review

Characteristics of human Ewing/PNET sarcoma models

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Ann Saudi Med 2011; 31(2): 174-182

PMID: **** DOI: 10.4103/0256-4947.78206

Ewing/PNET (peripheral neuroepithelioma) tumors are rare aggressive bone sarcomas occurring in young people. Rare-disease clinical trials can require global collaborations and many years. In vivo models that as accurately as possible reflect the clinical disease are helpful in selecting therapeutics with the most promise of positive clinical impact. Human Ewing/PNET sarcoma cell lines developed over the past 45 years are described. Several of these have undergone genetic analysis and have been confirmed to be those of Ewing/PNET sarcoma. The A673 Ewing sarcoma line has proven to be particularly useful in understanding the biology of this disease in the mouse. The chromosomal translocation producing the EWS/FLI1 fusion transcript characterizes clinical Ewing sarcoma. Cell lines that express this genetic profile are confirmed to be those of Ewing sarcoma. The A673 Ewing sarcoma line grows in culture and as a xenograft in immunodeficient mice. The A673 model has been used to study Ewing sarcoma angiogenesis and response to antiangiogenic agents. Many Ewing sarcoma clinical specimens express the cell surface protein endosialin. Several Ewing sarcoma cell lines, including the A673 line, also express cell surface endosialin when grown as subcutaneous tumor nodules and as disseminated disease; thus the A673 is a useful model for the study of endosialin biology and endosialin-directed therapies. With the advent of tools that allow characterization of clinical disease to facilitate optimal treatment, it becomes imperative, especially for rare tumors, to develop preclinical models reflecting disease subsets. Ewing PNET sarcomas are a rare disease where models are available.

Ewing sarcoma, the second most frequent bone cancer, is a rare aggressive tumor which occurs primarily in children, adolescents and young adults and is a member of the family of primitive neuroectodermal tumors.[1-3] There is a tendency for Ewing sarcoma to be a more deadly disease in young adults than in younger patients. Different chromosomal abnormalities are found in patients more than 15 years of age than in younger patients and correlate with disease outcome.[1,4] Ewing sarcoma is one of the small round blue cell tumors of the bone, characterized by strong membrane staining for CD99, and occurs primarily in Causasians.[5] Pathognomonic translocations involving the *EWS* gene on chromosome 22 and the ets-type gene *FLI1* on chromosome 11 occur in about 85% of cases.[6] The EWS/FLI1 fusion protein product of this translocation is a potent transcription factor which functions as an oncoprotein.[7,8] Therapy for Ewing sarcoma includes surgery, radiation therapy and chemotherapy comprised of cycles of combinations of vincristine, doxorubicin, cyclophosphamide, etoposide, ifosfamide, actinomycin D and topotecan. For patients with metastatic disease at presentation and patients with recurrent disease, chances of cure are less than 20%.[3,9] In rare diseases like Ewing/PNET (peripheral neuroepithelioma) sarcoma, where Phase III clinical trials often require global collaborations, selecting experimental therapeutics to move forward can benefit from preclinical models which as accurately as possible reflect the clinical disease.

EWING/PNET sarcoma model systems

Human tumor xenografts from established tumor lines or recent surgical explants remain the core models for tumor biology and cancer drug discovery.[10] Scientific understanding of the diseases that these models repre-

Table 1. Characteristics of human Ewing/PNET sarcoma cell line.

Cell line	Age	Gender	Region	Status	Prior treatment	P53 status	EWS/FLI1
SK-NEP-1	25 y	F	Pleural effusion	Relapse	Post-chemo	Mutant	+
EW5	16 y 9 mo	M	Paraspinal	Diagnosis	-	Mutant	+
EW8 (Rh1)	17 y 9 mo	M	Abdominal mass	Diagnosis	-	Mutant	+
TC-71	22 y	M	Humerus/bone marrow	Relapse	Post-chemo	Mutant	+
CHLA258	12 y	F	Lung met	Relapse	Vincristine/doxorubicin/ cyclophosphamide; etoposide/ifosphamide; Ifosphamide/carboplatin/ etoposide	Mutant	+
CHLA-9	14 y	F	Thoracic mass	Diagnosis	-	Wild-type	
CHLA-10	14 y	F	Thoracic lymph node	Relapse	Cisplatin/doxorubicin/ cyclophosphamide/ etoposide	Mutant	
SK-ES-1	18 y	M	Bone				
Hs 822.T	9 y	F	Bone				
A-673	15 y	F	Muscle		Unknown		+
Hs 863.T	5 y	F	Bone				
RD-ES	19 y	M	Bone				
CHLA-25	2.6 y	F	Unknown	Relapse	Etoposide / ifosphamide / vincristine/ cyclophosphamide		
CHLA-32	8.5 y	F	Pelvic	Diagnosis	-		
COG-E-352	17 y	M	Fibula	Relapse	Vincristine /adriamycin/ cyclophosphamide /ifosphamide/etoposide		
TC-32	17 y	F	Ileum	Diagnosis	-		
SK-N-MC	12 y	F	Retro-orbital met	Relapse	Vincristine/ cyclophosphamide/ doxorubicin/actinomycin		

