Translational Oncology 13 (2020) 100846

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon

Original research

Pediatric sarcomas display a variable EpCAM expression in a histology-dependent manner

Lucia Tombolan [a,*], Elisabetta Rossi [b,c], Angelica Zin [a], Luisa Santoro [d], Paolo Bonvini [a], Rita Zamarchi [c], Gianni Bisogno [e]

[a] Institute of Pediatric Research, Fondazione Città della Speranza, Padua, Italy
[b] Department of Surgery, Oncology and Gastroenterology, Oncology Section, University of Padova, Padova, Italy
[c] Veneto Institute of Oncology IOV - IRCCS, Padua, Italy
[d] University Hospital of Padova, Institute of Pathology, Padua, Italy
[e] Department of Woman's and Children's Health, Hematology and Oncology Unit, University of Padua, Padua, Italy

ARTICLE INFO

Article history:
Received 19 June 2020
Received in revised form 17 July 2020
Accepted 20 July 2020
Available online xxxx

ABSTRACT

EpCAM is a transmembrane glycoprotein typically overexpressed in cancer of epithelial origin and mainly involved in the epithelial-to–mesenchymal transition (EMT) of tumor cells that spread and disseminate. Strategies for the targeting and capture of EpCAM-expressing tumor cells are showing promise in cancers prone to metastatize, both as diagnostic tools and potential therapies. Sarcomas are among the most aggressive tumors in children, with a common mesenchymal origin that comprises both soft tissue sarcomas (STS) and bone sarcomas. The aim of this study was to assess EpCAM expression in pediatric sarcomas and correlate its expression with disease progression. To do so, we analyzed a set of cell lines and primary tumor tissues from rhabdomyosarcoma (RMS), Ewing sarcoma (ES), synovial sarcoma (SS) and desmoplastic small round cell tumor (DSRCT) STS, or osteosarcoma (OS) bone cancer. We demonstrated that EpCAM was variably expressed in pediatric sarcomas, with DSRCT, a rare, aggressive and almost fatal tumor type, characterized by the highest EpCAM expression levels. Interestingly, although EpCAM expression was lower in RMS tumors, high levels at diagnosis correlated with reduced patients' overall survival ($p < 0.05$). Indeed, membrane-bound EpCAM was detected in circulating sarcoma tumor cells, revealing its potential to be used as dissemination biomarker in this type of childhood cancers. This reinforces the concept that pediatric sarcomas do express both epithelial and mesenchymal markers and reside in an intermediate condition that most likely contributes to their aggressive phenotype and low survival rate.

Introduction

Sarcomas encompass a heterogeneous group of mesenchymal tumors with different pathological, molecular and clinical characteristics classified into two major subtypes: bone sarcomas and soft tissue sarcomas (STSs). In children, STSs represent approximately the 8% of all neoplastic malignancies, with rhabdomyosarcoma (RMS) accounting for 55–60% of them and the remaining composed by a variety of different histotypes usually termed as “non-rhabdomyosarcoma soft tissue sarcomas” (NRSTS). Likewise, osteosarcoma is the most frequent bone sarcoma affecting children, adolescents, and young adults. Survival of patients with localized disease is remarkable, with almost 80% of children with localized RMS and Ewing Sarcoma cured, as the vast majority of synovial sarcoma cases (90%) [1–3]. Nonetheless, the most important challenge in pediatric sarcomas remains the treatment of patients with metastasis at diagnosis. Despite the use of different intensive treatment strategies prognosis has not significantly changed, with a 3-year event-free survival (EFS) around 30–40% that drops to less than 20% when multiple negative prognostic factors are involved [1]. Studying and understanding the process of metastatic spread is an important goal to undercover the tumor dissemination mechanisms and develop possible target treatments able to improve the survival of cancer patients.

The capability of a malignant tumor to become metastatic require single cells or clusters of cancer cells with special plasticity to detach from the primary tumor, shed into bloodstream and grow into new environments. All these processes necessitate of a fine balance deregulation of gene and

Abbreviations: EpCAM, epithelial cell adhesion molecule; ARMS, alveolar rhabdomyosarcoma; ERMS, embryonal rhabdomyosarcoma; ES, Ewing sarcoma; OS, osteosarcoma; SS, synovial sarcoma; DSRCT, desmoplastic small-round-cell tumor; STS, soft tissue sarcoma; EMT, epithelial to mesenchymal transition; MET, mesenchymal to epithelial transition; IHC, immunohistochemistry; IRS, Intergroup Rhabdomyosarcoma Studies (Clinical Group); CTCs, circulating tumor cells.

* Corresponding author.
E-mail address: lucia.tombolan@unipd.it. (L. Tombolan).

http://dx.doi.org/10.1016/j.tranon.2020.100846

protein expression, which results in a dynamic change of cell phenotype known as epithelial-to-mesenchymal transition (EMT).

The epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein typically expressed in tumors deriving from epithelial cells. High levels of EpCAM are detected in human colorectal cancer stem cells [4] and tumorigenic breast cancer cells [5], likewise in undifferentiated human embryonic stem cells (hESCs) [6]. In this context, EpCAM is involved in the regulation of cell-cell adhesion, via the interaction with transmembrane cadherin proteins [7], and in cell proliferation through the up-regulation of c-Myc [8] and cyclin D1 [9].The dynamic expression of EpCAM in tumor cells strictly correlates with the EMT process during cancer spreading, as in the reverse process, known as the mesenchymal-to-epithelial transition (MET), that allows the acquisition of adhesion and proliferative properties of cancer cells regardless their histological origin. EpCAM is transiently lost during the early pro-migratory and migratory period of cancer cells and subsequently re-acquired in settled larger metastases [10] [11]. Of note, MET-related program occurs mainly in sarcomas that express epithelial markers, such as E-cadherin and β-catenin in synovial sarcomas [12] or in Ewing/PNET tumors that express tight junction proteins [13], but it also represents the ultimate step of EMT in carcinoma cells once metastases have invaded new tissues. Several sarcomas are characterized by the co-expression of both epithelial and mesenchymal markers, appearing to reside in the intermediate EMT state also termed “metastable phenotype”. Metastable phenotypes are more common in aggressive sarcomas and frequently contribute to drug resistance [14].

The expression of EpCAM on the cell surface of carcinoma cells makes it a useful marker for capturing circulating tumor cells (CTCs) in blood of cancer patients. Since CTCs represent a crucial step in disease progression and metastasis, CTCs are considered a promising minimally invasive biomarker to test the metastatic potential of tumors [15].

Based on these findings, we investigated EpCAM expression in pediatric sarcomas, at both transcript and protein level, reporting for the first time a strong tumor type-dependent variance between histotypes. We also investigated the possibility to use the expression of EpCAM on the cell surface for detecting circulating tumor cells (CTC) in sarcomas. Our findings disclosed a dynamic expression of EpCAM during tumor evolution and a likely correlation between EpCAM expression and patients' overall survival.

Methods

Cell culture

All cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin and streptomycin (100 μg/ml) (Life Technologies, Carlsbad, CA) at 37 °C in 5% CO2 in a humidified incubator. All cell lines were mycoplasma-free at time of collection.

