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Identification of side population cells (stem-like cell population) in pediatric solid tumor cell lines.

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Abstract

Purpose: Recent evidence has supported the "cancer stem cell" theory that cancer contains a small number of cancer stem cells (CSC) as a reservoir of cancer cells. Only the CSC, but not the remaining majority of constituent cancer cells, are thought to be responsible for tumorigenesis, progression, and metastasis as well as cancer relapse, suggesting that the CSC should be targeted to eradicate the cancer. Side population (SP) cells isolated by fluorescence activated cell sorting (FACS) using Hoechst dye are known to be enriched in stem cells in various normal tissues as well as cancers. The authors investigated whether such stem-like side population cells may exist in pediatric solid tumors (PST). **Materials and methods:** Sixteen pediatric tumor cell lines including 7 neuroblastomas, 4 rhabdomyosarcomas, and 5 Ewing's sarcomas were used for FACS analysis. Analysis of SP cells based on the exclusion of the DNA binding dye Hoechst 33342 with and without verapamil using FACS was performed. **Results:** One Ewing's sarcoma cell line did not show an SP fraction, and only a small fraction of SP cells (0.12-14.6%) was detected in the other 15 cell lines. These SP cells were all sensitive to verapamil. **Conclusions:** This study suggested that most PST would contain a small fraction of SP cells (possible stem-like population). Targeting the CSC will provide a novel treatment strategy to eradicate refractory PST.

Index words: Cancer stem cell, Side population, Neuroblastoma, Rhabdomyosarcoma, Ewing's sarcoma

Stem cells are characterized by the properties of self-renewal and multipotency which are called “stemness”. Normal tissue comprises a hierarchical organization composed of a small fraction of stem cells as a reservoir and their descendants. Recent evidence has shown that cancer may also be maintained by a small fraction of cancer stem cells (CSC) (stem-like cancer cells) which retain the properties common to normal stem cells.[1-4] It is believed that only the CSC but not the remaining majority of their descendants (constituent cancer cells) are responsible for tumorigenesis, progression, metastasis, and relapse after treatments. Recently, the existence of CSC has been proven for several cancers.[4-6] Stem cells have a long life span with the ability to survive in severe environments, staying quiescent in G0 phase most of the time and self-renewing occasionally. In contrast, progenitors and mature cells have a limited life span, and rapidly divide. This implies that multiple oncogenic gene abnormalities are more likely to accumulate in stem cells than in progenitors and mature cells. The CSC can generate their descendants, including progenitors and relatively differentiated cancer cells, producing the heterogeneity of the cancer. Furthermore, only the CSC can survive conventional anticancer therapies which target only the rapidly dividing cancer cells, although their descendants (the remaining majority of constituent cancer cells) can be killed. This could be strongly related to treatment failure as well as cancer relapse. Accordingly, the elimination of the CSC is an important goal for eradicating refractory cancers. Despite recent advances in treatment modalities, some refractory pediatric solid tumors (PST) remain a challenge for pediatric oncologists. It is possible that targeting the CSC in refractory PST may provide a novel treatment strategy to eradicate them completely. The aim of this study was to identify the possible stem-like population in PST.

Side population (SP) cells characterized by the efficient efflux of Hoechst 33342 dye are thought to be enriched for stem cells in many normal tissues.[7-15] Recently, SP cells that showed stem cell characteristics were isolated from several cancers. [16-20] SP cells express various ABC transporter family members which are responsible for drug resistance, including ABCG2 (BRCP1).[21] In this study, the authors investigated the existence of SP cells in PST cell lines by fluorescence activated cell sorting (FACS) analysis.

Materials and methods

Cell lines: Seven neuroblastoma (NB) cell lines (SK-N-AS, SK-N-DZ, GOTO、LAN1, LAN5, NB16, NB19), 4 rhabdomyosarcoma (RMS) cell lines (RMS, RD, SCMC-RM-2, KYM-1) and 5 Ewing's sarcoma (EWS) cell lines (ES-1-OT, UTP-ES-1, SCMC-ES-1, RD-ES, SK-ES) were cultured in appropriate media and used for FACS analysis. [22-24]

Isolation of side population (SP) cells: Pediatric tumor cells were adjusted to 10^6 cells/ml in HBSS. Hoechst 33342 dye was added to the cell suspension at a final concentration of 5 μg/ml and the mixture was incubated for 90 min at 37 °C. As a negative control, verapamil (Sigma, St. Louis, MO, USA), an inhibitor of ABC transporters, was also added to a final concentration of 50 or 500μM with Hoechst 33342. After Hoechst 33342 staining, the tumor cells were washed by centrifugation, resuspended at 1-2 x 10^7 cells/ml in HBSS, and kept on ice until use. Before FACS analysis, propidium iodide (PI) solution was added to a final concentration of 1μg/ml to identify non-viable cells. FACS analysis and sorting were performed on a dual laser

flow cytometer (Becton Dickinson FACS Vantage SE cell sorter). The Hoechst dye was excited by the 355 nm UV laser, and its fluorescence was measured at two wavelengths using a 424/44 (Hoechst blue) band-pass filter and a 585/42 (Hoechst red) band-pass filter. PI fluorescence was excited by 488 nm laser, and detected after passing through a 630/22 band-pass filter. PI-positive dead cells and debris were excluded.