sent is growing, and it is now possible to match the xenograft tumor with the clinical disease of interest based upon gene expression and protein target expression. The realization that the host tissue or organ in which the tumor is growing influences the characteristics of the disease, including the response to therapies, in a manner similar to those of clinical disease, has improved use of these models. The work of the Preclinical Pediatric Testing Program (PPTP) in characterizing the 47 human tumor xenografts that comprise the consortium exemplifies the best preclinical efforts.[11-14]

Seventeen human Ewing/PNET sarcoma cell lines that are currently in use to study these diseases are listed in **Table 1**. Some of the lines were established in the 1970s; and others, more recently. There is variable information on the origin of the lines as well as varied

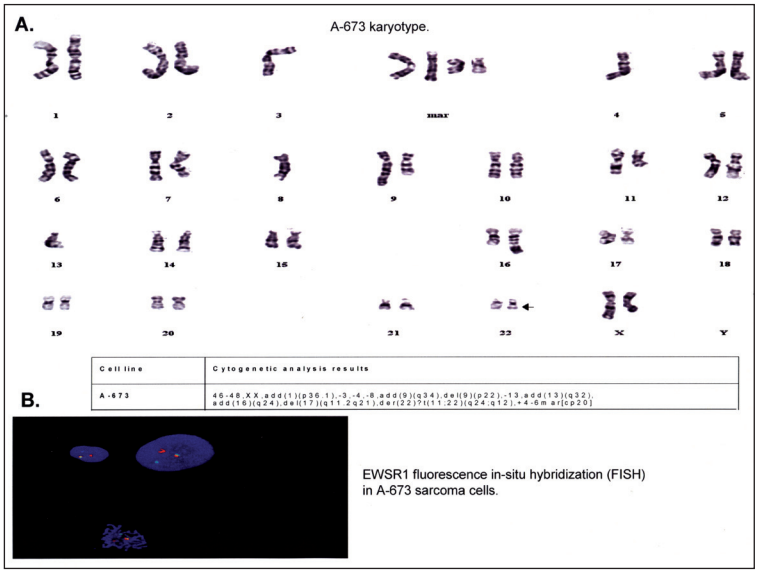


Figure 1a. Representative complex karyotype of the A673 cell line showing multiple rearrangements, including a chromosome 11 and 22 fusion, **b.** Fluorescence in situ hybridization (FISH) analysis for EWSR1 in A673 Ewing sarcoma cells.[19]