MCF-7, H228, A459, RH30 and RD cells were obtained from American Type Culture Collection (Manassas, VA); RH4 and CCA were gift from Prof. Pier Luigi Lollini (Dept. Medicina Specialistica, Diagnostica e Sperimentale, University of Bologna, Italy) [16]; RH36 and SMS were obtained from Dr. Maria Tsokos (National Cancer Institute, Bethesda, MD) [17]; RH28, CHP100 and TC32 were a gift from Dr. Peter J. Houghton (St. Jude Children's Hospital, Memphis, TN) [18–20]; synovial sarcoma cell line, SW982, was kindly provided by Dr. Andrea Ferrari (Istituto Nazionale Tumori IRCSS, Milan, Italy) [21] while osteosarcoma cell line SAOS-2 [22] was provide by Prof. Scorrano (Department of Biology, University of Padova).

Tumor samples, peripheral blood samples and ethical issue

Tumor specimens of sarcoma patients were retrieved from the sarcoma biobank part of Italian Association of Pediatric Hematology and Oncology (AIEOP). A total of 76 patients were analyzed: 21 Alveolar RMS (ARMS), 19 Embryonal RMS (ERMS), 16 Ewing Sarcoma (ES), 7 Synovial sarcoma (SS), 3 Osteosarcoma (OS), 10 Desmoplastic small-round-cell tumor (DSRCT). The cohort of 76 cases included also patients analyzed both at onset and at relapse of disease (Fig. 4A). Clinical features of patients enrolled in this study are summarized in Supplementary material, Tables S1 and S2.

Peripheral blood samples from 11 pediatric patients, who underwent to our observation for sarcomas, were also collected upon signed informed consent. The enrolled patients were representative of main sarcoma histologies: ARMS (n = 2), ERMS (n = 5), ES (n = 2), SS (n = 1) and OS (n = 1). For 8 out of 11 specimens processed for CTC detection, EpCAM expression was also evaluated. These 8 patients are included in the cohort of 76 patients analyzed for EpCAM.

This study was approved by the local Ethics Committee (protocol number 3347/AO/14) and the patients have signed informed consent.

Expression analysis by qRT-PCR

Total RNA was extracted from sarcoma fresh tumor biopsied using Trizol Reagent (Life Technologies, Thermofisher Scientific) following the manufacture's instruction. Superscript II (Life Technologies, Thermofisher Scientific) was used to retrotranscribed 1 μg of RNA to cDNA then used as template in qPCR experiments. Syber Green PCR master mix (Applied Biosystem, Thermofisher Scientific) were used to perform qPCR in a ViiA 7 Real-Time PCR System (Applied Biosystem, Thermofisher Scientific). EpCAM and GAPDH (housekeeping) primers were tested for efficiency before usage. Fetal skeletal muscle (FSA) was used as control and a relative expression ratio (RQ) was calculated by comparative delta Ct method ($2^{-\Delta\Delta Ct}$) [23] available in ViiA 7 Real-Time PCR System. A 95% confidence interval (IC) was calculated. Primers sequences are available on Supplemental data (Table S3).

Flow cytometry

We used flow cytometry to establish the EpCAM expression in sarcoma cell lines. One million of cells were harvested and collected, following fixation. Cells were incubated with anti EpCAM antibody (clone HEA125, cat. n. LS B3709 Life Span BioScience) or a mouse isotype control (mouse IgG1, cat.n. 555748, BD Bioscience) for 30 min. Afterwards, cells were rinsed with PBS for twice and the record collected using BD FACSCAlibur (at least 20,000 events) and the data were analyzed using Kaluza (Beckman Coulter).

Immunohistochemistry (IHC)

Overall, 6 STS cell lines and 11 STS patients (ARMS = 3 cases; ERMS = 2 cases, and ES = 4 cases, SS = 1 case, DSRCT = 1 case) were considered. The immunostaining was performed on 5 μm formalin fixed, paraffin-embedded tissue sections using an automated system (Bond-maX, Leica, Newcastle Upon Tyne, UK).

After dewaxing and rehydration, the sections were incubated with retrieval buffer solution (Leica) for antigen recovery. Then, the specimens were washed with phosphate-buffered saline (pH 7.0) and incubated with the Bond Polymer Refine Detection Kit (Leica). The expression of EpCAM was immunohistochemically assessed by applying monoclonal antibodies against EpCAM antigen (clone MOC-31; Cell Marque, Rocklin, CA-USA, dilution 1:50) and the staining was visualized with 3,3′-diaminobenzidine. Mayer's hematoxylin was used as counterstained.

Detection of circulating tumor cells (CTCs)

Enumeration of CTCs in whole blood was performed by the CellSearch System as previously reported [24]. According to manufacturer's instructions, we classified an event as a CTC when its morphological features were consistent with that of a cell and it exhibited the phenotype EpCAM+, CK (8, 18, and 19)+, DAPI+ and CD45−.

Moreover, to investigate the co-expression of both epithelial and mesenchymal markers in CTCs of pediatric sarcomas, we used the anti-Desmin Ab (clone Y66; abcam, UK), in conjunction with the CTC standard assay, as

previously reported for other integrated tests [25,26]. Results were expressed as the total number of CTCs and Desmin-positive CTCs per 7.5 ml of blood.

Statistical analysis

Prism7 software (Graphpad software, Inc.) was used to perform all statistical analysis. Mann-Whitney or Kruskal-Wallis test were applied to qPCR data. Kaplan–Meier survival curves with log-rank test were applied to cohort of RMS samples to test the prognostic value of EpCAM expression. The heatmap relative to onset and relapse was done using RQ data. Overall survival (OS) days were calculated from the date of diagnosis to the date of death for any cause or the last follow-up; indeed, a time frame between date of diagnosis and date of the first event or the last follow-up was used for progression-free survival (PFS).

Results

EpCAM is expressed in pediatric sarcoma cell lines and tumor cells

With the aim to explore the expression of EpCAM in the heterogeneous group of pediatric sarcomas, we analyzed a panel of 10 sarcoma cell lines representative of the RMS (RH30, RH28, RH4, RH36, RD, SMS-CTR), ES (TC32 and CHP100), SS (SW982) and OS (SAOS-2), and compared them to fetal and adult skeletal muscle samples (FSM and ASM, respectively), breast and lung carcinoma cell lines (MCF7, A549 and H2228) (Fig. 1A). We found that EpCAM expression was heterogeneous among the cell lines, regardless their epithelial or mesenchymal origin. As expected, MCF7 breast cancer cells overexpressed EpCAM, whereas A459 and H228 adenocarcinoma cell lines expressed EpCAM mRNA at similar level of sarcoma cells. Adult skeletal muscle cells, deriving from a typical differentiated tissue of mesenchymal origin, did not expressed EpCAM. Next, we measured EpCAM mRNA expression in 76 sarcoma primary tumors (Tables S1–S2) and, as for the cell lines, a variable expression was observed among samples (Fig. 1B, Kruskal-Wallis test $p = 0.0009$), with DSCRT tumors displaying the highest EpCAM mRNA levels. Accordingly, when EpCAM mRNA expression was analyzed by querying four public sarcoma gene expression datasets from R2 Genomic Analysis and Visualization platform (http://r2.amc.nl), DSCRT tumors still displayed the highest levels of EpCAM compared to the others (Fig. 1C Kruskal-Wallis test $p = 0.0001$). In addition, when a second epithelial cell marker, E-cadherin, was tested, the expression was found, even higher of the mesenchymal family member protein N-cadherin (Fig. 1D, Mann-Whitney test $p < 0.001$).