Results

SP cells in PST cell lines : One EWS cell line, RD-ES, did not show SP cells in this study (Fig.1). SP cells were detected in the remaining 15 cell lines including 7 NB, 4 RMS and 4 EWS (0.12-14.6%) (Table 1 and Fig.2).

Sensitivity of SP cells to verapamil : The SP fraction cells disappeared upon treatment with 50 μM verapamil in 6 cell lines (GOTO, NB16, SCMC-RM-2, ES-1-OT, SK-ES, UTP-ES-1) (Table 1, and Fig.2B and 2C). In the other 9 cell lines, the SP fraction disappeared in the presence of 500 μM of verapamil (Table 1 and Fig.2A).

Discussion

Macroscopically as well as microscopically, PST show a heterogeneous appearance, containing undifferentiated as well as differentiated cells. Furthermore, when the tumor is resected after aggressive chemotherapies and/or radiotherapies, it contains a heterogeneous mixture of tissues, showing not only some viable portions but also other non-viable portions in the same tumor. Certainly, not every cell in the bulk

of the tumor behaves in the same manner. How this heterogeneity is generated has been well answered by the "cancer stem cell theory". That is to say, cancer originates from a small fraction of CSC (stem-like cancer cells) with the abilities of self-renewal as well as multipotency as a reservoir. The cancer has a hierarchical organization with only a small number of CSC and a large number of their descendants (constituent cancer cells). Only the CSC are responsible for tumorigenicity, progression and metastasis in the cancer, while their descendants are not. Their descendants will differentiate in various ways, resulting in the heterogeneity of the cancer. Recently, CSC have been isolated from several cancers.[4-7] Normal stem cells and CSC show similar resistance to current therapies, because they both stay in a quiescent state and have a common drug efflux capacity. This means that a small fraction of CSC can survive aggressive therapies, even though the remaining majority of the constituent cancer cells are responsive to them. This eventually leads to relapse of the cancer. Accordingly, the real target determining the biological characteristics and the treatment strategy should be the CSC themselves instead of the bulk majority of the constituent cancer cells. If this theory is true in the case of PST, isolation of the CSC is the first step to better understand their characteristics and to develop novel treatment strategies for eradicating refractory PST.

The CSC in PST resembled normal stem cells morphologically as well as immunohistochemically, as found in AML, in which CSC were first identified.[4] The CSC are believed to retain properties similar to those of normal stem cells. That means that CSC may be isolated using the same procedures as used to isolate tissue stem cells. The major problems in isolating normal stem cells as well as CSC are their rarity and the absence of specific markers for purifying them. Various efforts have

been made to isolate CSC from various cancers.[4-7] CSC were first identified in AML that could reproduce the original cancer with heterogeneous phenotypes and expressed the stem cell surface markers CD34+, CD38-.[4] In cancers for which specific markers had not been identified, CSC or stem-like cancer cells have been isolated as side population (SP) cells that export Hoechst 33342 dye.[16-20] It has been shown that stem cells are enriched in SP cells in various normal tissues, including bone marrow, skeletal muscle, mammary gland, skin, lung, testis, brain, liver, and kidney.[7-15] Recently, SP cells have been isolated as the CSC that possess the stemness characteristics and are responsible for tumorigenesis in several cancers.[16-20] SP cells are characterized by the rapid efflux of Hoechst 33342 dye via ATP binding cassette (ABC) transporters. SP fractions are known to disappear upon treatment with inhibitors of ABC transporters such as verapamil and rapamycin. A variety of ABC transporters, including multiple drug resistance protein (MDR1, ABCB1), MDR related protein (MRP1, ABCC1), and breast cancer resistant protein (BRCP1, ABCG2), have been shown to contribute to the drug resistance in cancers.[25-29] Interestingly, some of these ABC transporters have also been shown to be expressed in various kinds of normal stem cells.[21, 25-30] In particular, BRCP1 (ABCG2) is known to contribute to the exclusion of Hoechst 33342 dye in SP cells, which are enriched in stem cells.[21] ABCG2 expressed in normal stem cells and SP cells is believed to play a physiological role in the protection of both of them. This suggests that the SP cells in cancers possess drug resistance due to expression of the same transporters. Transcriptional profiling studies of SP and non-SP cells in several tissues showed that the genes upregulated in SP cells are implicated in the quiescent status, the maintenance of pluripotency and the capacity to undergo asymmetric division.[31] SP cells have also been identified in several tumor cell lines as well as