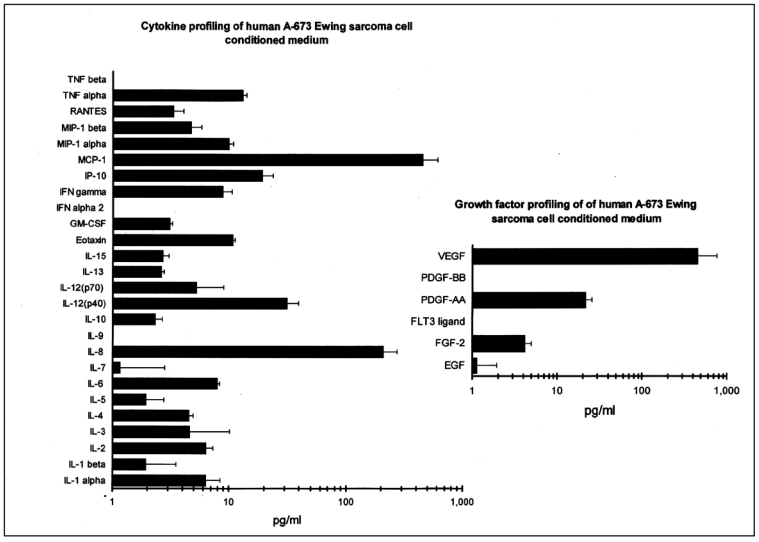


Figure 2a. Cytokine profiling of human A673 Ewing sarcoma cell–conditioned medium. **b.** Angiogenic growth factor profiling of human A673 Ewing sarcoma cell–conditioned medium.

levels of molecular characterization. For example, the SK-NEP-1 line established in 1971 was originally designated as Wilms tumor; however, through molecular profiling it was recently shown to express the EWS/FLI1 gene fusion transcript and thus is now known to be a Ewing sarcoma.[15] Similarly, the Rh1 xenograft which was derived from a patient whose diagnosis was rhabdomyosarcoma, also expresses an Ewing sarcoma gene expression profile and has confirmed expression of the EWS/FLI1 fusion transcript; thus it is an Ewing sarcoma and was renamed EW8(Rh1). Several other lines designated as Ewing sarcoma were confirmed expressers of the EWS/FLI1 fusion transcript. The cell line CHLA-9 was established at diagnosis from a 14-year-old female with a thoracic PNET, and the CHLA-10 line was established from the same patient after 4 cycles of chemotherapy, at which time the tumor cells had become p53 mutant.[16] The A673 cell line was described in 1973 as being from a patient with a possible rhabdomyosarcoma; however, recent cytogenetic testing and molecular profiling established that A673 cells express the EWS/FLI1 transcription factor and confirmed that A673 cells are Ewing sarcoma (**Figure 1**).[17-19] A673 cells grown as a human tumor xenograft in immunodeficient mice have proven to be a useful in vivo model to explore the biology of tumor growth and to identify therapeutic targets for sarcomas.

Angiogenic Targets

Vascular endothelial growth factor

Like other solid tumors, Ewing sarcoma requires formation of a vascular supply to grow; thus angiogenesis is a rational therapeutic approach for this tumor.[2,20] Tumors are dynamic, complex, living tissues undergoing varied processes of tissue growth under the guidance of aberrant malignant cells. The active involvement of normal cells in the vicinity of a malignant colony is required for a tumor mass to grow, and these normal cells become a major component of the malignant disease.[21] The corollary is that both the normal and the cancer cells that comprise malignant disease are valid targets for therapeutic intervention.

The A673 Ewing sarcoma human tumor xenograft was one of the first tumor models that demonstrated a marked response to the anti-human VEGF antibody A4.6.1, which became bevacizumab.[22] Using A4.6.1 in combination with a mouse VEGF neutralizing antibody showed that angiogenic factors secreted by the human malignant cells and by the murine host cells contributed to blood vessel growth in the tumor. Later, Gerber and Ferrara described the response of 20 human tumor xenografts representing 13 tumor types to treatment with bevacizumab, and the A673 Ewing sarcoma was one of the most responsive tumors to Vascular endothelial growth factor (VEGF) neutralization.[23] An in-depth study of the angiogenic properties of A673 cells found that the cells express and secrete VEGF and placental growth factor at high levels and that the tumor cells also express the VEGF receptors Flt-1/VEGFR1 and KDR/VEGFR2.[24] More recently,

multiplexing technology has allowed the determination of many secreted factors from small samples. Cytokine profiling of conditioned medium from A673 Ewing sarcoma cells grown in culture showed that MCP-1 and IL-8/CXCL8 were secreted at high levels (**Figure 2**). Among the growth factors profiled in this study, VEGF was secreted at the highest level. The EWS/FLI1 transcription factor oncoprotein may promote upregulation of proteins in the VEGF pathway.[20]