EpCAM protein is detectable by flow cytometry and immunohistochemistry

Epithelial cell adhesion molecule (EpCAM) is a glycosylated transmembrane cell surface protein typically expressed in carcinomas. Therefore, we evaluated EpCAM protein expression by flow cytometry in MCF7and A459 carcinoma cells and compared to that assessed in 6 sarcoma cell lines (RH4, RH30, RD, RH36, CHP100 and TC32). As for EpCAM transcript levels, protein expression was variable among the sarcoma cell lines: readily detectable in both ARMS cell lines (RH4 and RH30), weak (RH36 and TC32) or even absent (RD and CHP100) in either ERMS or Ewing Sarcoma cells (Fig. 2A). However, when protein expression was assessed by immunocytochemistry, only MCF7 breast cancer cells stained positive to anti-EpCAM antibody; while all the others, including both carcinoma and sarcoma cells were negative (Fig. 2B), due most likely to the lower sensitivity of the technique compared to flow cytometry. Accordingly, when EpCAM expression was assessed in STS specimens, distinguished based on the different EpCAM mRNA levels (Fig. 3A), protein detection by immunostaining was possible only in cases with high mRNA levels (Fig. 3B). Namely, the DSRCT sample found positive to anti-EpCAM antibodies expressed EpCAM mRNA at comparable levels than those measured in MCF7 carcinoma cells. In contrast, EpCAM protein was not detected in RMS and SS tumor samples when EpCAM immunostaining is chooses as detection method (Fig. 3B).

High levels of EpCAM correlate with a decreased survival

To investigate how EpCAM expression changes during tumor evolution we evaluated EpCAM mRNA levels in 13 STS samples at onset and relapse of disease: 6 RMS, 6 ES, 1 SS and 1 DSRCT. We observed a different expression of EpCAM during tumor evolution and progression (Fig. 4A, heatmap), however the levels were not significantly different at onset and relapse (Wilcoxon matched-pairs signed rank test $p = 0.56$). An extensive analysis was, hence, performed for the RMS group that counted 40 patients enrolled in the same clinical protocol. Association analysis between EpCAM expression and know prognostic factors, such as histology, fusion status and clinical stage, was carried out. As shown previously, high EpCAM mRNA levels were significantly associated with tumor histology (Fig. 1A, ERMS vs. ARMS Mann-Whitney $p = 0.0348$), whereas for any other variable considered a correlation was not demonstrated. We also tested whether an association between EpCAM levels and type of relapse (local vs. distal) existed. The analysis was performed on 14 RMS patients with localized disease at diagnosis that experienced an event during the follow-up, but no significant difference was observed between groups (Table S4). However, when Kaplan-Meier overall (OS) and progression free survival (PFS) analysis was carried out, dividing patients according to EpCAM median expression value, patients with higher levels of EpCAM were found to have a significantly poorer outcome compared to those with low EpCAM levels, while in PFS EpCAM levels were not significant (Fig. 4B and C, long-rank test, OS $p = 0.022$, PFS $p = 0.13$). Similarly, DSRCT patients overexpressing EpCAM had a dismal prognosis with very poor overall survival, suggesting that an association between high EpCAM levels and clinical outcome is likely (60% dead for progressive disease; 10% alive; 30% no data available).

Feasibility of exploiting EpCAM-based technologies to detect CTCs in pediatric sarcomas

The demonstration of EpCAM expression in primary tumors, although at different levels and depending on sarcomas histotype, offers the rationale to further investigate the expression of EpCAM in the peripheral blood of sarcoma patients, at CTCs level.

In fact, it has been already proved that EpCAM expression can be acquired during tumor progression [27] and EpCAM is also known to be highly expressed in cancer stem cells [28]. The association of EpCAM expression with patients' outcome, at least in RMS, supports the idea that EpCAM-positive tumor cells might play a role in metastatic cascade in sarcoma patients.

To test whether CTCs were detectable in sarcoma pediatric patients, blood samples from 11 patients were tested at diagnosis before starting the therapy, using the CellSearch platform as EpCAM-based technology for CTC capturing.

Besides EpCAM, we included additional epithelial and mesenchymal markers to isolate CTCs of pediatric sarcomas, such as cytokeratins (CK8/18/19) and Desmin.

The patient characteristics and the distribution of CTC number are shown in Table 1. Overall, 7 out of 11 (63, 6%) sarcoma patients had at least one CTC per 7.5 ml of peripheral blood, while 5 out of 11 (45%) patients had at least two CTCs. Moreover, 4 (57%) CTCs-positive patients had one Desmin–positive CTC. The number of total CTCs and Desmin-positive CTCs ranged from 1 to 7 (median 2) and 0 to 3 (median 1) cells, respectively.

Notably, the percentage of CTC-positive sarcoma patients and the number of total CTCs resembled data previously reported at diagnosis in other carcinomas [29]. In 8 out of 11 patients analyzed at CellSearch, EpCAM expression in the primary tumor was also assessed. Interestingly, we observed that 2 patients with metastasis at diagnosis (IRS group IV) showed detectable CTCs in peripheral blood (2 and 7 CTCs, respectively) and were positive for EpCAM expression (Table S5).

