Detection of Cancer Stem Cell Markers in Sarcomas

Detekce nádorových kmenových buněk v sarkomech

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Summary
The identification of cancer stem cell markers represents one of the very relevant research topics because cancer stem cells play important roles in tumour initiation and progression, as well as during metastasis formation and in relapse of the disease. This article summarises recent knowledge on well-known and putative cancer stem cell markers in various types of bone and soft-tissue sarcomas. Special attention is paid to the detection of CD133, ABC transporters, nestin and aldehyde dehydrogenase that have been intensively studied both in tumour tissues and in sarcoma cell lines during the past few years. Finally, an overview is given of the possible CSC phenotypes provided by functional assays of tumourigenicity.

Key words
cancer stem cells – osteosarcoma– rhabdomyosarcoma – CD133 – ABC transporters – nestin – aldehyde dehydrogenase – tumourigenicity

Souhrn
Identifikace nádorových kmenových buněk v současnosti představuje jednu z nejdůležitějších oblastí výzkumu, neboť nádorové kmenové buňky hrají důležitou úlohu v iniciaci a progresi nádoru, stejně jako v procesech metastazování a relapsu onemocnění. Tento článek shrnuje současné poznatky o známých i předpokládaných markerech nádorových kmenových buněk v různých typech sarkomů kostí i měkkých tkání. Zvláštní pozornost je věnována detekci CD133, ABC transportérů, nestinu a aldehyddehydrogenázy, které byly v posledních letech intenzivně zkoumány jak v nádorové tkáni, tak v sarkomových buněčných liniích. V závěru článku je uveden přehled možných fenotypů nádorových kmenových buněk, které byly prokázány funkčními testy tumorigenicity.

Klíčová slova
nádorové kmenové buňky – osteosarkom – rabdomyosarkom – CD133 – ABC transportéry – nestin – aldehyddehydrogenáza – tumorigenicita

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Introduction

At present, a theory concerning the role of cancer stem cells (CSCs) – sometimes termed tumour-initiating cells (TICs) – in initiation and progression of cancer is widely accepted. CSCs undoubtedly play an important role in the processes of tumour initiation and progression, as well as during metastasis formation and relapse of the disease [1,2]. Thus, a detailed understanding of the characteristics of CSCs in particular tumour types may play a key role in the development of new effective antineoplastic therapies because, in heterogeneous tumour tissue, only CSCs are supposed to initiate tumour growth after grafting into immunodeficient mice [3,4]. In this context, the biological features of CSCs represent one of the very important research topics in tumour biology and experimental oncology. Although a lot of papers concerning CSCs were published during past few years, especially in haematological malignancies, neurogenic tumours and most frequent carcinomas, relatively few studies have focused on the identification of CSCs in sarcomas. Therefore, the main aim of this paper is to summarise up-to-date knowledge on the identification of well-known and potential cancer stem cell markers in various types of human sarcomas.

Detection of Cancer Stem Cell Markers in Sarcomas

In general, detection of specific markers of CSCs can be performed in tissue sections from selected types of sarcomas can be followed by detection of the same markers in the corresponding cell lines derived from samples of the respective tumour tissues. The identification of CSC markers in tumour tissues enables us to determine the frequency of cells expressing individual markers as well as levels of co-expression of these markers; an exploratory analysis of CSC markers in relation to clinical characteristics of the cohort can be also performed using these data. Cell lines derived from the respective tumours can be used to determine the proportion of cells showing the CSC phenotype and then for cell sorting based on differences in expression of these markers. Subsequently, the sorted cell populations are analysed by functional assays of the tumourigenic potential both *in vitro* (colony forming assay, sphere formation assay, invasion assay) and *in vivo* (tumourigenicity in immunodeficient mice).

Methodological approaches to the identification of expression of individual stem cell markers or their combinations in both sarcoma tissue and sarcoma cell lines are based on the detection of the mRNA in question (RT-PCR or real-time PCR) or on immunodetection of the respective protein (immunohistochemistry – IHC; immunofluorescence – IF; western blotting – WB; flow cytometry – FC; fluorescence activated cell sorting – FACS).

