Endosialin Protein Expression and Therapeutic Target Potential in Human Solid Tumors: Sarcoma versus Carcinoma

Cecile Rouleau,1 Maritza Curiel,2 William Weber,1 Robert Smale,2 Leslie Kurtzberg,1 James Mascarello,3 Carol Berger,4 Gina Wallar,2 Rebecca Bagley,1 Nakayuki Honna,6 Kazumasa Hasegawa,6 Isao Ishida,6 Shiro Kataoka,6 Beth L. Thurberg,1 Khodadad Mehraein,2 Bruce Horten,5 Glenn Miller,2 and Beverly A. Teicher1

Abstract

Purpose: Endosialin/CD248/tumor endothelial marker 1 is expressed in stromal cells, endothelial cells, and pericytes in various tumors; however, few studies have focused on expression in malignant cells.

Experimental Design: We studied expression of endosialin in clinical specimens, cell culture, and animal models and designed an anti-endosialin therapeutic prototype.

Results: Fifty human tumor cell lines and 6 normal cell types in culture were assayed by reverse transcription-PCR and/or flow cytometry for endosialin. Cell surface protein was found on 7 sarcoma lines, 1 neuroblastoma, and 4 normal cell types in culture. A fully human anti-endosialin antibody bound to human A-673 Ewing’s sarcoma cells and SK-N-AS neuroblastoma cells but not HT-1080 cells. Exposure of cells to an anti-human IgG conjugated to saporin resulted in growth inhibition only of endosialin-expressing cells. Endosialin expression was assessed by immunohistochemistry in 250 clinical specimens of human cancer including 20 cancer subtypes. Endosialin is frequently found in human cancers. Endosialin expression is mainly a perivascular feature in carcinomas, with some expression in stromal cells. In sarcomas, endosialin is expressed by malignant cells, perivascular cells, and stromal cells. Development and characterization of experimental models for studying endosialin biology in sarcomas and evaluating anti-endosialin therapies is presented.

Conclusions: Findings suggest that an anti-endosialin immunotoxin might be a promising therapeutic approach for endosialin-positive neoplasia, especially synovial sarcoma, fibrosarcoma, malignant fibrous histiocytoma, liposarcoma, and osteosarcoma. Thus, a diagnostic/therapeutic targeted therapeutic approach to treatment of endosialin-expressing tumors may be possible.

Endosialin/CD248/tumor endothelial marker 1 (TEM1) was first identified in 1992 as the antigen of an antibody produced by mice after immunization with human fetal fibroblasts (1). In culture, the antibody designated FB5 reacted with human fibroblasts and neuroblastoma cell lines but did not react with human melanoma, glioma, sarcoma, carcinoma, or leukemia cell lines or with human normal primary endothelial cells. In tissues, FB5 reacted strongly with vascular endothelial cells in malignant tumors in 67% of the specimens tested and weakly with tumor stromal fibroblasts in a small subset of carcinomas. The FB5 antibody also reacted with malignant cells in a subset of sarcomas and failed to react with normal tissues. This initial study provided evidence that endosialin was expressed during development, being a fetal antigen, that it was overexpressed in cancer tissues, and that its expression patterns varied in carcinomas and sarcomas.

Subsequent reports of endosialin protein expression confirmed that endosialin expression was limited in normal tissues and that it was mainly a developmental and pathologic feature. However, in 2001, endosialin transcript was found to be ubiquitously expressed by Northern blot analysis in both human and mouse normal adult tissues in addition to somatic tissues during development (2). Indeed, endosialin protein expression may be limited in normal tissues, whereas endosialin transcript remains detectable. In fact, Dolznig et al. indicated that tissues with high levels of endosialin transcript were positive for endosialin protein, whereas tissues with lower levels of the transcript were negative for endosialin protein (3). Dolznig et al. showed that endosialin protein in normal tissues was limited to endometrial stroma (3). In 2006, Rupp et al. cloned and studied expression of the murine orthologue of endosialin during development and during tumor growth in adult mice (4). During embryo development, endosialin

Human Cancer Biology

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Requests for reprints: Cecile Rouleau, Genzyme Corporation, 49 New York Avenue, Framingham, MA 01701. Phone: 508-270-2031; Fax: 508-872-4091; E-mail: Cecile.Rouleau@Genzyme.com.

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Translational Relevance

Our work shows the prevalence of endosialin expression in human cancer. The main finding that endosialin is expressed frequently and at high levels in three cellular compartments of sarcomas (malignant, perivascular, and stromal cells) and across subtypes, based on a survey of 92 clinical specimens, has fundamental implications for clinical medicine. Endosialin now emerges as a diagnostic marker and as a therapeutic target for sarcomas, a set of diseases critically lacking both of those. This discovery bears the promise of personalized medicine for sarcoma patients through a diagnostic/therapeutic application whereby patients whose disease expresses endosialin, regardless of subtype, may potentially be eligible for endosialin-directed therapy. Our work presents the details of a diagnostic immunohistochemical assay for endosialin and describes a potential therapeutic approach using a model prototype therapy for soft-tissue and bone sarcoma patients.

protein was restricted to vascular endothelium and fibroblast-like cells but was largely absent in adult mice, except in uterine fibroblasts and in the stroma of implanted tumors. MacFadyen et al., using a rabbit polyclonal antibody, observed highly restricted endosialin expression in adult mouse tissues and found that endosialin was expressed in fibroblasts in both tumor and normal tissues but that expression in pericytes was observed only in tumors and not in normal tissues (5). Expression in fibroblasts was detected in synovium, bone marrow, salivary gland, and thyroid gland. Using the same rabbit polyclonal antibody, Lax et al. detected endosialin in fibroblasts and pericytes in human thymus, lymph nodes, and spleen and observed that endosialin expression was high during lymphoid tissue development but mostly absent in the adult (6). In addition, increased endosialin was observed in stromal cells in a Salmonella-induced model of splenic enlargement, making endosialin a pathologic feature and suggesting a possible role in repair. In summary, endosialin expression in normal tissues appears limited to fibroblasts and/or pericytes in certain organs and tissues including endometrium, synovium, bone marrow, salivary gland, thyroid gland, thymus, lymph nodes, and spleen.