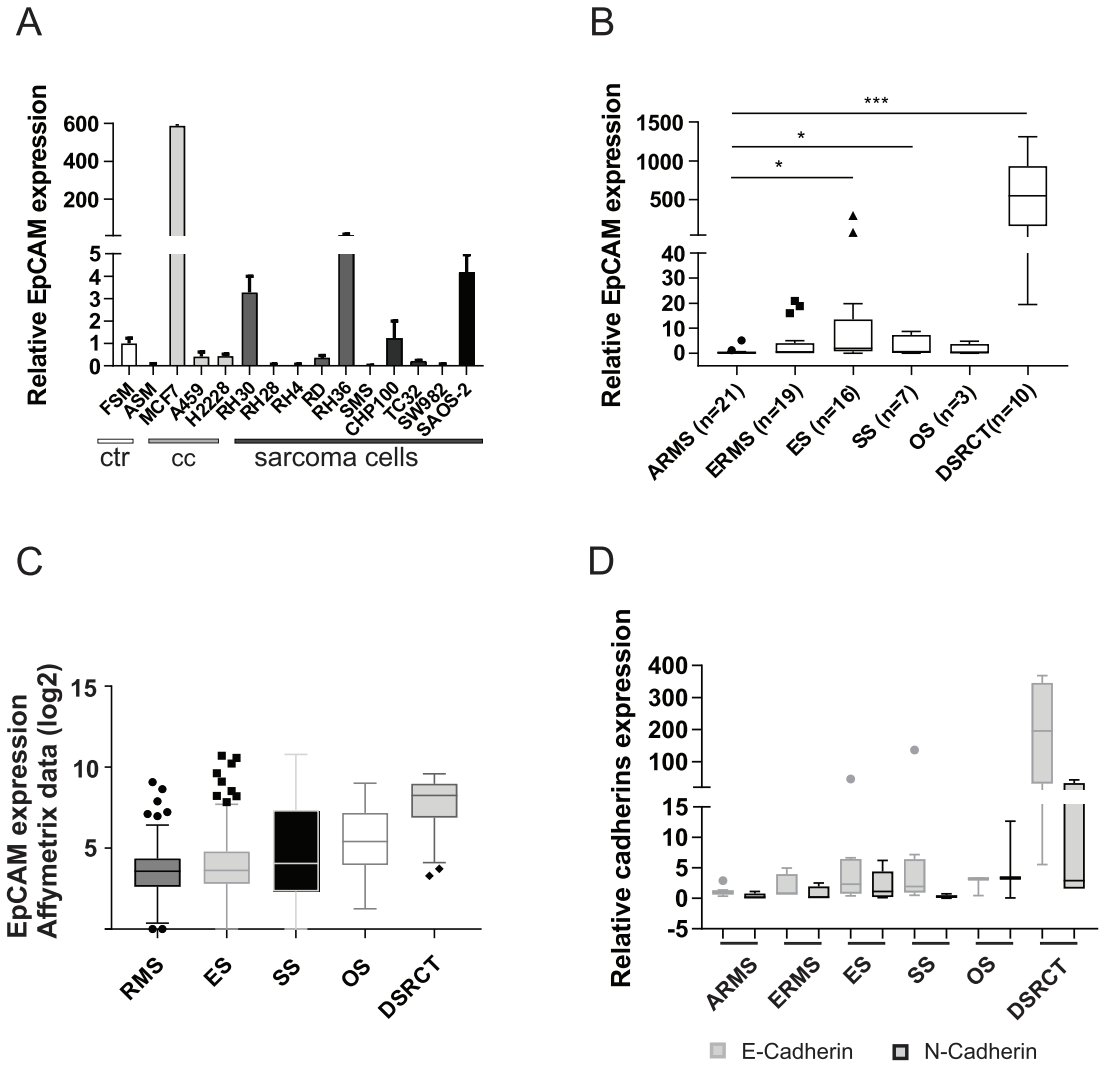


Fig. 1. Evaluation of EpCAM and E-/N-cadherins expression by qPCR. (A) We analyzed EpCAM expression in a set of tumor cell lines; carcinoma-derived cells (CC, carcinoma cells: MCF7, A459, H2228) and sarcoma cell lines representative of different histotypes that include RMS (RH30, RH4, RH28, RD, RH36, SMS-CTR), ES (TC32, CHP100), SS (SW982) and OS (SAOS-2). In (B) EpCAM expression was evaluated in a cohort of 76 primary tissue of pediatric sarcomas. A statistically different expression was observed across sarcoma histotypes (Kruskal-Wallis test $p = 0.0009$). In (C) are represented EpCAM levels (log2) based on gene expression datasets analyzed using R2 Genomic Analysis and Visualization platform. A significant different expression of EpCAM was observed between histotypes (Kruskal-Wallis test $p = 0.0001$) (Fillion dataset = 137 samples, DSCRT, ES, SS; Davicioni dataset = 147 RMS cases, Dellatre = 117 ES patients, Kobayashi = 27 OS samples). (D) A subgroup of sarcoma tissue samples was analyzed for cadherin N- and E- expression. Epithelial E-cadherin is more expressed than mesenchymal N-cadherin in sarcoma patients (Mann-Whitney test $p < 0.001$). All qPCR data are reported as expression value relative to normal tissues used as controls (fetal, FSM, and adult skeletal muscle, ASM). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference transcript. Vertical bars represent confidence interval at 95%.

Besides, among 5 patients who experienced an event during tumor evolution, 4 were positive for EpCAM expression and 3 had detectable CTCs at diagnosis. Unfortunately, 2 out of 3 patients that exhibited the highest EpCAM levels experienced progressive disease and had a fatal outcome.

Discussion

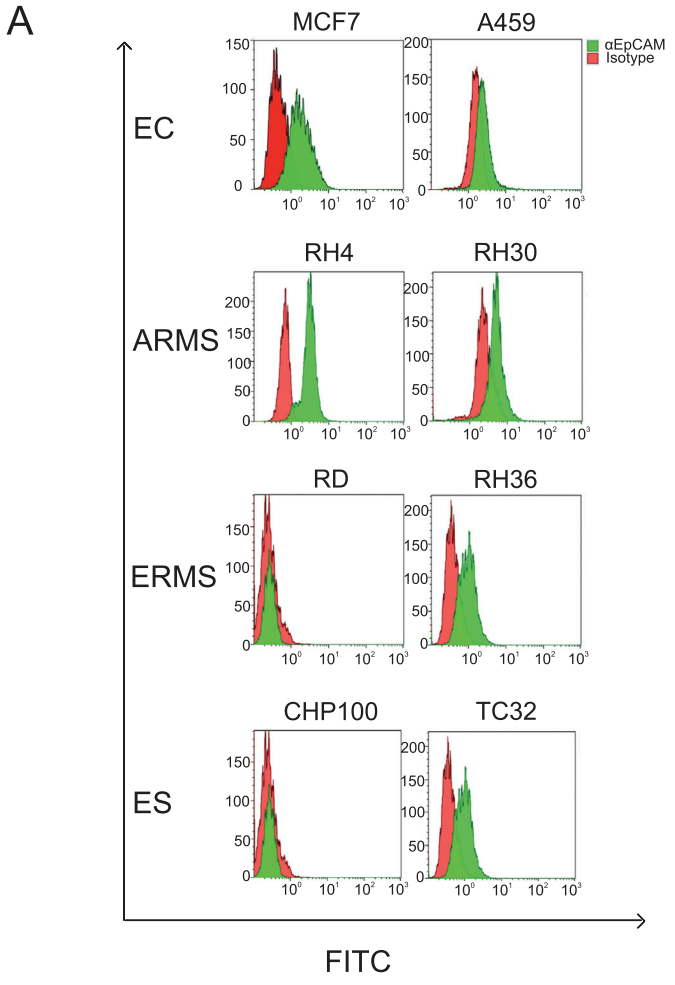
The current study investigated the expression of Epithelial Cell Adhesion Molecule (EpCAM) in pediatric sarcomas. EpCAM is a transmembrane protein typically expressed in normal epithelial tissues and overexpressed in cancer of epithelial origin. It is involved in cell adhesion processes, by interacting with transmembrane cadherin proteins, and it plays a role in the epithelial-to-mesenchymal transition (EMT) program, a key process in cancer dissemination and metastasis formation.

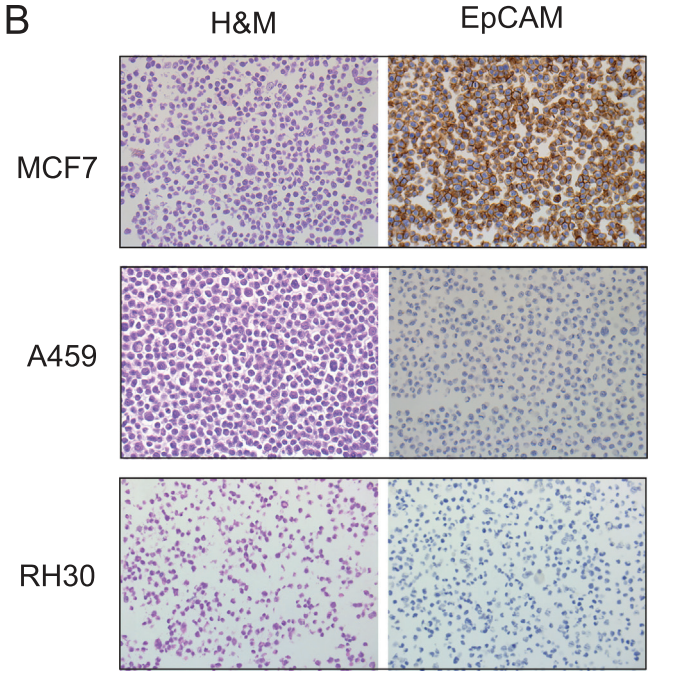
Our data support the concept that bone and soft tissue sarcomas may reside in an intermediate metastable phenotype and shift from a more proliferative (epithelial-like) to a more invasive (mesenchymal) state, maintaining the combined expression of epithelial and mesenchymal markers.