Furthermore, expression profiling can also be employed to identify differences in expression of genes participating in regulatory pathways in tumour cells. The results obtained should be compared with those from tissue sections as well as with the data concerning the clinical course of the disease in the respective patients to help us to determine the clinical importance of the examined individual marker or cell phenotype. Other potential markers of CSCs can also be selected on the basis of the obtained expression profiles compared with clinical data.

In addition to commonly known stem cell markers (Oct3/4, Sox2, Nanog, etc.), special attention is paid to finding specific markers that enable us to detect CSCs positively in specific types of sarcomas [5]. As given in the subheadings below, expression of the widely accepted and putative markers of CSCs is intensively studied both in tumour tissues and cell lines derived from various types of bone and soft-tissue sarcomas. The following overview is focused particularly on CSC markers that were identified in sarcomas by more than one research group. The last subheading is dedicated to describe possible CSCs phenotypes (i.e. combinations of various CSCs markers) as identified in various sarcomas by functional assays of tumourigenicity.

CD133 (Prominin-1)

CD133 glycoprotein (also known as prominin-1) is a cell surface antigen with five transmembrane domains. CD133 and namely its AC133 epitope are widely discussed to be putative "universal" markers of CSCs in various human malignancies; however, its biological function still remains unclear [6].

Expression of CD133 was detected using real-time PCR and FC in Saos-2 reference osteosarcoma cell line for the first time [7]. This finding in the same cell line was later confirmed using IF; strong expression of CD133 was also reported in four other in-house osteosarcoma cell lines [8]. CD133-positive side population was further confirmed by FACS in Saos-2, U2OS and MG-63 osteosarcoma cell lines; all these cell populations were simultaneously positive also for Ki-67 that is expressed in proliferating cells only [9]. The next study of this research group confirmed a CD133 positive subpopulation in primary cultures of 21 sarcomas (two osteosarcomas, six chondrosarcomas, one osteochondrosarcoma, four fibrosarcomas, three synovial sarcomas, three liposarcomas, one leiomyosarcoma and one chordoma), as well as in HT1080 reference fibrosarcoma cell line using FACS [10]. From all of these primary cultures, two osteosarcoma and two chondrosarcoma cell lines were successfully established; all of them show only low levels (up to 7.8%, similarly to those in primary tumors) of CD133 expression, as detected by FACS. Moreover, sorted CD133-positive cell populations were able to regenerate the original not-sorted cell populations, i.e. the mixture of CD133-positive and CD133-negative cells [10]. Strong expression of CD133 was also found in 3AB-OS osteosarcoma cell line by employment of IF, FC and RT-PCR, which was reported as a new in-house cancer stem-like cell line. In contrast, the above mentioned MG-63 osteosarcoma cell line was described as CD133-negative by this research group [11]. Most recently, CD133 expression was identified in monolayers of Saos-2, CHA59 and HuO9 osteosarcoma cell lines but significantly decreased in spheres formed from Saos-2 and CHA59 cells; in HuO9 sarcospheres CD133 expression remains at the same levels as in monolayer [12].

The clinical relevance of the CD133 expression in osteosarcoma tumour tissue

was recently reported in a cohort of 70 patients diagnosed with primary osteosarcoma. CD133 expression was found in 46 (65.7%) tumours and correlated positively with the occurrence of lung metastases [13]. Interestingly, CD133 expression in MG-63 cells was also analysed in this study using FACS and WB and this cell line was found to express CD133, in accordance with results given by Tirino and colleagues [9] but in contrast to the results by DiFiore and colleagues [11].