The response of human A673 Ewing sarcoma xenografts grown subcutaneously in immunodeficient mice to treatment with varied doses of bevacizumab is shown in **Figure 3**. Three doses of bevacizumab were tested: 10, 18.6 and 37.2 mg/kg. Bevacizumab was administered by intraperitoneal injection twice weekly. In the group receiving 37.2 mg/kg, the initiation of treatment was delayed until the tumors reached 300 mm^3. Immunohistochemical analysis of the tumors showed that the vehicle-treated control tumors had well-formed vessels with endothelial cell-lined lumens surrounded by pericytes, while the bevacizumab treatment groups had poorly formed vessels that were often collapsed and missing endothelial cells and scattered endothelial cells with an absence of lumens (**Figure 4**). Combining bevacizumab/A4.6.1 with doxorubicin, topotecan, paclitaxel, docetaxel or radiotherapy resulted in additive or synergistic tumor growth inhibition. Changes in vascular functions were frequently reported in response to treatment. In some studies, these improvements resulted in an increase in intratumoral uptake of chemotherapy.[22,23] Bevacizumab/A4.6.1 treatment in combination with radiation therapy increased tumor oxygenation and tumor growth delay.

Bevacizumab is now an integral therapeutic component in the treatment of several major malignancies.[25-29] The benefits of bevacizumab are clear, and the potential adverse events are known. Bevacizumab has been combined with numerous cytotoxic chemotherapy regimens, usually without untoward increases in toxicity. Other promising biological VEGF-directed agents include aflibercept, a VEGF-trap or decoy receptor; and ramucirumab, an antibody-targeting VEGF receptor 2.[29,30] Both are in Phase III clinical trials. The antiangiogenic kinase inhibitors sorafenib, sunitinib and pazopanib have received regulatory approval in renal cell carcinoma (sunitinib, pazopanib), gastrointestinal stromal tumors (sunitinib) and hepatocellular carcinoma (sorafenib) as single agents.[31-35] The adverse events that occur with these drugs are understandable and frequent.[30,36,37] Although some preclinical studies combining small molecule antiangiogenic agents with standard treatments have been promising,[38,39] clinically

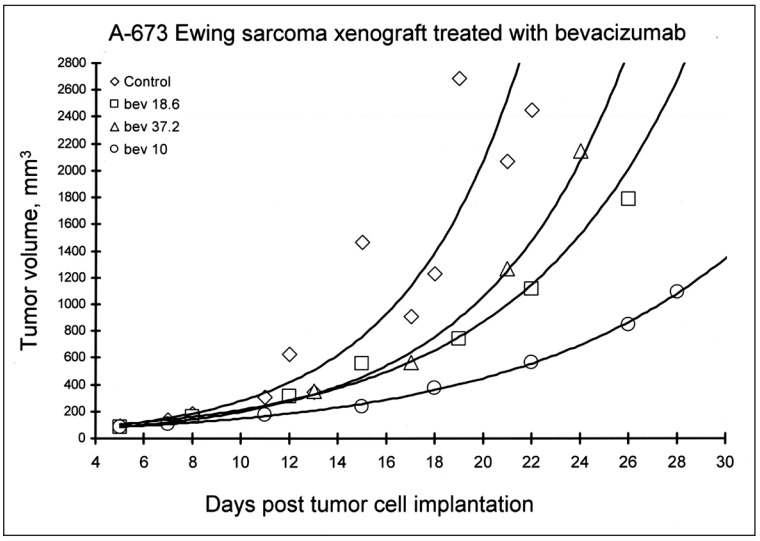


Figure 3. Growth delay of subcutaneously implanted A673 human Ewing sarcoma xenografts. Mice were treated with intraperitoneal injections of bevacizumab (10, 18.6 or 37.2 mg/kg) twice weekly. The lower-dose treatments (10 and 18.6 mg/kg) were initiated when tumors were 100 mm^3, and the high-dose treatment (37.2 mg/kg) was initiated when the tumors were 300 mm^3.