In 2000, St Croix et al. compared the gene expression profile of vascular cells captured by anti-CD146 P1H12 freshly isolated from a colon tumor with those isolated from normal adjacent colon mucosa (7). The transcript most differentially expressed in tumor endothelial cells was called TEM1 and was identical to endosialin (1, 8). Using a rabbit polyclonal antibody, Brady et al. found expression of endosialin in vascular endothelium, vascular smooth muscle cells, and fibroblasts of human brain tumor specimens including astrocytoma, anaplastic astrocytoma, glioblastoma multiforme, meningioma, oligodendroglioma, ependymoma, and carcinoma (9). MacFadyen et al. studied breast cancer and normal breast specimens by immunohistochemistry with a mouse anti-endosialin and showed that endosialin colocalized with the pericyte marker NG2 in tumors but not with the endothelial marker CD31 (5).

Dolznig et al. reported expression of endosialin transcript and protein in a variety of tumors, including carcinomas and sarcomas. In carcinomas, endosialin protein was detected in tumor capillaries and fibroblasts, suggesting that endosialin might be a suitable target for experimental therapies aimed at disrupting the stromal compartment of tumors (3). Tentori et al. found expression of endosialin in SV40-transformed human umbilical vein endothelial cells (HUVEC), which proliferate faster than parental HUVEC and were proposed as a model of tumor-associated neoangiogenic endothelium (10). Huber et al. detected endosialin in the blood vessels of melanoma metastases and squamous cell carcinomas with the FB5 antibody (11). Rupp et al. reported that FB5 antibody stained tumor endothelial cells in frozen carcinoma specimens. The same investigators found that, in tumor-bearing adult mice, host endosialin could be detected in the tumor stroma in both vascular endothelium and stromal fibroblasts (12). Recently, MacFadyen et al. detected endosialin protein in the stromal fibroblasts of the mesenchyme and developing vasculature of mouse embryos (13). Expression in vasculature did not colocalize with CD31 staining, but rather with NG2 staining, indicating that pericytes, rather than endothelial cells, were positive for endosialin. Using a mouse monoclonal anti-endosialin, Virgintino et al. observed that endosialin staining localized with NG2-positive cells (pericytes) and clarified the subcellular localization of endosialin in pericytes in human fetal telencephalon (5, 14). Endosialin was detected in the pericyte body and finger-like processes, whereas NG2 colocalized with collagen IV staining.

Endosialin has epidermal growth factor and thrombomodulin domains, suggesting a role in protein-protein interaction (8). Murine endosialin has been knocked out, and although the animals appear normal, tumors implanted on the cecum grew more slowly in endosialin/TEM1 knockout mice compared with wild-type, suggesting that host endosialin/TEM1-positive stroma promotes malignancy (15). Endosialin may play a role in cell-cell adhesion and in adhesion to extracellular matrix proteins (16, 17).

Since 1992, when Rettig et al. observed that endosialin could be detected in malignant cells in some sarcomas, Dolznig et al. found expression of endosialin transcript in sarcomas and, most importantly, expression of the protein in malignant cells in one malignant fibrous histiocytoma (MFH) and one liposarcoma (1, 3). Our report is the first large survey of endosialin protein expression in clinical cancer specimens and the first large study of endosialin protein and mRNA expression in human cancer cell lines. We also present data supporting the potential for endosialin-targeted therapy for treatment of endosialin-expressing sarcomas.

Materials and Methods

Materials. The anti-endosialin used in these studies is a fully human monoclonal antibody isolated after inoculation of the KM mouse with human endosialin protein (18, 19). The control antibody is a fully human monoclonal antibody raised against dinitrophenol (DNP) in the KM mouse. For production, CHO cells were transfected with plasmid constructs with the genes for anti-DNP or anti-endosialin. Stably transfected CHO cells were grown in CD-CHO simplified chemically defined basal medium (Invitrogen) supplemented with glucose and essential amino acids. The antibodies secreted into

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conditioned medium were captured over a protein A-Sepharose column (GE Healthcare) that was equilibrated with 50 mmol/L phosphate, 25 mmol/L NaCl (pH 7.1) and then eluted with 10 mmol/L succinate (pH 3.75). The eluates containing the antibodies were sterile filtered through a 0.22 µm PES membrane (Whatman). The antibodies were formulated in 20 mmol/L histidine buffer (pH 6.0). 0.005% P80 and concentrated.

**Cell culture.** All human tumor cell lines were purchased from the American Type Culture Collection and grown in RPMI with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen). Human umbilical vein endothelial cells (HUVEC), human mesenchymal stem cells, primary lung fibroblasts, and aortic smooth muscle cells were purchased from Cambrex. HUVEC and HMSC were grown in EGM2-MV, mesenchymal stem cells were grown in MSCGM, fibroblasts were grown in FGM-2 medium, and aortic smooth muscle cells were grown in SmGM-2 (Cambrex). Human brain vascular pericytes were purchased from ScienCell and grown in PM growth medium (ScienCell).

All cells were maintained at 37°C in 5% CO2 humidified atmosphere.

