Aminopeptidase N (CD13): Expression, Prognostic Impact, and Use as Therapeutic Target for Tissue Factor Induced Tumor Vascular Infarction in Soft Tissue Sarcoma

Abstract

Aminopeptidase N (CD13) is expressed on tumor vasculature and tumor cells. It represents a candidate for targeted therapy, e.g., by truncated tissue factor (tTF)-NGR, binding to CD13, and causing tumor vascular thrombosis. We analyzed CD13 expression by immunohistochemistry in 97 patients with STS who were treated by wide resection and uniform chemo-radio-chemotherapy. Using a semiquantitative score with four intensity levels, CD13 was expressed by tumor vasculature, or tumor cells, or both (composite value, intensity scores 1-3) in 93.9% of the STS. In 49.5% tumor cells, in 48.5% vascular/perivascular cells, and in 58.8%, composite value showed strong intensity score 3 staining. Leiomyosarcoma and synovial sarcoma showed low expression; fibrosarcoma and undifferentiated pleomorphic sarcoma showed high expression. We found a significant prognostic impact of CD13, as high expression in tumor cells or vascular/perivascular cells correlated with better relapse-free survival and overall survival. CD13 retained prognostic significance in multivariable analyses. Systemic tTF-NGR resulted in significant growth reduction of CD13-positive human HT1080 sarcoma cell line xenografts. Our results recommend further investigation of tTF-NGR in STS patients. CD13 might be a suitable predictive biomarker for patient selection.

Introduction

Soft tissue sarcomas (STSs) in adult patients represent a group of malignant mesenchymal tumors with an estimated incidence between 4 and 5/100,000/year in Europe [1]. These tumors may occur anywhere in the organism; common sites are extremities, the trunk, retroperitoneum, and head/neck. Exact histopathological diagnosis and grading as well as tumor staging by imaging are important since they influence multidisciplinary therapy, best carried out in experienced reference centers. Whenever possible, wide tumor
resection is the treatment of choice with curative intent, combined with radiotherapy and/or chemotherapy in specific situations [2]. In advanced disease, the therapeutic approach is also multidisciplinary, with chemotherapy gaining high importance [2]. In cases with widespread metastatic disease, the therapeutic aim often is restricted to palliation and/or prolongation of progression-free survival time instead of cure. First-line systemic therapy is based on doxorubicin alone or in several combinations such as doxorubicin and ifosfamide or doxorubicin and olaratumab. The combination of gemcitabine and docetaxel seems to be equally effective as doxorubicin alone but induces more severe hematotoxicity and is usually reserved for second-line therapy [2]. Besides conventional chemotherapy, trabectedine [3], pazopanib [4], and eribulin [5] are alternatives for second- and further-line therapy, eribulin only in liposarcoma. Especially the tyrosine kinase inhibitor pazopanib [4] and the monoclonal antibody olaratumab directed against platelet-derived growth factor receptor alpha are examples for drugs targeting tumor angiogenesis [6]. However, overall survival of patients with advanced STS in general remains poor. Thus, there is an unmet medical need for new therapeutic targets and agents in this group of diseases.

Angiogenesis and neovascularization are important for sufficient nutrient and oxygen supply into growing tumors and removal of metabolic waste products [7]. Among other components of the tumor microenvironment, the tumor vasculature presents targets for therapeutic approaches such as antiangiogenic therapies to interfere with vessel formation or vascular disruption and vascular infarction to target already existing tumor vessels. Denekamp et al. were the first to propose existing tumor vessels and endothelial cells as target for antivascular therapy [8]. The Thorpe laboratory introduced the concept of tumor vessel infarction by targeted tissue factor (TF) [9]. Among other targeting molecules in tumor vasculature, Pasqualini et al. published small NGR (asparagine-glycine-arginine)-containing peptides binding to aminopeptidase N (APN, CD13) as a tumor vascular target [10]. NGR-human tumor necrosis factor (h-TNF)–binding assays showed that, in particular, a CD13 isoform predominantly expressed on tumor vessels could function as a vascular binding site for NGR peptides with specific amino acid flanking regions [11].

CD13 [12] has been shown to promote angiogenesis, tumor growth, and metastasis [13] and has also been shown to be of prognostic relevance for patients with cancer of some but not all histologies examined [14–18]. We have previously performed several studies with fusion proteins carrying NGR peptides at the C-terminus of truncated tissue factor (tTF) to induce tumor vasculature infarction [19–25]. These fusion proteins inhibit human tumor xenograft growth in vivo independent of tumor histology through tumor vascular thrombosis, as evidenced by various imaging modalities. The lead protein rTF-NGR is currently in phase I clinical testing (NCT02902237).

Since HT1080 STS cell line xenografts revealed sensitivity towards the in vivo therapeutic efficacy of rTF-NGR [20,25], patients with these types of tumors are a candidate target population for phase II clinical trials with this agent. Aoki et al. reported CD13 expression on tumor cells with variability according to the STS subtype in a small series [26], but there is no investigation published differentiating between vascular and perivascular presence of CD13 versus presence of CD13 on sarcoma cells in human tissues. CD13 has been described to contribute to osteosarcoma migration and invasion [27,28], but overall, only little is known on the functional role of CD13 in sarcoma cells.

