: (1) only a small portion of the cancer cells in the tumor tissue has tumorigenic potential; (2) the TSC subpopulation can be separated from the other cancer cells through distinctive cell surface markers; (3) tumors resulting from the TSC contain mixed tumorigenic and nontumorigenic cells of the original tumor; and (4) the TSC subpopulation can be serially transplanted through multiple generations, indicating that it is a self-renewing population [16–18]. TSC identification is the first step to investigating their further characteristics. Cells within the CD44+ population of human cancers were reported to possess the unique properties of cancer stem cells, including HNSCC [19–21]. By applying CD44 as a specific marker, we investigated 43.33% and 13.89% CD44-positive samples ($P<0.05$) in 66 primary supraglottic carcinomas, which indicated the possible role of TSC in lymphatic metastasis. There were also 5~10% cells positive for CD44 in each slide, which was consistent with the characteristics of TSC in a small portion. RT-PCR and Western blot inspected and verified the same results.

CD133 is one of the markers for TSC in human laryngeal tumors of the Hep-2 cell line. Applying magnetic cell sorting technology, CD133 positive cells from a Hep-2 cell line have been reported to be tumorigenic [9]. CD133 has also been reported for TSC identification in various tumors [22–27]. In this study, 93.33% and 44.44% CD133-positive samples were detected for N+ and N0, respectively, which also suggested the important role of CD133 in lymphatic metastasis. Further, 70~85% cells were CD133 positive in the same sample compared with CD44, which indicated that 60~70% CD133-positive cells were negative for CD44. This suggested that CD133 expression was not specific for TSC. RT-PCR and Western blot exhibited the same results. As we know, experiments in vitro could not fully reflect the status in vivo due to the various limitations of cell lines, especially their separation from the complex microenvironment in vivo. Another study reported that CD133 expression is not restricted to stem cells, and that both CD133+ and CD133− metastatic colon cancer cells initiat tumors [28]. We hypothesize that CD133 may be an unspecific marker for TSC identification in hypostatic supraglottic carcinoma.

TSC, as identified by CD44 positive cells, may be responsible for lymph node metastasis in supraglottic carcinoma, providing a new perspective for cancer therapy. CD133, another possible TSC marker, may be unspecific for TSC identification in hypostatic supraglottic carcinoma.

References

1. Kyzas PA, Geleff S, Batistatou A et al (2005) Evidence for lymphangiogenesis and its prognostic implications in head and neck squamous cell carcinoma. J Pathol 206(2):170–7
2. Zhao D, Pan J, Li XQ et al (2008) Intratumoral lymphangiogenesis in oral squamous cell carcinoma and its clinicopathological significance. J Oral Pathol Med 37(10):616–25

3. Dalerba P, Cho RW, Clarke MF (2007) Cancer stem cells: models and concepts. Annu Rev Med 58:267–84
4. Clarke MF, Fuller M (2006) Stem cells and cancer: two faces of eve. Cell 124:1111–5
5. Wicha MS, Liu S, Dontu G (2006) Cancer stem cells: an old idea: a paradigm shift. Cancer Res 66:1883–1890
6. Pardal R, Clarke MF, Morrison SJ (2003) Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 3(12):895–902
7. Prince ME, Ailles LE (2008) Cancer stem cells in head and neck squamous cell cancer. J Clin Oncol 26(17):2871–5
8. Ailles L, Prince M (2009) Cancer stem cells in head and neck squamous cell carcinoma. Methods Mol Biol 568:175–93
9. Wei XD, Zhou L, Cheng L et al (2009) In vivo investigation of CD133 as a putative marker of cancer stem cells in Hep-2 cell line. Head and neck 31:94–101
10. Audet N, Beasley NJ, MacMillan C et al (2005) Lymphatic vessel density, nodal metastases, and prognosis in patients with head and neck cancer. Arch Otolaryngol Head Neck Surg 131:1065–70
11. Koskinen WJ, Bono P, Leivo I et al (2005) Lymphatic vessel density in vocal cord carcinomas assessed with LYVE-1 receptor expression. Radiother Oncol 77:172–5
12. Gao P, Zhou GY, Yin G et al (2006) Lymphatic vessel density as a prognostic indicator for patients with stage I cervical carcinoma. Hum Pathol 37:719–25
13. Mori D, Yamasaki F, Shibaki M et al (2007) Lateral peritumoral lymphatic vessel invasion can predict lymph node metastasis in esophageal squamous cell carcinoma. Mod Pathol 20:694–700
14. Maula SM, Luukkaa M, Grénman R et al (2003) Intratumoral lymphatics are essential for the metastatic spread and prognosis in squamous cell carcinomas of the head and neck region. Cancer Res 63:1920–6
15. Gao P, Zhou GY, Zhang QH et al (2008) Clinicopathological significance of peritumoral lymphatic vessel density in gastric carcinoma. Cancer Lett 263:223–230
16. Wang J, Guo LP, Chen LZ et al (2007) Identification of cancer stem cell-like side population cells in human nasopharyngeal carcinoma cell line. Cancer Res 67:3716–24
17. Bianchini C, Ciorba A, Pelucchi S et al (2008) Head and neck cancer: the possible role of stem cells. Eur Arch Otorhinolaryngol 265:17–20
18. Harper LJ, Piper K, Common J et al (2007) Stem cell patterns in cell lines derived from head and neck squamous cell carcinoma. J Oral Pathol Med 36:594–603
19. Pries R, Witrkopf N, Trenkle T et al (2008) Potential stem cell marker CD44 is constitutively expressed in permanent cell lines of head and neck cancer. In Vivo 22:89–92
20. Wright MH, Calcagno AM, Salcido CD et al (2008) Brca1 breast tumors contain distinct CD44+/CD24−and CD133+ cells with cancer stem cell characteristics. Breast Cancer Res 10:R10
21. Du L, Wang H, He L (2008) CD44 is of functional importance for colorectal cancer stem cells. Clin Cancer Res 14(21):6751–60
22. Zito G, Richiusa P, Bommarito A et al (2008) In vitro identification and characterization of CD133 (pos) cancer stem-like cells in anaplastic thyroid carcinoma cell lines. PLoS One 3: e3544
23. Choi D, Lee HW, Hur KY et al (2009) Cancer stem cell markers CD133 and CD24 correlate with invasiveness and differentiation in colorectal adenocarcinoma. World J Gastroenterol 15 (18):2258–64
24. Griguer CE, Oliva CR, Gobin E et al (2008) CD133 is a marker of bioenergetic stress in human glioma. PLoS One 3(11):e3655
25. Richardson GD, Robson CN, Lang SH et al (2004) CD133, a novel marker for human prostatic epithelial stem cells. J Cell Sci 117:3539–45
26. Olempska M, Eisenach PA, Ammerpohl O et al (2007) Detection of tumor stem cell markers in pancreatic carcinoma cell lines. Hepatobiliary Pancreat Dis Int 6:92–7
27. Immervoll H, Hoem D, Sakariassen PØ et al (2008) Expression of the "stem cell marker" CD133 in pancreas and pancreatic ductal adenocarcinomas. BMC Cancer 8:48
28. Shmelkov SV, Butler JM, Hooper AT et al (2008) CD133 expression is not restricted to stem cells, and both CD133+ and CD133−metastatic colon cancer cells initiate tumors. J Clin Invest 118:2111–2120