In synovial sarcomas, strong expression of CD133 was originally found using IHC in five samples and in three cell lines derived from these tumours [14]. In rhabdomyosarcomas, CD133 was originally detected using IHC and IF in ten FFPE samples of rhabdomyosarcoma tissue. Moreover, the same study reported CD133 expression also in five in-house cell lines derived from these tumours, demonstrated using IF, FC and WB [15]. Another study showed strong CD133 expression in five cell lines derived from embryonal rhabdomyosarcomas as well as in rhabdospheres formed from these cell lines using real-time PCR, IF, FC and WB [16]. Another five rhabdomyosarcoma cell lines (both embryonal and alveolar) were then analysed for CD133 expression by FACS and isolated CD133-positive subpopulations were obtained from all of these cell lines [17]. Results achieved using FC also indicate an increase in CD133 expression in rhabdomyosarcoma cell populations during culture and IF showed both membranous and cytoplasmic localisations of this molecule [15]; a similar finding was previously reported in an osteosarcoma cell line and this effect should be explained by deposition of CD133 into cytoplasmic vesicles, as identified by confocal microscopy [9].

ABC Transporters

ATP-binding cassette (ABC) transporters are found in the plasma membrane of numerous cell types giving them protection against xenobiotics. Their expression in transformed cells determines the multidrug-resistance (MDR) phenotype because they can actively exclude antineoplastic agents from the cytoplasm and the same mechanism is considered to cause the resistance of CSCs to chemotherapy [18].

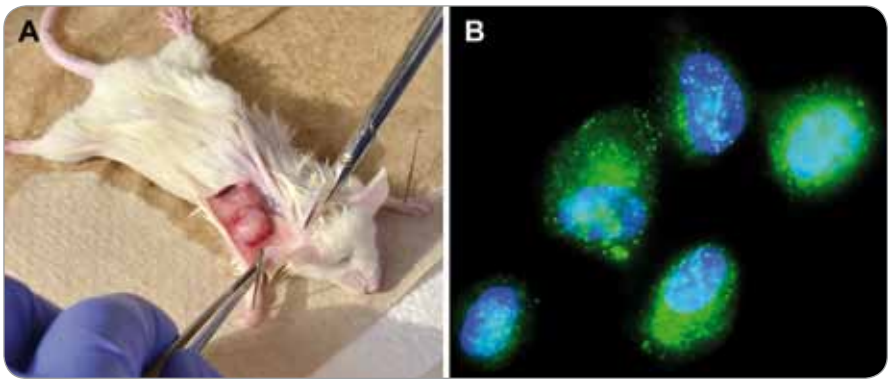

Fig. 1. Examples of our results on identification of CSCs in rhabdomyosarcomas. A. Subcutaneous xenograft tumour in NOD/SCID mice injected with NSTS-11 embryonal rhabdomyosarcoma cells. B. Expression of CD133 (green) as detected by indirect immunofluorescence in NSTS-11 cell line; counterstaining: DAPI (blue). Original magnification: 1,000×.

Results concerning expression of ABC transporters in sarcomas seem to be partly controversial. Strong expression of ABCG2 transporter was detected by FACS in Saos-2, U2OS and MG-63 osteosarcoma cell lines at the first time [9]. Surprisingly, one of these cell lines, Saos-2 cell line used as reference cell line in another study, was previously found ABCG2 negative by quantitative real-time RT-PCR analysis as well as by FC [7]. Furthermore, both MG-63 osteosarcoma cell line and 3AB-OS in-house osteosarcoma cell line were reported as ABCG2 positive using IF, FC and RT-PCR. In contrast, expression of ABCB1 transporter was found only in MG-63 cell line but not in 3AB-OS cell line in the same study [11].

Saos-2 cell line, as well as two other osteosarcoma cell lines, CHA59 and HuO9, were recently analysed for the expression of selected ABC transporters in detail using transcriptome and proteome analysis, real-time PCR and FACS. In this study, expression of four ABC transporters – ABCA5, ABCB1, ABCC1 and ABCG2 – was found in all three cell lines, but some marked differences were identified if expression in monolayers and in sarcospheres were compared. Nevertheless, only ABCG2 transporter showed a significant increase in sarcospheres of all three examined cell lines compared with the respective monolayers [12].

The up-regulation of ABCG2 was also shown in side population of MHF2003 malignant fibrous histiocytoma cell line using expression profiling [19].