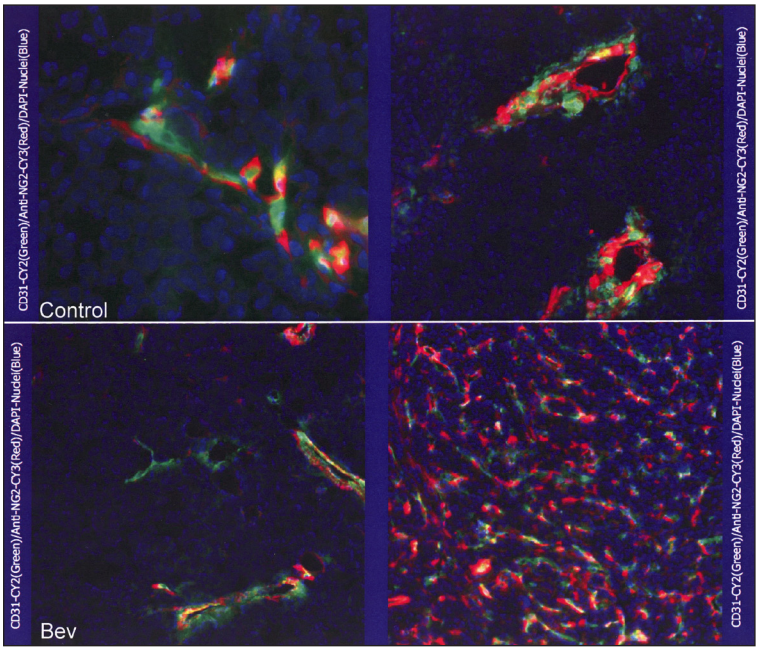


Figure 4. Immunohistochemical staining of human A673 human tumor xenografts after treatment with bevacizumab or control buffer is shown. The staining for CD31 to visualize endothelial cells is green, and the staining for NG2 to visualize pericytes is red.

it has been more difficult to incorporate the small-molecule kinase inhibitors into combination chemotherapy regimens than it has been to incorporate bevacizumab into these regimens. The clinical evaluation of anti-angiogenic agents for Ewing/PNET sarcoma is at an early stage[20]; however, the Children's Oncology Group (COG) has initiated a randomized Phase 2 trial of retrieval chemotherapy with or without bevacizumab for patients with first recurrence of Ewing sarcoma.

CXCL8 (IL-8)/ CXCR2

Over the past 15 years, VEGF and its signal transduction pathway have been the focus of antiangiogenic therapeutics in cancer.[40,41] Among the many other angiogenic factors, interleukin-8 (CXCL8) is a mitogen for endothelial cells and stimulates angiogenesis in vivo. CXCL8 is secreted by macrophages, leading to macrophage-associated angiogenesis in malignant disease. Interestingly, in addition to CXCL8, A673 Ewing sarcoma cells secrete MCP-1(CCL2), monocyte chemotatic protein-1, which recruits macrophage to the tumor (**Figure 2**). The receptor for CXCL8 is CXCR2. There are 7 known proangiogenic chemokines that are ligands for the chemokine receptor CXCR2.[42] A positive correlation has been found between the levels of tumor-associated macrophage (TAM) (macrophage index) in many human cancers and tumor angiogenesis.[43,44] Overexpression of CXCL8 correlates with tumor stage as well as disease progression and recurrence. Serum levels of CXCL8 have been considered a potential tractable clinical biomarker in melanoma.[45] CXCL8 was evaluated as a therapeutic target using a fully human anti-CXCL8 (ABX-IL8) neutralizing antibody. Although the antibody had little effect in cell culture, it produced a significant decrease in the growth of human tumor xenografts, indicating a potential effect on the tumor microenvironment. However, ABX-IL8 antibody was discontinued due to limited activity in a clinical trial. More recently, in preclinical studies, CXCR2 was targeted with an antagonistic antibody.[46,47] Small-molecule inhibitors of CXCR2 have been tested in inflammatory diseases; dual CXCR1/ CXCR2 inhibitors such as SCH-479833 and SCH-527123 have been evaluated preclinically in cancer.[43,48]

Sarcoma Targets

Endosialin/CD248/TEM-1

Endosialin/CD248/TEM-1 was first identified in 1992 as the antigen of an antibody designated FB5 that was raised in mice inoculated with human fetal fibroblasts.[49-51] In tissues, FB5 reacted strongly with vascular cells in 67% of malignant tumor specimens and more weakly with stromal fibroblasts in a subset of specimens. This initial study provided the evidence that endosialin was expressed during development, being a fetal antigen, that it was overexpressed in cancer tissues and that its expression varied between carcinomas and sarcomas.