**RNA extraction and real-time reverse transcription-PCR.** Total RNA was isolated by chloroform extraction using TriZol (Invitrogen). Integrity and purity was assessed using 2100 Bioanalyzer (Agilent). Reverse transcription was done using cDNA Archive (Applied Biosystems) for human endosialin cDNA quantification, real-time PCR was done in duplicate wells on a 7700 real-time TaqMan thermal cycler (Applied Biosystems) for 40 cycles using 900 nmol/L primers (Integrated DNA Technologies). 250 nmol/l fluorogenic probe (Applied Biosystems), and TaqMan Universal PCR Master Mix (Applied Biosystems). Forward primer sequence was 5′-CGACGTGCCAGCACTGTGCCT-3′. Reverse primer sequence was 5′-GCCGAGGCCGCCTGAAGCCA-3′. Probe sequence was 5′-CGCTGCTGTGCGCCGACCTGCAGCAGC-3′. Loading was measured by 18S mRNA detection using rRNA Control Reagents (Applied Biosystems) in multiplex reactions with endosialin primers and probe. 18S probe final concentration was 250 nm/L and 50 nmol/L of 18S primers.

Quantification of endo- sialin transcript copy number was done using a cDNA standard curve using 50 nmol/L of each 18S primer. Absolute quantification of endosialin transcript copy number was done using a cDNA standard curve with full-length human endosialin cDNA. Reverse transcription reactions were done in both the presence and the absence of reverse transcriptase. Reverse transcription reactions done without reverse transcriptase were used as templates in the reverse transcription-PCR experiment to assay genomic DNA contamination in total RNA preparations and to distinguish it from cDNA amplification.

**Immunocytochemistry.** Human A-673 Ewing's sarcoma cells (1.25 × 10^4 per well) were plated on a collagen I precoated 96-well plate (BD) in 10% FBS and 1% penicillin/streptomycin (Invitrogen). Human umbilical vein endothelial cells (HUVEC), human mesenchymal stem cells, primary lung fibroblasts, and aortic smooth muscle cells were purchased from Cambrex. HUVEC and HMSC were grown in EGM2-MV, mesenchymal stem cells were grown in MSCGM, fibroblasts were grown in FGM-2 medium, and aortic smooth muscle cells were grown in SmGM-2 (Cambrex). Human brain vascular pericytes were purchased from ScienCell and grown in PM growth medium (ScienCell).

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**Fluorescence in situ hybridization.** Fluorescence in situ hybridization was done on cells grown and harvested in situ on glass coverslips using standard cytogenetic methods. Coverslips were mounted cell-side-up on slides, pretreated, and denatured with a dual-color, break-apart

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probe for EWSR1 on chromosome 22 at q12 (Abbott Molecular). Slides were hybridized overnight at 37°C and washed in 0.4 × SSC, 0.3% NP-40 at 70°C for 2 min followed by a room temperature wash in 2 × SSC, 0.1% NP-40 for 1 min. Slides were air dried and counterstained with diamino-2-phenylindole. Using a fluorescence microscope, 200 interphase nuclei were analyzed independently by two observers and scored for the percentage of nuclei with specific hybridization patterns.

**Immunohistochemistry.** For formalin-fixed, paraffin-embedded (FFPE) tissues, an indirect immunohistochemistry was done using a tyramide immunohistochemistry procedure. FFPE sections (5 μm) were deparaffinized in three baths of xylene for 15 min each and transferred to two 100% alcohol baths for 5 min each followed by three graded alcohol (95%, 70%, and 50%) baths for 3 min each and then rinsed for 5 min in distilled H2O. Slides were pretreated in citrate buffer (Zymed Laboratories/Invitrogen) diluted to 1:20 in distilled H2O and placed inside the pressure cooker chamber already filled with 500 mL distilled H2O. Slides were incubated for 1 h. The first 20 min was pressurized incubation at 120°C at 15 p.s.i. and then cooling at room temperature for 60 min. Slides were then rinsed in PBS-Tween 20 and incubated at room temperature for 30 min in a universal peroxidase blocking solution (Dako). Slides were rinsed for 3 min in a wash solution [100 mL of 1 mol/L Tris (pH 7.5), 30 mL of 5 mol/L citrate, 867.5 mL distilled H2O, and 2.5 mL Triton X-100]. Slides were incubated at room temperature for 30 min using a blocking solution [100 mL of 1 mol/L Tris (pH 7.5), 3 mL of 5 mol/L citrate, 62.67 mL distilled H2O, 10 mL goat serum, 13.33 mL of 7.5% bovine serum albumin, and 1 mL Triton X-100].

![Fig. 1. A, endosialin mRNA expression in human cancer cell lines was determined by real-time PCR. Carcinoma cell lines and melanoma cell lines were negative for endosialin transcript. One of three neuroblastoma cell lines, SK-N-AS, was positive for endosialin transcript. B, endosialin protein expression by human sarcoma and hematopoietic cancer cell lines, primary fibroblasts, mesenchymal stem cells, HUVEC, HMVEC, pericytes, and smooth muscle cells was determined by flow cytometry. Endosialin protein was detected on the cell surface on 7 of 15 sarcoma cell lines and on primary fibroblasts, mesenchymal stem cells, pericytes, and smooth muscle cells. The hematopoietic cancer cell lines, HUVEC and HMVEC, were negative for endosialin protein.](image-url)
Endosialin Protein in Human Cancer

Table 1. Endosialin cell surface expression of 15 human sarcoma cell lines was assessed by flow cytometry