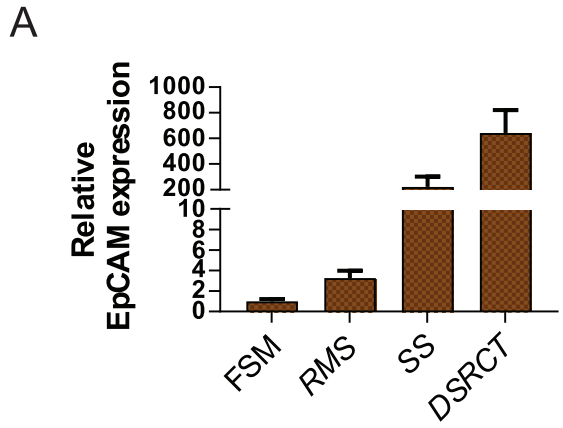
Indeed, we demonstrated that EpCAM is expressed in pediatric sarcomas, though levels varied significantly in a histology-dependent manner: from the highest levels of DSRCT tumors to the lowest of RMS ones, through the intermediate values of ES, OS and SS. Differences were observed between cases as well, with primary embryonal RMS expressing more EpCAM than alveolar rhabdomyosarcomas, in accordance with the different gene and protein expression profiles of the two major RMS subtypes [33–35]. Of note, EpCAM was found to be expressed in the same histology-dependent manner reported into publicly available pediatric cancer datasets, supporting the concept that the expression of epithelial tumor markers in mesenchymal sarcomas is likely. Desmoplastic small round cell tumors (DSRCT) are the examples, as they show expression of multiple markers of both epithelial and mesenchymal origin, such as keratin, vimentin and desmin [36]. Deregulation of EMT-related genes in DSRCT tumors would explain their extreme inter-tumor heterogeneity, as their propensity to disseminate and resist to drug treatment [37,38].

Similarly, Synovial Sarcoma displays epithelial differentiation in restricted areas of the primary tumor mass, perhaps sites of spontaneous

EMT. Indeed, epithelial-like synovial sarcoma cells do express E-cadherin and other typical epithelial markers, most likely as a consequence of *SYT-SSX1* or *SYT-SSX2* inhibitory activity on E-cadherin repressor Snail [12]. Such a variable degree of epithelial/mesenchymal differentiation has been observed in Ewing Sarcoma and epithelioid sarcomas as well. ES tumor cells characterized by strong *EWSR1-FLI1* transcription activity display an epithelial-like phenotype, whereas low-level *EWSR1-FLI*-positive cells mostly express cell-matrix proteins which give advantages in terms of motility and invasive capacity but reduce their proliferative activity







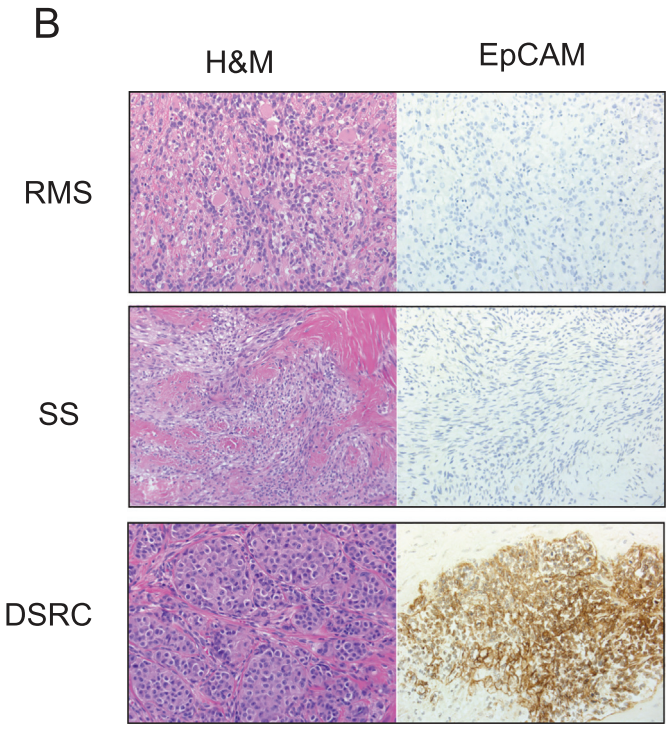


Fig. 3. Evaluation of EpCAM status in selected STS primary tumor. (A) qPCR quantitation of EpCAM mRNA in tumor biopsies of STS patients. As example, we reported 3 cases with different level of EpCAM from low to very high. Expression value is relative to fetal skeletal muscle use as control while, GAPDH was used as housekeeping gene. Error bars are the 95% confidence interval. (B) Immunodetection of EpCAM in paraffin-embedded sections of tumor samples from the same STS patients analyzed above. Only the sample with high EpCAM expression at transcript level (DSCTR) has a positive staining. Original magnification 20×. Hematoxylin was used as counterstain. H&M, hematoxylin staining; RMS, rhabdomyosarcoma; SS, synovial sarcoma; DSRCT, Desmoplastic small-round-cell tumor; FSM, fetal skeletal muscle.

Fig. 2. Evaluation of EpCAM protein in tumor cell lines. (A) Cell surface expression of EpCAM levels by flow cytometry of the indicated cell lines, in red isotype antibody and in green EpCAM specific antibody. (B) Immunodetection of EpCAM in paraffin-embedded section of 3 different cell lines representative of breast cancer (MCF7) adenocarcinoma (A459) and pediatric rhabdomyosarcoma (RH30). Original magnification, 20×. Hematoxylin was used as counterstain.