Subsequently, several reports of endosialin protein expression concurred that endosialin expression was limited to a few cell types in normal tissues and was mainly a developmental and pathologic feature. However, endosialin transcript was found to be ubiquitously expressed in normal adult tissues in addition to somatic tissues during development, both in humans and mice.[52] Tissues with high levels of endosialin transcript seemed to express the protein, while tissues with lower levels of the transcript seemed negative for the protein.[53] High endosialin was detected in fibroblasts and pericytes in human thymus, lymph nodes and spleen during lymphoid tissue development but mostly absent in the adult except during secondary lymphoid organ remodeling during adaptive immune responses.[54,55] In normal adults, endosialin protein expression appears to be limited to normal endometrial stroma and occasional fibroblasts.[53,56,57] The murine ortholog of endosialin was cloned and found to be expressed during development and during implanted tumor growth in the adult mouse.[58,59]

In 2000, St. Croix et al found that the mRNA most upregulated in a sample of human colon cancer vascular cells was the message for endosialin (TEM1).[60,61] Later, endosialin was found to be expressed in the vasculature and fibroblasts of human brain tumor specimens, including astrocytoma, anaplastic astrocytoma, glioblastoma multiforme, meningioma, oligodendroglioma, ependymoma and carcinoma brain metastasis.[62,63] By immunohistochemistry, endosialin co-localized with the pericyte marker NG2 in breast cancer specimens but not with the endothelial marker CD31.[56,57] In carcinomas, endosialin protein was detected in tumor capillaries and fibroblasts.[53] Although there was some controversy regarding the vascular cells that express endosialin, it is now clear that endosialin stained NG2-positive cells, i.e., pericytes with subcellular localization of endosialin on the surface of the pericyte cell-body and finger-like processes.[58,59,64-66] Tumors grow more slowly in endosialin/TEM1 KO mice, suggesting that host endosialin/TEM1-positive stroma promotes malignancy.[67] Endosialin may play a role in cell-cell adhesion and in adhesion to extracellular matrix proteins.[68-70] The endosialin protein sequence has EGF and thrombomodulin domains, suggesting a role in protein-protein interactions.[61]

Endosialin/CD248/TEM-1 is expressed in stromal cells, endothelial cells and pericytes in various tumors;

however, a few studies focused on expression in malignant cells. In 2005, Dolznig et al showed expression of endosialin transcript in sarcomas, and expression of the protein in malignant cells in one malignant fibrous histiocytoma and one liposarcoma.[53] More recently, endosialin protein expression was assessed by immunohistochemistry in 250 clinical specimens of human cancer, including 20 cancer subtypes.[19] The results showed that endosialin protein is frequently found in human cancers. Endosialin expression was mainly a perivascular feature in carcinomas, with some detectable expression in stromal cells; however, in sarcomas endosialin was expressed by malignant cells, perivascular cells and stromal cells. The expression of endosialin in an Ewing sarcoma specimen expressing the protein with 3+ intensity (high) is shown in **Figure 5**. When 9 clinical specimens of Ewing sarcoma were tested, endosialin expression was ≥50% in 5 specimens, and several maintained high levels of expression at the 3+ intensity expression level (**Figure 5**).

Fifty human tumor cell lines and six normal cell types in culture were assayed by RT-PCR and/or flow cytometry for endosialin.[19] Endosialin cell surface protein was found on 7 sarcoma lines, 1 neuroblastoma and 4 normal cell types in culture. The flow cytometric histograms of 5 human Ewing sarcoma cell lines immunostained for endosialin are shown in **Figure 6**. Four of the 5 cell lines expressed high levels of cell surface endosialin. Images of the endosialin immunostaining of A673 Ewing sarcoma cells illustrate the characteristic pattern for cell surface protein expression (**Figure 6**).

CD146, also known as the melanoma cell adhesion molecule (MCAM) or cell surface glycoprotein MUC18, is an adhesion molecule currently used as a marker for endothelial cell lineage. CD146 has been seen as a marker for mesenchymal stem cells isolated from multiple adult and fetal organs. CD146 expression may be linked to multipotency; mesenchymal stem cells with greater differentiation potential express higher levels of CD146 on the cell surface. In addition to expression in the vascular compartment, CD146 malignant cell expression is associated with an advanced tumor stage in melanoma, prostate, ovarian and breast cancers.[71] CD146 is expressed by SK-ES-1 and A673 human Ewing sarcoma cells in culture; however, the expression of endosialin by these cells is stronger (**Figure 6**). The question remains whether endosialin will continue to be expressed by sarcoma cells in vivo. Human A673 Ewing sarcoma cells were injected intravenously into immunodeficient mice to produce disseminated disease. Tumor nodules grew in several organs in the mice, including ovary, lymph node, spine and lung (**Figure 7**). The tumors that arose in each tissue environment continued to express endosialin. The intensity of the