<table>
<thead>
<tr>
<th>Cell line designation</th>
<th>Sarcoma subtype</th>
<th>Source</th>
<th>Age of patient (y)</th>
<th>Endosialin status</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-673</td>
<td>Ewing’s sarcoma*</td>
<td>Muscle</td>
<td>15</td>
<td>Positive</td>
</tr>
<tr>
<td>HOS</td>
<td>Osteosarcoma</td>
<td>Bone</td>
<td>13</td>
<td>Positive</td>
</tr>
<tr>
<td>Hs 414.T</td>
<td>Fibrosarcoma</td>
<td>Connective tissue</td>
<td>13</td>
<td>Positive</td>
</tr>
<tr>
<td>Hs 93.T</td>
<td>Fibrosarcoma</td>
<td>Connective and soft tissue</td>
<td>12</td>
<td>Positive</td>
</tr>
<tr>
<td>Hs729T</td>
<td>Rhabdomyosarcoma</td>
<td>Connective tissue</td>
<td>74</td>
<td>Positive</td>
</tr>
<tr>
<td>SW872</td>
<td>Liposarcoma*</td>
<td>Connective tissue</td>
<td>36</td>
<td>Positive</td>
</tr>
<tr>
<td>SW982</td>
<td>Synovial sarcoma*</td>
<td></td>
<td>25</td>
<td>Positive</td>
</tr>
<tr>
<td>A204</td>
<td>Rhabdomyosarcoma</td>
<td>Muscle</td>
<td>1</td>
<td>Negative</td>
</tr>
<tr>
<td>HT-1080</td>
<td>Fibrosarcoma</td>
<td>Connective tissue</td>
<td>35</td>
<td>Negative</td>
</tr>
<tr>
<td>RC13</td>
<td>Rhabdomyosarcoma</td>
<td>Muscle</td>
<td>17</td>
<td>Negative</td>
</tr>
<tr>
<td>RD</td>
<td>Rhabdomyosarcoma</td>
<td>Muscle</td>
<td>Fetus</td>
<td>Negative</td>
</tr>
<tr>
<td>SK-LMS-1</td>
<td>Leiomyosarcoma</td>
<td>Vulva</td>
<td>43</td>
<td>Negative</td>
</tr>
<tr>
<td>SK-UT-1</td>
<td>Leiomyosarcoma</td>
<td>Uterus</td>
<td>75</td>
<td>Negative</td>
</tr>
<tr>
<td>SW1353</td>
<td>Chondrosarcoma</td>
<td>Bone, right humerus</td>
<td>72</td>
<td>Negative</td>
</tr>
<tr>
<td>VA-ES-BJ</td>
<td>Epithelioid carcinoma</td>
<td>Bone marrow metastasis</td>
<td>41</td>
<td>Negative</td>
</tr>
</tbody>
</table>

NOTE: Seven of the 15 lines were positive for endosialin. There was no correlation with sarcoma subtype, source of tumor tissue, or patient age. *Cell lines that underwent cytogenetic analysis (Table 2).

Results

Endosialin expression in human tumor cell lines and primary cells. Twenty-seven human cancer cell lines, including 5 melanoma, 3 neuroblastoma, and 19 carcinoma lines were assayed for endosialin transcript by reverse transcription followed by real-time PCR (Fig. 1A). The 5 melanoma lines were A-375, WM-115, SK-MEL-28, MEL-624, and MDA-MB-435. The 3 neuroblastoma lines were the IMR-32, SH-SY5Y, and SK-N-AS. The 19 carcinoma lines included MCF7, MDA-MB-231, SK-BR-3 breast adenocarcinoma and BT-474 breast carcinoma, SK-OV-3 ovarian adenocarcinoma, PC-3 prostate adenocarcinoma, DU 145 and LNCaP prostate carcinoma, 786-O renal cell adenocarcinoma, Hep-3B hepatocellular carcinoma, PAN-C1 pancreatic epithelioid carcinoma, MIA PaCa-2 pancreatic carcinoma and AsPC-1 pancreatic adenocarcinoma, DLD-1, HT-29, LS 174T, SW-480, and SW-837 colorectal adenocarcinoma, and HCT-116 colorectal carcinoma lines. Of these tumor lines, only the SK-N-AS neuroblastoma expressed endosialin mRNA.

Four human leukemia cell lines, 4 human lymphoma lines and 15 human sarcoma lines were assayed for endosialin protein by flow cytometry done on ice with ice-cold reagents without membrane permeabilization to inhibit endosialin internalization; thus, a positive signal indicated cell surface...
expression of endosialin (Fig. 1B). The 4 leukemia cell lines included HL-60 acute promyelocytic leukemia, K-562 chronic myelogenous leukemia, MOLT-4 acute lymphoblastic leukemia, and Jurkat acute T-cell leukemia. The 4 lymphoma lines included Daudi, DND39, NAMALWA, and Raji Burkitt’s lymphoma. The 15 sarcoma lines assayed included A-673 Ewing’s sarcoma, HOS osteosarcoma, Hs 414.T, Hs 93.T, and HT-1080 fibrosarcoma, A-204, Hs 729.T, RC13, and RD rhabdomyosarcoma, SW-872 liposarcoma, SW-982 synovial sarcoma, SK-LMS-1 and SK-UT-1 leiomyosarcoma, SW-1353 chondrosarcoma, and VA-ES-B1 epithelioid sarcoma.

The neuroblastoma line SK-N-AS and 7 of 15 sarcoma lines were positive for endosialin protein (Table 1). The positive sarcoma lines included the A-673 Ewing’s sarcoma, HOS osteosarcoma, Hs 414.T, Hs 93.T, and HT-1080 fibrosarcoma, A-204, Hs 729.T, RC13, and RD rhabdomyosarcoma, SW-872 liposarcoma, SW-982 synovial sarcoma, SK-LMS-1 and SK-UT-1 leiomyosarcoma, SW-1353 chondrosarcoma, and VA-ES-B1 epithelioid sarcoma.