Nestin

Nestin (= neuronal stem cell protein) belongs to class VI of the intermediate filaments. This protein is expressed primarily in nervous tissue during embryonic development and especially in neuronal stem cells. Nevertheless, nestin expression has also been detected in various types of human solid tumours, as well as in the corresponding established cell lines. Co-expression of nestin together with other stem cell markers, namely CD133, is discussed to be a possible marker of cancer stem cells [20].

The first study concerning nestin in sarcomas showed expression in samples of various paediatric rhabdomyosarcomas (sixteen embryonal rhabdomyosarcomas, six alveolar rhabdomyosarcomas, five pleomorphic rhabdomyosarcomas, one spindle cell rhabdomyosarcoma, one dense rhabdomyosarcoma and two embryonal sarcomas) using IHC [21]. In contrast, the same study did not find nestin in one sample of fibrosarcoma and in two samples of Ewing's sarcoma [21]. The presence of nestin in ten samples both of embryonal and alveolar rhabdomayosarcomas; as well as in five rhabdomyosarcoma cell lines derived from these tumours was later confirmed by IHC and/or IF with another anti-nestin antibody [15].

Regarding bone sarcomas, nestin was originally detected in all eighteen samples of osteosarcoma (fourteen osteoblastic osteosarcomas, three chondroblastic osteosarcomas and one teleangiectatic osteosarcoma) using IHC, as well as in three osteosarcoma cell lines derived from these tumours using IF [8]. A subsequent study of the same research group aimed to determine the prognostic value of nestin expression in 45 high-grade osteosarcomas but the results were partly controversial. Although nestin-positive tumour cells were detected in all of the examined FFPE samples using both IHC and IF, the proportion of positive neoplastic cells varied in individual samples. Moreover, high levels of nestin as measured by IF were significantly associated with worse clinical outcomes and the similar results achieved with IHC also showed a trend to shorter patient survival rates but these results did not reach statistical significance. Therefore nestin does not seem to be a powerful prognostic marker in high-grade osteosarcomas [22].

Nestin expression was also detected by RT-PCR in sphere-forming cell subpopulations of two osteosarcoma and two chondrosarcoma in-house cell lines, as well as the HT1080 fibrosarcoma reference cell line. In contrast, adherent cell populations of the same cell lines were obviously nestin negative [10]. Similar results were obtained by analysis of spheres and adherent populations of CHA59 cells by real-time PCR and WB. A reverse pattern of nestin expression (i.e. down-regulation of nestin in sarcospheres) was described in HuO9 cell line. Surprisingly, both spheres and adherent cell populations were found to be nestin negative in Saos-2 cells [12].

Aldehyde Dehydrogenase

Aldehyde dehydrogenase (ALDH) is the enzyme catalysing the oxidation of intracellular aldehydes in many cell types. High ALDH activities were detected in neuronal and haematopoietic stem cells as well as in CSCs of some human solid tumours, especially carcinomas [23].

Four human osteosarcoma cell lines – Saos-2, MG-63, HuO9, and OS99-1 – showed high levels of ALDH in cell subpopulations that substantially differ in size among these cell lines: whereas they were minor in Saos-2, MG-63, and HuO9 cell lines, OS99-1 contained about 45% of cells with high ADLH activities as detected by FC [24]. Surprisingly, a significant decrease in total number of cells with high levels of ALDH was found in OS99-1 xenografts grown in NOD/SCID mice but these cells were much more tumourigenic if compared to those with low activities of ALDH [24]. In contrast, very low activities of ALDH were identified both in Saos-2 and HuO9 cell lines (mentioned above as ALDH positive) by another research group. Nevertheless, ALDH activities were higher in spheres of CHA59 cells compared with adherent population of this same cell line [12]. CSCs exhibiting high levels of ALDH and characterised by marked chemoresistance were also identified in Ewing's sarcoma. This cell subpopulation was also successfully tested as tumourigenic using clonogenicity assay, sphere formation assay and in NOD/SCID mice [25].