fresh tumor samples, including NB.[16-20] Hirschmann-Jax et al. demonstrated that SP cells constituted 0.8-51% of the cells in 15 of 23 NB tumor samples as well as 4-37% of the cells in five NB cell lines. SP cells in NB were Gd2+, c-kit+, CD133-, CD71-, CD56± and expressed ABC transporter proteins ABCG2 and ABCA3 at high levels, supporting the possibility that they were CSCs.[16] We have investigated SP cells in PST cell lines, including NB, RMS, and ES. Unlike fresh tumor samples, cell lines do not contain any contaminating non-cancer stem cells such as bone marrow-derived stem cells. Accordingly, the SP cells identified in cell lines are definitely derived from cancer cells. The stem-like population in PST may be enriched in SP cells. Our data showed that 15 of 16 pediatric solid tumor cell lines contained a small fraction of SP cells (0.12~14.6%). The percentage of SP fractions is lower than that in the previous study on NB.[16] This may be partly due to the differences in the gated regions for the SP fraction or in the types of the cell lines used. It is likely that the cells with "stemness" constitute a smaller fraction. One Ewing's sarcoma cell line did not show an SP fraction. SP formation was blocked by 50 or 500 μM verapamil (an inhibitor of ABC transporters), although the sensitivity of the cell lines varied. The absence of SP cells in the presence of verapamil confirmed the identity of the SP cells as an enriched stem-like population. This also suggested that verapamil-sensitive ABC transporters are involved in the Hoechst dye efflux of these SP cells. Nine of 15 cell lines were more resistant to verapamil because verapamil is generally used at a concentration of 50 μM (Table 1). Different mechanisms may be involved in the Hoechst dye efflux activity of these cell lines. Further characterization of the SP cells as candidates of the CSC will be essential to understand the mechanisms of tumorigenesis, progression, metastasis, treatment failure, and tumor relapse in PST. SP analysis should also be

done in fresh tumor samples, because the cell lines may be a collection of immortalized and rather modified cancer cells after many passages.

Our study demonstrated that most PST contained SP cells (possible stem-like population). Conventional treatments used for PST have targeted rapidly growing cancer cells, which are synthesizing DNA. The survival of the CSC in PST despite such treatments may be responsible for treatment failure and tumor relapse. In other words, the target of future treatments for PST should be the CSC themselves. New treatment strategies for targeting the CSC will be required to eradicate the refractory PST completely. Although how to attack only the CSC selectively without any influence on the normal tissue stem cells will be a difficult problem, complete removal of the CSC pool as a reservoir will result in the ability to eradicate refractory PST in the near future.

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Table 1. SP cells in pediatric solid tumor cell lines and their sensitivity to verapamil.

Cell lines	SP cells (%)	Verapamil-induced disappearance of SP fraction (Concentration of verapamil))
Neuroblastoma		
SK-N-AS	+ (0.43)	+ (500μM)
SK-N-D Z	+ (3.26)	+ (500μM)
GOTO	+ (0.55)	+ (50μM)
LAN1	+ (0.54)	+ (500μM)
LAN5	+ (9.88)	+ (500μM)
NB16	+ (1.76)	+ (50μM)
NB19	+ (8.71)	+ (500μM)
Rhabdomyosarcoma		
RMS	+ (0.53)	+ (500μM)
RD	+ (1.51)	+ (500μM)
SCMC-RM-2	+ (0.83)	+ (50μM)
KYM-1	+ (14.6)	+ (500μM)
Ewing's sarcoma		
ES-1-OT	+ (0.33)	+ (50μM)
UTP-ES-1	+ (5.53)	+ (50μM)
SCMC-ES-1	+ (0.63)	+ (500μM)
RD-ES	−	
SK-ES	+ (0.12)	+ (50μM)

Figure legends

Fig.1 A Ewing's sarcoma cell line, RD-ES, did not show an SP fraction in the absence (left) or presence (right) of verapamil.

Fig.2 Sp cells found in pediatric solid tumor cell lines. SP cells identified in a neuroblastoma cell line, LAN 5 (A, left), a rhabdomyosarcoma cell line, SCMC-RM-2 (B, left), and a Ewing's sarcoma cell line, UTP-ES-1 (C, left). Every SP fraction disappeared with co-treatment of 50 (B and C, right) or 500 μM (A, right) verapamil plus Hoechst 33342.