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Table 2. Cytogenetic analysis of human SK-N-AS neuroblastoma and A-673, SW-872, and SW-982 sarcoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cytogenetic analysis results</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-673</td>
<td>46-48,XX,add(1)(p36.1),-3,-4,8,add(9)(q34),del(9)(p22),-13,add(13)(q32),add(16)(q24),del(17)(q11.2q21), der(22)(?11;22)(q24;q12),+4-6mar[cp20]</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>44-47,XX,+1,psu dic(1;14)(p22;q22),der(?)(5;?;q11.2),der(6)(p23)add(6)(q275),add(8)(q24.1),add(9)p22, add(11)(q13),add(16)(q274),-17,add(22)(q12),+0-4mar[cp20]</td>
</tr>
<tr>
<td>SW-872</td>
<td>79-99&lt;4n&gt;,X,del(X)(q25),X,-X,-1,add(1)(q44)x2,-2,-3,-4,add(4)(q31.1),add(4)(q35),+5,add(5)(q35)x2,add(5)(q35)x2, add(6)(q23),+7,add(7)(q36)x2,-10,-12,13,add(15)(p10)x2,-16,-16,-19,+20,+21,-22,+2-3mar[cp20]</td>
</tr>
<tr>
<td>SW-982</td>
<td>47-49,XX,(1;4;9)(q12;q11;p24),del(5)(q22q33),del(9;13)(q10;q10),+der(20)(t5;20)(q11.1;p13),+1-3mar[cp19]/94,idemx2, nonclonal changes[1]</td>
</tr>
</tbody>
</table>
osteosarcoma, Hs 729.T rhabdomyosarcoma, Hs 93.T and Hs 414.T fibrosarcoma, SW-872 liposarcoma, and SW-982 synovial sarcoma lines (negative sarcoma cell line: data not shown). Given the frequent endosialin expression by human cancer cells of mesenchymal lineage, human mesenchymal stem cells and human primary lung fibroblasts growing in cell culture were tested for cell surface expression of endosialin by flow cytometry. Both cell types were strong endosialin expressers.

Vascular cells growing in cell culture including HUVEC, HMVEC, human primary vascular pericytes, and vascular smooth muscle cells were assayed for cell surface endosialin expression by flow cytometry. HUVEC and HMVEC were negative for endosialin protein, whereas pericytes and vascular smooth muscle cells were strongly positive (Fig. 1B).

Localization of endosialin protein. The cell surface expression of endosialin protein was confirmed by staining A-673 Ewing's sarcoma cells, which showed a staining pattern outlining the perimeter of the cytoplasm and consistent with cell surface expression (Fig. 2A).

Cell line characterization. The histiocytes of 4 human tumor cell lines found positive for endosialin protein were analyzed by karyotyping (Table 2; Supplementary Figs. S2-S5). The A-673 cell line exhibited a diploid pseudodiploid karyotype with monosomy 13, a chromosome 16 that had unidentified chromatin replacing part of the long arm and a chromosome 22 that resembled the chromosome 22 derived from the t(11;22) translocation seen in Ewing's sarcoma (20). The reciprocal chromosome 11 product of this translocation was not observed (Table 2; Supplementary Fig. S2; refs. 21, 22).

The SK-N-AS line chromosomal abnormalities were highly complex and not specific. Abnormalities included a pseudodicentric chromosome derived from chromosome 1 and 14, monosomy 17, and structural abnormalities involving chromosomes 5, 8, 9, 11, 16, and 22. Three of 20 cells had loss of the X chromosome (Table 2; Supplementary Fig. S3). SK-N-AS cells were analyzed by fluorescence in situ hybridization for the EWSR1 gene. No evidence of EWS translocation was detected in SK-N-AS cells; thus, SK-N-AS cell line is not from a Ewing's sarcoma (Supplementary Fig. S1).

All of the 20 SW-872 metaphase cells examined had a hypotetraploid karyotype with numerous numerical and structural abnormalities and were karyotypically heterogeneous, suggesting that the line either is karyotypically unstable or contains an unknown number of subclones, although some abnormalities were consistently observed (23). The abnormalities detected were not specific to liposarcoma, although recurring cytogenetic abnormalities have only been described for myxoid liposarcomas. Structural abnormalities were...
observed for chromosomes 5 to 7 and numerical abnormalities for chromosomes 20 and 21 (Table 2; Supplementary Fig. S4).

All of the 20 SW-982 metaphase cells examined had a female karyotype and complex numerical and structural chromosome abnormalities observed in every cell (Table 2; Supplementary Fig. S5). The t(X;18) translocation associated with synovial sarcoma was not observed.

Internalization assay. A-673 Ewing’s sarcoma cells were exposed to anti-endosialin for 2 h at 37°C or on ice and then stained with secondary antibody. The staining pattern observed on ice indicated cell surface localization, whereas the staining pattern observed after incubation at 37°C indicated intracellular localization; thus, the anti-endosialin had internalized (Fig. 2B).

Cytotoxicity of anti-endosialin anti-human IgG-saporin conjugate. Endosialin-positive A-673 Ewing’s sarcoma and SK-N-AS neuroblastoma cells and endosialin-negative HT-1080 fibrosarcoma cells were exposed to anti-endosialin followed by anti-human IgG secondary antibody conjugated to the ribosome-inactivating protein saporin. The A-673 and SK-N-AS cells were sensitive to the anti-endosialin toxin conjugate, whereas endosialin-negative HT-1080 cells were unresponsive. All three cell lines were similarly sensitive to paclitaxel growth inhibition (Fig. 3).