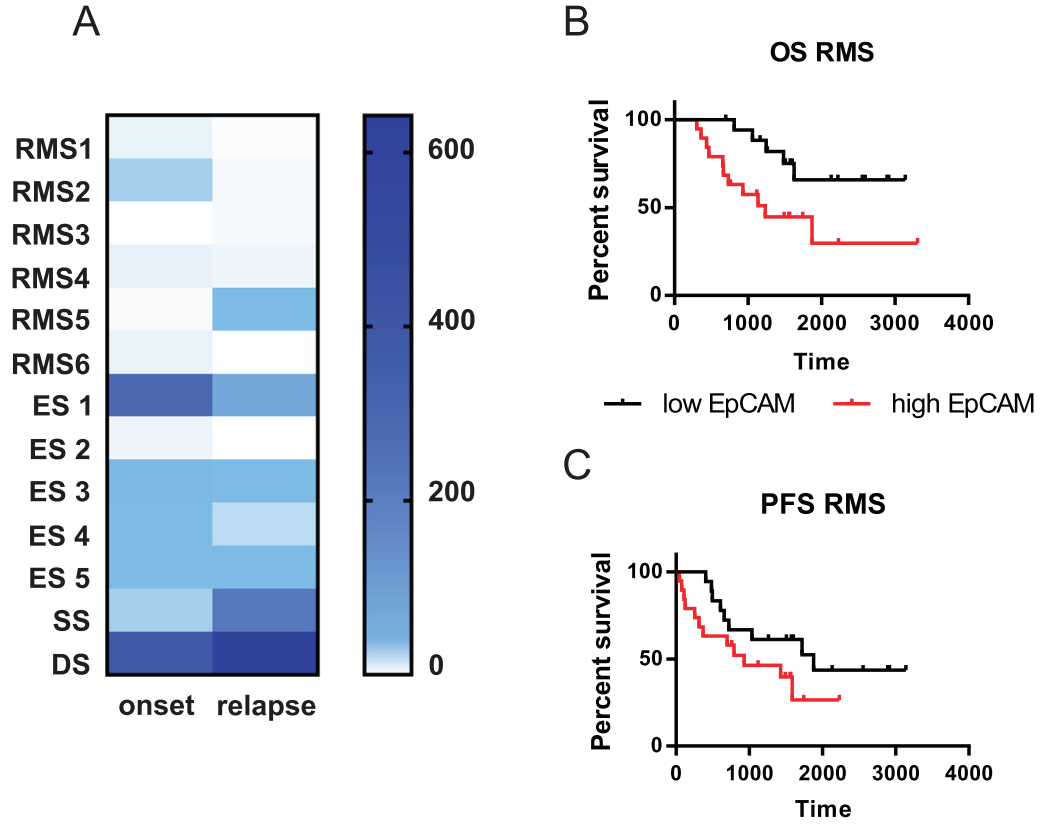


Fig. 4. EpCAM expression in tumor evolution context. (A) The heatmap shows the expression of EpCAM transcript in selected STS patients evaluated at onset and relapse of disease. Kaplan-Meier and long-rank analysis for overall survival, OS (B) and progression free survival, PFS (C) was performed for the cohort of RMS patients (n = 40) based on low or high EpCAM levels at diagnosis (median value). The subgroup with high EpCAM level at onset of disease shows a significantly different outcome in OS (OS, p = 0.022, PFS = p = 0.13).

[39]. In osteosarcoma tumor cells, combined expression of both epithelial and mesenchymal markers, such as cadherins and vimentin has been well documented [40,41]. Many reports, however, have elucidated an active role of miRNAs (i.e. miR-130a, miR-126, miR-145) in the modulation of cadherins as well as other epithelial cell markers, resulting in a shift from a proliferative state to a more invasive phenotype of osteosarcoma cells [42,43]. Finally, a regulation of EMT/MET-related genes in RMS tumors has also been described. The guanine nucleotide exchange factor T(GEFT) appears to be involved in this program, throughout the activation of the Rac1/Cdc42-PAK signaling pathway, the loss of E-cadherin and the increased expression of N-cadherin [44].

The fact that PAX3-FOXO1 transcription factor antagonizes wild-type Pax3-induced cell aggregation and epithelioid morphologic changes in RMS cells explains why this type of tumor expresses the lowest levels of both EpCAM and E-cadherin among all the sarcoma histotypes assessed in our study.

Targeting EpCAM to capture in the bloodstream of cancer patients the cells that detach from the primary tumor and are considered to be the seed of metastasis, the circulating tumor cells (CTCs), has been proven successful in many prospective clinical studies, since a strong correlation between EpCAM expression, increased risk of relapse and decreased overall survival has been widely demonstrated [24,30,31]. Nowadays, a rationale to use EpCAM as a marker of cancer spreading and metastasis formation in carcinoma patients exists, whereas for patients with tumors of mesenchymal origin, such as children with bone or soft tissue sarcomas, biomarkers suitable for monitoring disease progression are not available. Mesenchymal markers, such as N-Cadherin and vimentin, are frequently expressed in peripheral blood mononuclear cells and, hence, are not suitable for CTC detection and prediction of metastasis occurrence [32].

Accordingly, by using an EpCAM-based automated platform we got the proof-of-principle that CTCs are detectable in the peripheral blood of sarcoma patients, they are not of hematopoietic origin (all the circulating cells were CD45-negative), and express markers for either an epithelial or mesenchymal phenotype.

Besides, although the sample size was limited, the prevalence of CTC-positive cases and the levels of detectable CTCs resembled that observed in other malignancies, mainly carcinomas of adult patients. This finding was only apparently in contrast with low EpCAM expression in primary STS tissues assessed by IHC, as immunostaining is commonly used for investigating robust antigen expression on paraffin-embedded solid tumor tissues rather than measuring weak antigen expression at single-cell level. Technical hitches may explain the differing results obtained by IHC and CellSearch, leaning more toward the latter than the former to use EpCAM as a target for detection and isolation of single circulating tumor cells [27].

This finding warrants further investigation in ad hoc designed study, however it supports CTC enumeration as improvement in management of sarcoma patients.

In conclusion, our data demonstrated that EpCAM is an epithelial cell marker detectable in pediatric bone and soft tissue sarcoma specimens and cell lines. In pediatric sarcomas EpCAM expression levels are tumor type-dependent and correlate with adverse patients' outcome. Our results reinforce the concept that sarcoma cells express both epithelial and mesenchymal cell markers, giving them the capability to adapt well to different growth conditions. The knowledge of why certain proteins, like EpCAM, change their expression during tumor progression in pediatric sarcoma cells remains to be clarified, as the detection, enumeration and molecular characterization of CTCs, their clinical and therapeutic significance merits more attention.

Our data contribute to define the complex picture of pediatric sarcomas, highlighting as the comprehension of tumor biology may have important clinical implications to control and cure these rare, aggressive and heterogeneous malignancies.

Table 1
Main clinical features of pediatric sarcoma patients analyzed for CTCs. All patients were evaluated at "baseline" prior to any treatment or surgery. (-, indicates a data not available, numbers indicate patients for each group.)

Variable	ARMS	ERMS	ES	OS	SS
Age, years					
≤10 years	2	4	1	0	0
>10 years	0	1	1	1	1
Sex					
Male	1	3	2	0	1
Female	1	2	0	1	0
Size					
≤5 cm	0	3	1	-	-
>5 cm	2	2	1		
IRS group					
I–II–III	1	4	2	-	-
IV	1	1	0		
Translocation					
PAX3-FOXO1	2	0	0	0	0
EWS-FLI1	0	0	1	0	0
SYT-SSX1	0	0	0	0	1
No	0	5	1	1	0
Event					
Yes	1	1	1	1	1
No	1	4	1	0	0
Status of disease					
Alive	1	4	1	1	1
Dead	1	1	1	0	0
CTCs detection					
CTC = 0 (none cell)	0	2	1	0	1
CTC = 1 (1 cell)	1	1	0	0	0
CTCs ≥ 2 (2 or more cells)	1	2	1	1	0

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2020.100846.

CRediT author statement

Lucia Tombolan: Conceptualization, Methodology, Investigation, Writing-original draft
Elisabetta Rossi: Investigation, Methodology, Visualization
Angelica Zin: Investigation
Luisa Santoro: Investigation
Paolo Bonvini: Writing-review & editing
Rita Zamarchi: Formal analysis, Conceptualization, Writing-review & editing
Gianni Bisogno: Supervision, Writing-review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors wish to thank Ilaria Zanetti, Angela Scagnellato and Julia Daragjati for clinical data management.

Funding

This work is supported by Fondazione AIRC per la ricerca sul cancro (AIRC) grant IG-15813 (GB). PB and AZ were supported by Fondazione Città della Speranza (the City of Hope Foundation).