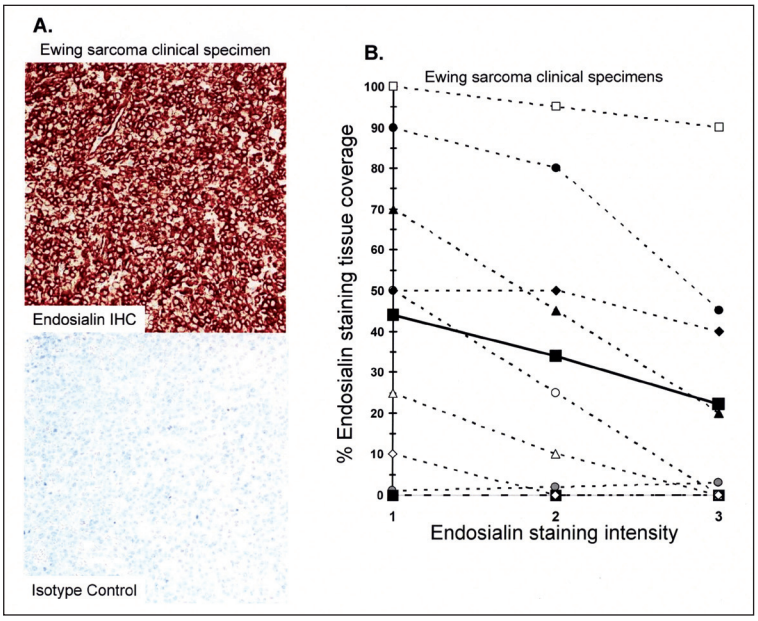


Figure 5a. Histologic and immunohistochemical features of Ewing sarcoma. Classic Ewing sarcoma appears as sheet of monotonous round cells. The cells have little cytoplasm and round nuclei. The cells show strong plasma-membrane staining for endosialin. There is no staining with the isotype control antibody, **b.** Endosialin staining intensities in 9 human Ewing sarcoma clinical specimens. Each dotted line is a clinical specimen showing the endosialin staining intensity at 1+, 2+ and 3+ levels. The solid line is the mean staining intensity for the 9 clinical specimens at each level (19).

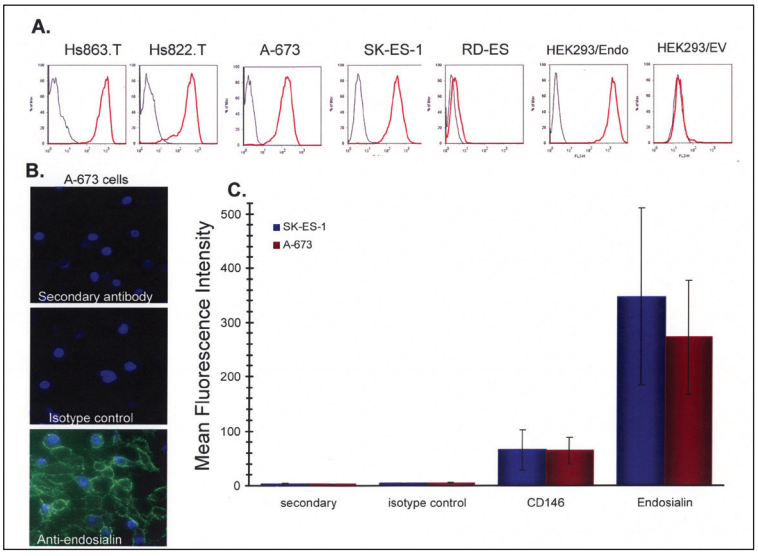
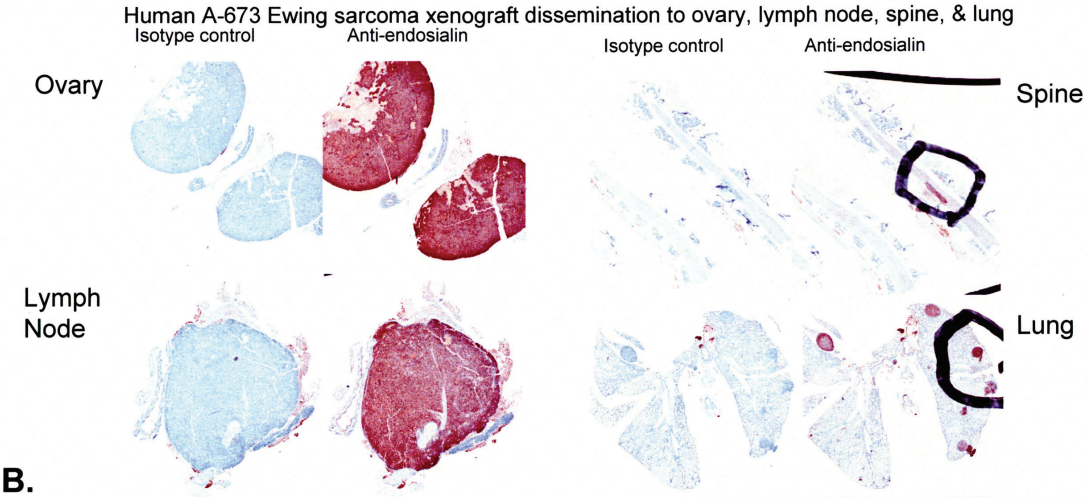