Other Putative Markers of CSCs in Sarcomas

In addition to the four markers discussed above, some other molecules have been proposed as putative markers of CSCs in various sarcoma types. Nevertheless, the most important results were achieved using osteosarcoma cell lines. For example, overexpression of MET oncogene is involved in regulation of self-renewal and cell differentiation [26]. Double positivity for both CD117 (c-kit) and Stro-1 (a marker of osteogenic progenitors in bone marrow) is also considered to indicate a tumourigenic phenotype in osteosarcoma cells [27]. Furthermore, the previous study as well as other findings suggest the CXCR4 chemokine receptor to be one of the putative CSCs markers in this tumour type [13,27].

Possible CSCs Phenotypes in Sarcomas Identified by Functional Assays

Cell subpopulations isolated from two osteosarcoma and two chondrosarcoma in-house cell lines, as well as the HT1080 fibrosarcoma reference cell line, were analysed in detail using sphere-formation assay and tumourigenic assay in NOD/SCID mice [10]. Strong expressions of CD44 and CD29 cell surface antigens, as well as expression of Oct3/4, Nanog, Sox2 and nestin, were found in all CD133-positive cell populations capable of forming spheres and to induce tumour xenografts. These cell populations were also able to differentiate into mesenchymal lineages, such as osteoblasts and adipocytes [10].

The 3AB-OS cells belonging to another osteosarcoma in-house cell line were identified as CD133 and ABCG2 positive, with strong expression of stem cell markers Oct3/4, Nanog, nucleostemin, hTERT and several apoptosis inhibitors. Both MG-63 and 3AB-OS cell lines were capable of sarcosphere formation, but these spheres differed in number and volume during growth [11]. Although the previous study reported the MG-63 cell line to be CD133 negative, He et al successfully isolated a CD133-positive subpopulation from this cell line; this subpopulation was identified as positive for Oct4, Nanog and CXCR4, and showed increased migration and invasive potential [13]. Similarly, a cell subpopulation characterised by high ALDH activity was positive for Oct3/4, Nanog and Sox2 [24].

The sarcospheres rich in CSCs isolated from Saos-2, CHA59 and HuO9 osteosarcoma cell lines were shown to be positive for ABCG2 transporter and chromobox protein homolog 3 (CBX3); this phenotype was accompanied by decreased expression of CD24, CD44 and CD326 compared with monolayer culture [12].

In rhabdomyosarcomas, Sana et al showed using functional assays that NSTS-11 in-house cell line positive for CD133, nestin, nucleostemin and Oct3/4 is able to form colonies *in vitro* and tumour xenografts in NOD/SCID mice [15]. Similarly, a tumourigenic potential of rhabdomyosarcoma cell populations forming rhabdospheres *in vitro* was proved; CD133, Oct4, Nanog, c-Myc, Pax3 and Sox2 stem cell markers were up-regulated in these cell populations [16].

CD133-positive subpopulations isolated from RD and RH30 rhabdomyosarcoma cell lines were shown to be myo-

genically primitive cells with enhanced ability to form colonies. Interestingly, both of these cell lines were identified as resistant to chemotherapy but sensitive to genetically engineered HSV oncolytic virotherapy [17].

Moreover, Walter et al found by expression profiling that tumourigenic populations of rhabdomyosarcoma cells showed apparently more similarities with neuronal stem cells compared with expression profiles of haematopoietic or mesenchymal stem cells [16]. These findings are in accordance with all published results of Veselska and colleagues that found CD133/nestin positive cell populations – previously described as typical cancer stem cell phenotype in neurogenic tumours [28,29] – in both osteosarcoma [8] and rhabdomyosarcoma [15] cell lines.

Conclusion

To conclude, the findings on various types of sarcoma cells as summarised above suggest that putative CSCs markers such as CD133, ABC transporters, nestin and ALDH are of importance also in sarcoma cells. Nevertheless, despite published results especially on various osteosarcoma and rhabdomyosarcoma cell lines, the characteristic phenotype of CSCs allowing their unambiguous identification for diagnostic or therapeutic purposes remains unclear.

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