References

[1] O. Oberlin, A. Rey, E. Lyden, G. Bisogno, M.C.G. Stevens, W.H. Meyer, et al., Prognostic factors in metastatic rhabdomyosarcomas: results of a pooled analysis from United States and European cooperative groups, J. Clin. Oncol. 26 (2008) 2384–2389, https://doi.org/10.1200/JCO.2007.14.7207.
[2] C.A.S. Arndt, J.A. Stoner, D.S. Hawkins, D.A. Rodeberg, A.A. Hayes-Jordan, C.N. Paidas, et al., Vincristine, actinomycin, and cyclophosphamide compared with vincristine, actinomycin, and cyclophosphamide alternating with vincristine, topotecan, and cyclophosphamide for intermediate-risk rhabdomyosarcoma: Children's Oncology Group Study D9803, J. Clin. Oncol. 27 (2009) 5182–5188, https://doi.org/10.1200/JCO.2009.22.3768.
[3] A. Ferrari, G.L. De Salvo, B. Brennan, M.M. van Noesel, A. De Paoli, M. Casanova, et al., Synovial sarcoma in children and adolescents: the European Pediatric Soft Tissue Sarcoma Study Group prospective trial (EpSSG NRSTS 2005), Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 26 (2015) 567–572, https://doi.org/10.1093/annonc/mdu562.
[4] P. Dalerba, S.J. Dylla, I.K. Park, R. Liu, X. Wang, R.W. Cho, et al., Phenotypic characterization of human colorectal cancer stem cells, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 10158–10163, https://doi.org/10.1073/pnas.0703478104.
[5] M. Al-Hajj, M.S. Wicha, A. Benito-Hernandez, S.J. Morrison, M.F. Clarke, Prospective identification of tumorigenic breast cancer cells, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 3983–3988, https://doi.org/10.1073/pnas.0530291100.
[6] T.Y. Lu, R.M. Lu, M.Y. Liao, J. Yu, C.H. Chung, C.F. Kao, et al., Epithelial cell adhesion molecule regulation is associated with the maintenance of the undifferentiated phenotype of human embryonic stem cells, J. Biol. Chem. 285 (2010) 8719–8732.
[7] S.V. Litvinov, M. Balzar, M.J. Winter, H.A.M. Bakker, I.H.B. Bruijn, F. Prins, et al., Epithelial cell adhesion molecule (Ep-CAM) modulates cell–cell interactions mediated by classic cadherins, J. Cell Biol. 139 (1997) 1337–1348, https://doi.org/10.1083/jcb.139.5.1337.
[8] M. Münz, C. Kieu, B. Mack, B. Schmitt, R. Zeidler, O. Gires, The carcinoma-associated antigen EpCAM upregulates c-myc and induces cell proliferation, Oncogene. 23 (2004) 5748–5758, https://doi.org/10.1038/sj.onc.1207610.
[9] A. Chaves-Pérez, B. Mack, D. Maetzel, H. Kremling, C. Eggert, U. Harréus, et al., EpCAM regulates cell cycle progression via control of cyclin D1 expression, Oncogene. 32 (2013) 641–650, https://doi.org/10.1038/onc.2012.75.
[10] M. Jojović, E. Adam, U. Zangemeister-Wittke, U. Schumacher, Epithelial glycoprotein-2 expression is subject to regulatory processes in epithelial-mesenchymal transitions during metastases: an investigation of human cancers transplanted into severe combined immunodeficient mice, Histochem. J. 30 (1998) 723–729, https://doi.org/10.1023/a:1003486630314.
[11] P. Massoner, T. Thomm, B. Mack, G. Untergasser, A. Martowicz, K. Bobowski, et al., EpCAM is overexpressed in local and metastatic prostate cancer, suppressed by chemotherapy and modulated by MET-associated miRNA-200c/205, Br. J. Cancer 111 (2014) 955–964, https://doi.org/10.1038/bjc.2014.366.
[12] T. Saito, M. Nagai, M. Ladanyi, SYT-SSX1 and SYT-SSX2 interfere with repression of E-cadherin by snail and slug: a potential mechanism for aberrant mesenchymal to epithelial transition in human synovial sarcoma, Cancer Res. 66 (2006) 6919–6927, https://doi.org/10.1158/0008-5472.CAN-05-3697.
[13] A.N. Schuetz, B.P. Rubin, J.R. Goldblum, B. Shehata, S.W. Weiss, W. Liu, et al., Intercellular junctions in Ewing sarcoma/primitive neuroectodermal tumor: additional evidence of epithelial differentiation, Mod. Pathol. 18 (2005) 1403–1410, https://doi.org/10.1038/modpathol.3800435.
[14] G. Sannino, A. Marchetto, T. Kirchner, T.G.P. Grünewald, Epithelial-to-mesenchymal and mesenchymal-to-epithelial transition in mesenchymal tumors: a paradox in sarcomas? Cancer Res. 77 (2017) 4556–4561, https://doi.org/10.1158/0008-5472.CAN-17-0032.
[15] F.-C. Bidard, D.J. Peeters, T. Fehm, F. Nolé, R. Gisbert-Criado, D. Mavroudis, et al., Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data, Lancet Oncol. 15 (2014) 406–414, https://doi.org/10.1016/S1470-2045(14)70069-5.
[16] D.N. Shapiro, J.E. Sublett, B. Li, J.R. Downing, C.W. Naeve, Fusion of PAX3 to a member of the forkhead family of transcription factors in human alveolar rhabdomyosarcoma, Cancer Res. 53 (1993) 5108–5112http://www.ncbi.nlm.nih.gov/pubmed/8221646. (Accessed 22 March 2016).
[17] G. Kouraklis, T.J. Triche, R. Wesley, M. Tsokos, Myc oncogene expression and nude mouse tumorigenicity and metastasis formation are higher in alveolar than embryonal rhabdomyosarcoma cell lines, Pediatr. Res. 45 (4 Pt 1) (1999) 552–558, https://doi.org/10.1203/00006450-199904010-00015.
[18] B.J. Hazelton, J.A. Houghton, D.M. Parham, E.C. Douglass, P.M. Torrance, H. Holt, et al., Characterization of cell lines derived from xenografts of childhood rhabdomyosarcoma, Cancer Res. 47 (1987) 4501–4507http://www.ncbi.nlm.nih.gov/pubmed/3607778. (Accessed 9 July 2020).
[19] R.K. Srivastava, A.R. Srivastava, Y.S. Cho-Chung, Synergistic effects of 8-chlorocyclic-AMP and retinoic acid on induction of apoptosis in Ewing's sarcoma CHP-100 cells, Clin. Cancer Res. 4 (1998) 755–761http://www.ncbi.nlm.nih.gov/pubmed/9533545. (Accessed 9 July 2020).
[20] J.A. Toretsky, Y. Connell, L. Neckers, N.K. Bhat, Inhibition of EWS-FLI-1 fusion protein with antisense oligodeoxynucleotides, J. Neuro-Oncol. 31 (1997) 09–16, https://doi.org/10.1023/A:1005716926800.
[21] M. Shabahang, A.E. Buffan, J.M. Nolla, L.M. Schumaker, R.V. Brenner, R.R. Buras, et al., The effect of 1,25-dihydroxyvitamin D3 on the growth of soft-tissue sarcoma cells as mediated by the vitamin D receptor, Ann. Surg. Oncol. 3 (1996) 144–149, https://doi.org/10.1007/BF02305793.
[22] M. Vilien, H. Wolf, The specificity of the microcytotoxicity assay for cell-mediated immunity in human bladder cancer, J. Urol. 119 (1978) 338–342, https://doi.org/10.1016/S0022-5347(17)57484-4.