Figure 6a. Endosialin cell surface protein expression in 5 human Ewing sarcoma cell lines and Hek293 cells transfected to express high levels of endosialin. Four of the 5 human Ewing sarcoma cell lines express endosialin. **b.** Human A673 Ewing sarcoma cells immunostained with an antibody to endosialin showing a cell surface staining pattern. c. The mean fluorescence intensity from flow cytometric histograms for SK-ES-1 and A673 human Ewing sarcoma cells showing the relative fluorescence intensity for expression of CD146.



B.

Tissue Type	Histologic Review	TUMOR CELL intensity and percent coverage				
		3+	2+	1+	0	Long H-score
Lung - A-673 xenograft	Tumor in lung	100	0	0	0	300
Lung - A-673 xenograft	Scant tumor in lung	100	0	0	0	300
Lymph node - A-673 xenograft	Tumor nodule	80	20	0	0	280
Ovary - A-673 xenograft	Tumor mass & adjacent uterus	100	0	0	0	300
Spine - A-673 xenograft	Tumor in spinal cord & bone	40	20	40	0	200

Figure 7. Immunohistochemical staining for endosialin in tissue from immunodeficient mice with disseminated A672 Ewing sarcoma xenografts, along with the scoring of the intensity of endosialin tumor staining for each specimen, is shown.

endosialin staining was scored on the same scale as the one used for the human clinical specimens, and most of the tumor nodules showed a high percentage of 3+ staining intensity (**Figure 7**). A fully human anti-endosialin monoclonal antibody bound to human A673 Ewing sarcoma cells and SK-NA-S neuroblastoma cells but not HT-1080 fibrosarcoma cells. Exposure of the cells to an anti-human IgG conjugated to saporin resulted in growth inhibition only of the endosialin-expressing cells.[19]

Although the biological function of endosialin is incompletely understood beyond evidence that endosialin may interact with the tumor microenvironment[67-70,72] and play a role in the expression of PDGF and in pericyte function,[70] data support the notion that endosialin may play a role in malignancy. Most importantly, endosialin is expressed in sarcomas with poor prognosis and in advanced sarcoma, opening up the possibility that targeting endosialin could offer a therapeutic avenue for the more than 50% of children and adults suffering from sarcomas whose disease cannot be cured with existing treatment modalities.[73,74]

Conclusion

The treatment of cancer is moving toward therapeutics selected based upon the molecular characteristics of the specific disease. Thus the potential therapeutic targets of each malignancy must be known and addressed. Preclinical models that as accurately as possible reflect the clinical disease will have an important role in facilitating drug discovery in this new era. Ewing sarcoma is a rare tumor; however, the human Ewing/PNET sarcoma cell lines available have allowed the elucidation of angiogenic and malignant cell targets that can be confirmed in clinical specimens of the disease. This knowledge should promote the development of new treatments and treatment regimens for this family of neuroectodermal tumors.

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