Endosialin protein expression in human clinical tumor specimens. Endosialin immunohistochemistry staining was carried out on 250 human clinical tumor specimens on glass slides: 186 FFPE sections including 100 carcinomas and 86 sarcomas and 64 frozen sections including 58 carcinomas and 6 sarcomas (Table 3; Figs. 4 and 5). FFPE carcinoma samples included bladder, breast, colon, endometrial, gastric, head and neck, liver, lung, ovarian, and prostate carcinomas. FFPE sarcoma samples included angiosarcoma, Ewing’s sarcoma, fibrosarcoma, gastrointestinal stromal tumors (GIST), liposarcoma, MFH, osteosarcoma, rhabdomyosarcoma, and synovial sarcoma. Frozen carcinoma samples included bladder, breast, colon, endometrial, gastric, lung, ovarian, and renal carcinomas. Frozen sarcomas included synovial and spindle sarcomas.

FFPE tumor tissue staining analysis determined that 33% (33 of 100) of carcinomas were endosialin positive in vasculature (Table 3; Supplementary Tables S1-S4). In FFPE carcinomas, endothelial cells were rarely positive for endosialin and perivascular cells such as pericytes were almost exclusively the source of the signal. In FFPE carcinomas, vasculature was the only tissue component where endosialin was detected. Bladder

<table>
<thead>
<tr>
<th>Tissue preservation</th>
<th>Tissue type</th>
<th>Percentage of specimens with endosialin-positive vasculature</th>
<th>Percentage of specimens with endosialin-positive tumor cells</th>
<th>Percentage of specimens with endosialin-positive stromal cells</th>
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<td>50 (5/10)</td>
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<td>70 (7/10)</td>
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<td>40 (4/10)</td>
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<td>100 (7/7)</td>
<td>0 (0/7)</td>
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<tr>
<td></td>
<td>Lung cancer</td>
<td>80 (4/5)</td>
<td>0 (0/5)</td>
<td>20 (1/5)</td>
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<td>71 (2/7)</td>
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<tr>
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<td>Total frozen sarcoma</td>
<td>100 (6/6)</td>
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NOTE: The percentage of samples of each tumor type having endosialin-positive vasculature, malignant cells, and stromal cells is listed for each cancer subtype surveyed by immunohistochemistry staining for endosialin. Each tumor specimen was scored and examined independently by two pathologists.
cancers had highest frequency of endosialin-positive vasculature in FFPE carcinoma specimens; 100% (10 of 10) of bladder cancers had endosialin-positive vasculature followed by endometrial cancers (50% or 5 of 10), lung cancers (40% or 4 of 10), gastric cancers (33% or 3 of 9), breast cancers (30% or 3 of 10), colon cancers (30% or 3 of 10), ovarian cancers (27% or 3 of 11), head and neck cancers (10% or 1 of 10), liver cancers (10% or 1 of 10), and prostate cancers (0% or 0 of 10). In FFPE carcinomas, bladder cancer had the highest endosialin staining intensity and the highest endosialin vascular coverage followed by endometrial cancer. Bladder cancer and endometrial cancer were the only carcinoma subtypes of the 10 surveyed that had extensive endosialin vasculature with 3+ staining intensity (Fig. 4).

Fig. 4. Immunohistochemical staining intensity and percent vascular coverage for endosialin in FFPE human clinical carcinoma specimens. The scoring for vascular staining intensity was done independently by two pathologists and scored as 0, 1+, 2+, and 3+, with 3+ being the greatest intensity. Each point represents a single independent tumor specimen. Mean of each tumor type group. Numbers in parentheses, number of tumor specimens in the group.
In FFPE sarcomas, endosialin staining was found in malignant sarcoma cells and stromal cells in addition to vasculature (Table 3; Supplementary Tables S5-S7). In most cases, tumor cell staining was located on the cell surface, whereas in some cases it could also be detected in the cytoplasm. In total, 51% (54 of 86) of FFPE sarcoma specimens had endosialin-positive malignant cells, 78% (67 of 86) had endosialin-positive vasculature, and 22% (19 of 86) had

![Graphs showing immunohistochemical staining intensity and percent coverage of endosialin in FFPE human clinical sarcoma specimens. The scoring for staining intensity was done independently by two pathologists and scored as 0, 1+, 2+, and 3+, with 3+ being the greatest intensity. Each point represents a single independent tumor specimen. Mean of each tumor type group. Numbers in parentheses, number of tumor specimens in the group.](https://www.aacrjournals.org)
endosialin-positive stromal cells. Eighty-percent (8 of 10) of synovial sarcomas, 75% (6 of 8) of fibrosarcomas, 73% (8 of 11) of MFH, 70% (7 of 10) of liposarcomas, 64% (7 of 11) of osteosarcomas, 33% (3 of 9) of Ewing's sarcomas, 30% (3 of 10) of GIST, 14% (1 of 7) of angiosarcomas, and 10% (1 of 10) of rhabdomyosarcomas expressed endosialin in malignant sarcoma cells (Figs. 5 and 6).

Endosialin expression frequency in malignant cells in FFPE sarcomas varied widely from 80% of synovial sarcomas to 10% of rhabdomyosarcomas; however, endosialin expression frequency in vasculature in sarcomas was high in all 9 sarcoma subtypes, ranging from 100% (11 of 11) osteosarcomas to 91% (10 of 11) of MFH, 90% (9 of 10) of rhabdomyosarcomas, 80% (8 of 10) of liposarcomas, 75% (6 of 8) of fibrosarcomas, 70% (7 of 10) of synovial sarcomas, 67% (6 of 9) of Ewing's sarcomas, 60% (6 of 10) of GIST, and 57% (4 of 7) of angiosarcomas (Table 3; Fig. 5). Endosialin expression frequency in stromal cells was variable among the 9 sarcoma subtypes with 73% (8 of 11) of osteosarcomas, 75% (7 of 10) of synovial sarcomas, 27% (3 of 11) of MFH, 20% (2 of 10) of GIST, 20% (2 of 10) of synovial sarcomas, 11% (1 of 9) of Ewing's sarcomas, 10% (1 of 10) of liposarcomas, 0% (0 of 8) of fibrosarcomas, and 0% (0 of 10) of rhabdomyosarcomas having endosialin-positive stromal cells (Table 3). Synovial sarcoma, fibrosarcoma, MFH, liposarcoma, and osteosarcoma had the highest endosialin expression frequency in malignant cells; however, all sarcoma subtypes had intense and extensive endosialin coverage of three cellular compartments, malignant...
cells, stromal cells, and perivascular cells, with the exception of rhabdomyosarcoma where endosialin expression, although frequent and intense, was restricted to vasculature (Table 3).