[23] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method, Methods. 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.
[24] M. Cristofanilli, G.T. Budd, M.J. Ellis, A. Stopeck, J. Matera, M.C. Miller, et al., Circulating tumor cells, disease progression, and survival in metastatic breast cancer, N. Engl. J. Med. 351 (2004) 781–791.
[25] E. Rossi, U. Basso, R. Celadin, F. Zilio, S. Pucciarelli, M. Aieta, et al., M30 neoepitope expression in epithelial cancer: quantification of apoptosis in circulating tumor cells by CellSearch analysis, Clin. Cancer Res. 16 (2010) 5233–5243, https://doi.org/10.1158/1078-0432.CCR-10-1449.
[26] F. Pantano, E. Rossi, M. Iuliani, A. Facchinetti, S. Simonetti, G. Ribelli, et al., Dynamic changes of receptor activator of nuclear factor-κB expression in circulating tumor cells during denosumab predict treatment effectiveness in metastatic breast cancer, Sci. Rep. 10 (2020) 1288, https://doi.org/10.1038/s41598-020-58339-2.
[27] E. Rossi, M. Fassan, M. Aieta, F. Zilio, R. Celadin, M. Borin, et al., Dynamic changes of live/apoptotic circulating tumour cells as predictive marker of response to Sunitinib in metastatic renal cancer, Br. J. Cancer 107 (2012) 1286–1294, https://doi.org/10.1038/bjc.2012.388.
[28] J.E. Visvader, G.J. Lindeman, Cancer stem cells in solid tumours: accumulating evidence and unresolved questions, Nat. Rev. Cancer 8 (2008) 755–768, https://doi.org/10.1038/nrc2499.
[29] W.J. Allard, J. Matera, M.C. Miller, M. Repollet, M.C. Connelly, C. Rao, et al., Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases, Clin. Cancer Res. 10 (2004) 6897–6904.
[30] S.J. Cohen, C.J.A. Punt, N. Iannotti, B.H. Saidman, K.D. Sabbath, N.Y. Gabrail, et al., Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer, J. Clin. Oncol. 26 (2008) 3213–3221, https://doi.org/10.1200/JCO.2007.15.8923.
[31] K. Schulze, C. Gasch, K. Staufer, B. Nashan, A.W. Lohse, K. Pantel, et al., Presence of EpCAM-positive circulating tumor cells as biomarker for systemic disease strongly correlates to survival in patients with hepatocellular carcinoma, Int. J. Cancer 133 (2013) 2165–2171.
[32] S. Werner, A. Stenzl, K. Pantel, T. Todenhöfer, Expression of epithelial mesenchymal transition and cancer stem cell markers in circulating tumor cells, Adv. Exp. Med. Biol. 994 (2017) 205–228, https://doi.org/10.1007/978-3-319-55947-6_11.
[33] E. Davicioni, F.G. Finckenstein, V. Shahbazian, J.D. Buckley, T.J. Triche, M.J. Anderson, Identification of a PAX-FKHR gene expression signature that defines molecular classes and determines the prognosis of alveolar rhabdomyosarcomas, Cancer Res. 66 (2006) 6936–6946, https://doi.org/10.1158/0008-5472.CAN-05-4578.
[34] C. De Pittà, L. Tombolan, G. Albiero, F. Sartori, C. Romualdi, G. Jurman, et al., Gene expression profiling identifies potential relevant genes in alveolar rhabdomyosarcoma pathogenesis and discriminates PAX3-FKHR positive and negative tumors, Int. J. Cancer 118 (2006) 2772–2781, https://doi.org/10.1002/ijc.21698.
[35] D. Williamson, E. Missiaglia, A. de Reyniès, G. Pierron, B. Thuille, G. Palenzuela, et al., Fusion gene-negative alveolar rhabdomyosarcoma is clinically and molecularly indistinguishable from embryonal rhabdomyosarcoma, J. Clin. Oncol. 28 (2010) 2151–2158, https://doi.org/10.1200/JCO.2009.26.3814.
[36] K. Thway, J. Noujaim, S. Zaidi, A.B. Miah, C. Benson, C. Messiou, et al., Desmoplastic small round cell tumor, Int. J. Surg. Pathol. 24 (2016) 672–684, https://doi.org/10.1177/1066896916668637.
[37] T. Negri, S. Brich, F. Bozzi, C.V. Volpi, A.V. Gualeni, S. Stacchiotti, et al., New transcriptional-based insights into the pathogenesis of desmoplastic small round cell tumors (DSRCTs), Oncotarget 8 (2017) 32492–32504, https://doi.org/10.18632/oncotarget.16477.
[38] A. Devecchi, L. De Cecco, M. Dugo, D. Penso, G. Dagrada, S. Brich, et al., The genomics of desmoplastic small round cell tumor reveals the deregulation of genes related to DNA damage response, epithelial-mesenchymal transition, and immune response, Cancer Commun (Lond) 38 (1) (Nov 28 2018) 70, https://doi.org/10.1186/s40880-018-0339-3.
[39] G.A. Franzetti, K. Laud-Duval, W. Van Der Ent, A. Brisac, M. Irondelle, S. Aubert, et al., Cell-to-cell heterogeneity of EWSR1-FLI1 activity determines proliferation/migration choices in Ewing sarcoma cells, Oncogene. 36 (2017) 3505–3514, https://doi.org/10.1038/onc.2016.498.
[40] Lv Y. Fan, H. Dai, Yan G. Ning, G. Meng, X. Zhang, Guo Q. Nan, Downregulation of tumor suppressing STF cDNA 3 promotes epithelial-mesenchymal transition and tumor metastasis of osteosarcoma by the Wnt/GSK-3β/β-catenin/Snail signaling pathway, Cancer Lett. 373 (2016) 164–173.
[41] W. Liu, R.H. Qiao, D.M. Wang, X.W. Huang, B. Li, D. Wang, UHRF1 promotes human osteosarcoma cell invasion by downregulating the expression of E-cadherin in an Rb1-dependent manner, Mol. Med. Rep. 13 (2016) 315–320, https://doi.org/10.3892/mmr.2015.4515.
[42] J. Chen, D. Yan, W. Wu, J. Zhu, W. Ye, Q. Shu, Micro RNA-130a promotes the metastasis and epithelialmesenchymal transition of osteosarcoma by targeting PTEN, Oncol. Rep. 35 (2016) 3285–3292, https://doi.org/10.3892/or.2016.4719.
[43] R. Jiang, C. Zhang, G. Liu, R. Gu, H. Wu, MicroRNA-126 inhibits proliferation, migration, invasion, and EMT in osteosarcoma by targeting ZEB1, J. Cell. Biochem. 118 (2017) 3765–3774, https://doi.org/10.1002/jcb.26024.
[44] C. Liu, L. Zhang, W. Cui, J. Du, Z. Li, Y. Pang, et al., Epigenetically upregulated GEFT-derived invasion and metastasis of rhabdomyosarcoma via epithelial mesenchymal transition promoted by the Rac1/Cdc42-PAK signalling pathway, EBioMedicine. 50 (2019) 122–134.