Frozen tumor tissue staining and FFPE tissue staining concurred with endosialin staining of malignant cells as feature of sarcomas and not carcinomas (Table 3; Supplementary Tables S8-10). Signal intensity of frozen tissue immunohistochemistry was higher than FFPE tissue immunohistochemistry due to the lower degree of epitope modification in frozen tissues. A higher and more uniform observed frequency of endosialin vascular expression was seen in frozen carcinoma versus FFPE carcinoma (Table 3). Overall, 93% (54 of 58) of frozen carcinoma specimens had endosialin-positive vasculature compared with 33% (33 of 100) of FFPE carcinoma specimens. One hundred percent (7 of 7) of frozen bladder cancer specimens had endosialin-positive vasculature, in agreement with FFPE bladder cancer staining. One hundred percent (5 of 5) of frozen colon cancers, 100% (10 of 10) frozen endometrial cancers, 100% (7 of 7) of frozen gastric cancers, 100% (7 of 7) of frozen ovarian cancers, 100% (7 of 7) of frozen renal cancers, 80% (4 of 5) of lung cancers, and 70% (7/10) of frozen lung cancers had endosialin-positive vasculature (Table 3).

Frozen tumor tissue staining detected endosialin expression in stromal cells in carcinomas. Overall, 53% (31 of 58) of frozen carcinoma specimens tested had endosialin-positive stromal cells (Table 3). Specifically, 100% (5 of 5) of frozen colon cancers, 100% (7 of 7) of frozen gastric cancers, 71% (2 of 7) of frozen ovarian cancers, 50% (5 of 10) of frozen endometrial cancers, 40% (4 of 10) of frozen breast cancers, 29% (2 of 7) of frozen bladder cancers, 29% (2 of 7) of frozen renal cancers, and 20% (1 of 5) of lung cancers had endosialin-positive stromal cells.

Six frozen sarcoma specimens originally diagnosed as synovial sarcoma were immunocytochemistry stained for endosialin. On histologic review, 4 tumors appeared to be spindle cell tumors. All 6 (100%) frozen sarcoma specimens had endosialin-positive vasculature and all 6 (100%) had endosialin-positive stromal cells. Three (50%) had endosialin-positive malignant cells (Table 3; Supplementary Table S10). One of 2 confirmed synovial sarcomas had endosialin staining in mesenchymal tumor cells and in pockets of epithelial tumor cells (Fig. 6).

Overall, cancer subtypes with the greatest endosialin expression extent and intensity were synovial sarcoma, fibrosarcoma, MFH, liposarcoma, and osteosarcoma as well as bladder cancer and endometrial cancer. Endosialin expression extent and intensity varied sufficiently among cancer subtypes to identify the potentially most promising indications for anti-endosialin therapy. The clarity of the expression profile for certain tumors should not obscure the heterogeneity of expression in individual tumors in carcinomas or sarcomas. One example of the heterogeneity of endosialin expression among patients with the same disease is ovarian cancer specimen FFPE-P090, which has high vascular expression of endosialin, both in extent/coverage of vasculature and in signal intensity. Eighty percent of the vasculature in specimen FFPE-P090 had 3+ staining intensity and 20% had 2+ staining intensity. On the other hand, the other 10 ovarian cancer specimens had very low level to no vascular endosialin (Fig. 4; Supplementary Table S3). Similar heterogeneity of endosialin staining was observed in angiosarcomas where 80% of the vasculature in specimen FFPE-P106 had 3+ staining intensity and 20% had 2+ staining intensity, whereas most of the other 6 angiosarcoma specimens surveyed had a lower extent and level of endosialin or no endosialin. The same observation is true for Ewing's sarcoma.

Fig. 7. Endosialin immunohistochemical staining of human SK-N-AS neuroblastoma and human A-673 Ewing's sarcoma grown as subcutaneous xenografts in nude mice indicates that endosialin protein expression is maintained during tumor growth in vivo.
in vivo neuroblastoma cells were grown subcutaneously in BALB/c endosialin-positive A-673 Ewing's sarcoma cells and SK-N-AS neurosarcoma, and osteosarcoma (Supplementary Tables S5-S7). Also observed in synovial sarcoma, fibrosarcoma, MFH, liposarcoma, GIST, and rhabdomyosarcoma. Heterogeneity was also observed in synovial sarcoma, fibrosarcoma, MFH, liposarcoma, and osteosarcoma (Supplementary Tables S5-S7).

**Endosialin expression in human tumor xenografts.** Human endosialin-positive A-673 Ewing's sarcoma cells and SK-N-AS neuroblastoma cells were grown subcutaneously in BALB/c nude mice as tumor xenografts. As tumors, these cells maintained expression of endosialin, thus providing in vivo models for potential endosialin-directed therapeutics (Fig. 7).

**Discussion**

A survey of endosialin protein expression in human cancer was conducted. Two hundred fifty clinical specimens were surveyed encompassing 158 carcinomas and 92 sarcomas and representing 20 cancer subtypes including 11 carcinoma subtypes and 9 sarcoma subtypes. Endosialin is frequently expressed in human cancers; 19 of the 20 cancer subtypes surveyed had endosialin-positive specimens. Prostate cancer was the only cancer subtype surveyed (FFPE only) where endosialin was not detected.

Endosialin is broadly expressed in human cancer. Its frequency, extent, and intensity vary among cancer subtypes as well as among individual tumors within subtypes. An outstanding finding is the prominence of endosialin protein expression in sarcomas. Endosialin was detected in 9 of 9 (100%) sarcoma subtypes surveyed and in 76 of 92 (83%) of sarcoma specimens surveyed, suggesting that endosialin is a very frequent feature of sarcomatous disease. Endosialin was detected in several cellular compartments including malignant sarcoma cells, stromal cells, and vasculature. Endosialin levels in sarcomas were high: 23 of 86 (27%) FFPE sarcoma specimens surveyed displayed at least a 50% vascular and tumor cell reactivity with a minimum of 2+ staining intensity, whereas 12 (14%) displayed at least 50% vascular and tumor cell reactivity with 3+ staining intensity. Sarcoma subtypes with the greatest frequency, extent, and intensity of endosialin expression and potentially the most promising therapeutic potential were synovial sarcoma, fibrosarcoma, MFH, liposarcoma, and osteosarcoma.

High endosialin expression frequency was observed in vasculature of carcinomas, with bladder cancer emerging as an outstanding carcinoma subtype for endosialin expression with the highest frequency, extent, and intensity of endosialin vascular expression among the 10 carcinoma subtypes surveyed in the FFPE panel. The restriction of endosialin expression in carcinomas to vasculature observed in FFPE specimens, and to vasculature and stromal cells observed in frozen tumor specimens, has implications for potential endosialin-directed therapeutics, which could be expected to have an antiangiogenic or vascular-disrupting mechanism of action. In contrast, in sarcomas, an endosialin-directed therapeutic could have direct anticancer effects, antiangiogenic and/or vascular disrupting (24).

In cell culture, endosialin was present on the surface of primary perivascular cells (smooth muscle cells and pericytes), primary stromal cells (fibroblasts), primary mesenchymal stem cells, 7 human sarcoma cell lines, and 1 human neuroblastoma cell line. No carcinoma, melanoma, or hematopoietic cancer cell lines expressed endosialin. Data suggest that endosialin may be a mesenchymal marker.

Before selecting sarcoma cell lines derived from clinically relevant sarcoma subtypes for preclinical experimental model development, it became clear that the tumor subtype of several lines was in question. The SW-982 and SW-872 cell lines, respectively, seemed suitable choices, given our data showing that 80% of synovial sarcoma clinical specimens and 70% of liposarcoma clinical specimens express endosialin in malignant cells, and were selected for further characterization. Chromosome analysis could not confirm the reported diseases of origin for these lines. Characterization of the SK-N-AS cell line failed to reveal features specific to any particular neoplasm but ruled out Ewing's sarcoma. The A-673 and SK-N-AS cell lines were selected for in vivo model development. When grown as xenograft tumors in immunodeficient mice, both lines continued to express endosialin on the cell surface and may be useful in vivo models in which to test the efficacy of endosialin-directed therapies.

In cell culture, anti-endosialin internalized but did not alter the growth of endosialin-positive A-673 and SK-N-AS cells. However, exposure to anti-endosialin and to a saporin-conjugated secondary anti-human IgG antibody selectively inhibited the growth of endosialin-positive cells and did not alter the growth of endosialin-negative H1T-1080 fibrosarcoma cells, suggesting that an antibody-toxin conjugate might be a possible therapeutic approach for endosialin-positive cancers.

The pattern of endosialin expression in clinical specimens suggests that patients diagnosed with synovial sarcoma, fibrosarcoma, MFH, liposarcoma, or osteosarcoma could potentially benefit from an endosialin-directed therapy. However, despite the high frequency, extent, and intensity of endosialin expression observed in many tumors in these sarcoma subtypes, some tumors within these subtypes have low endosialin and might be unsuitable for endosialin-directed therapy. Conversely, among sarcoma subtypes with lower endosialin frequency, tissue coverage, and levels, there are some strongly endosialin-positive tumors that may benefit from anti-endosialin therapy. This heterogeneity of individual tumors within disease subtypes may be an obstacle to successful disease-specific clinical trials. Targeted therapies bring the promise of overcoming this obstacle through diagnostic/therapeutic applications and personalized medicine selection of patients by expression of the therapeutic target. Current clinical practice with Herceptin suggests that patients whose breast cancer displays moderate (2+) to strong (3+) circumferential cytoplasmic membrane HER-2 overexpression by immunohistochemistry should be candidates for trastuzumab therapy (25–27). HER-2 amplification measured by fluorescence in situ hybridization is also a criterion for patient selection for trastuzumab therapy (25, 26). Approximately 20% of breast cancers show HER-2 amplification or overexpression. The level of endosialin overexpression in sarcomas, with 27% of sarcomas reaching 50% vascular and tumor cell reactivity with 2+ staining intensity and 14% of sarcomas reaching 50% vascular and tumor cell reactivity with 3+ staining intensity (variably cytoplasmic membrane or cytoplasmic), is in line with the HER-2 experience in breast cancer (28).

The promise of endosialin-directed therapy in sarcoma is to bring a molecular target and therapy to a set of diseases with poor survival rates for unresectable or advanced disease (29–34). The reported low levels of endosialin in normal tissues (3) and the high levels measured in several sarcoma...
subtypes suggest that endosialin may be a suitable target protein for selective therapeutic intervention.

Disclosure of Potential Conflicts of Interest


References


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Endosialin Protein Expression and Therapeutic Target Potential in Human Solid Tumors: Sarcoma versus Carcinoma


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