Here, we investigated the expression of CD13 in human STS tumor samples, its prognostic impact, and its relevance as a target for rTF-NGR in human STS xenografts in vivo. We show that CD13 is present on tumor cells in 70.1% and on tumor vascular and perivascular cells in 92.8% of the human STS samples tested. The presence of CD13 on STS tumor cells or in the tumor vasculature had significant prognostic impact in univariate analysis for relapse-free survival (RFS) and overall survival (OS), where higher expression levels of CD13 indicated a better prognosis. After adjusting for patient age, tumor stage, and tumor site in multivariable analyses, CD13 expression on tumor cells retained its prognostic significance. Human STS sarcoma xenografts staining positive for CD13 in nude mice showed good sensitivity to systemic treatment with rTF-NGR which significantly inhibited sarcoma growth at nontoxic doses.

Materials and Methods

Patient Cohort

Tumor samples of 97 patients with local STS, including 3 patients with operable oligometastasis, who underwent surgical wide resection and obtained adjuvant “sandwich” chemo-radio-chemotherapy with four cycles of doxorubicin and ifosfamide and radiotherapy between cycles 2 and 3 in curative intent in our institution were included in our immunohistochemical analysis. Patient selection was on the basis of sufficient pathological material present for evaluation. This retrospective analysis on archived histological material was approved by the Ethical Board of the Physician’s chamber of Westphalia-Lippe and the Westphalian Wilhelms University of Muenster (AZ 2017-493-F-S). Baseline characteristics of STS patients (n = 97) included in this study are summarized in Table 1. The large group of undifferentiated sarcomas comprised 39 undifferentiated pleomorphic sarcomas, 10 undifferentiated spindle cell sarcomas, and 1 undifferentiated epithelial sarcoma. In the group of nine patients with lipo-sarcoma, the following subtypes were included: five dedifferentiated liposarcomas, three myxoid liposarcomas (high grade), and one pleomorphic liposarcoma. Myxoid liposarcomas showing a round cell component of >5% were categorized as high grade and thus included in the G3 group in Table 1. The group of 16 patients summarized in the category “other” in Table 1 included 3 epitheloid sarcomas, 3 angiosarcomas, 3 malignant peripheral nerve sheath tumors, 5 pleomorphic rhabdomyosarcomas, 1 spindle cell rhabdomyosarcoma, and 1 alveolar rhabdomyosarcoma. All 94 patients characterized as R0 in Table 1 had macroscopically and histologically clean margins upon wide excision; 3 patients had R1 as final surgical result with tumor cells found histologically at the margin, of which 1 patient was not unequivocally classifiable as either R1 or R2.

Follow-up investigations on the patients were done every 3 months for the first 2 years, every 6 months for the next 3 years, and once a year thereafter. Appointments not followed by the patient were substituted by a phone call. All patients “lost to follow-up” were censored at the time of last contact.

Immunohistochemical and Immunofluorescence Staining

Staining and evaluation procedures of paraffin-embedded archived STS tissue samples were essentially performed as published using a Ventana Benchmark Ultra instrument [29]. For CD13 immunohistochemistry, tissue slides were deparaffinized and pretreated for 8 minutes at 95°C in CC1 buffer (pH 8.4) followed by an incubation with 100 μl anti-CD13 antibody for 32 minutes (clone: SP187,
rabbit monoclonal antibody, Cell Marque, Rocklin, CA, USA; prediluted in a concentration of 0.59 μg/ml in Tris Buffer pH 7.3-7.7 with 1% BSA). For CD163 staining, after pretreatment at 95°C in CC1 buffer for 32 minutes, the slides were incubated for 32 minutes with 100 μl anti-CD163 antibody (clone MRQ-26, mouse monoclonal antibody, Cell Marque, Rocklin, CA, USA; prediluted in a concentration of 0.23 μg/ml in Tris Buffer pH 7.3-7.7 with 1% BSA). A biotinylated secondary antibody and DAB (OptiView DAB IHC Detection Kit, Ventana Medical Systems, Inc., Tucson, USA) as chromogen were used for the visualization of the immunoreaction according to the manufacturer's recommendations. Finally, tissue slides were counterstained with hematoxylin and covered with Cytoseal (Thermo Fisher Scientific, Inc., Waltham, USA). Immunohistochemical evaluation of CD13 expression was carried out essentially as described before by two independent investigators (A.B. and W.H.). Briefly, CD13 expression was evaluated separately in the mesenchymal tumor cells and in the vascular/perivascular compartment employing a semiquantitative score to assess staining intensity and proportion. Differentiation of tumor cells from CD13 positive macrophagocytic infiltrates was ascertained by close evaluation of cytological features; in selected cases, immunohistochemical analysis with antibodies against CD163 was complemented. Establishing a reference framework for the analysis, prototypical examples of four different staining intensities (0, none; 1, weak; 2, moderate; 3, strong) were assembled through an initial screen of the stained set of samples in analogy to a method published before [30]. A dichotomized evaluation system was used (intensities 0, 1, 2 versus 3) for further statistical investigation of the prognostic impact of CD13.

To further document the patterns of tumor cell and vascular/perivascular CD13 expression, additional immunofluorescence analysis was performed for CD31 as described [29]. CD31 was used as an endothelial cell marker to show staining overlap with CD13 on vascular architecture and distinguish between tumor cell and tumor vascular cell positivity for CD13. Paraffin sections (4 μm) were deparaffinized and steam heated for 30 minutes at 95°C (citrate buffer pH 6.1) for antigen retrieval followed by a 10% bovine serum albumin (BSA) blocking step. Subsequently, sections were incubated over night at 4°C with a CD13 antibody (SP187, Cell Marque; dilution 1:300) and a CD31 antibody (JC70A, DAKO; dilution 1:200) followed by a 60-minute incubation with a Cy3-labeled goat anti-rabbit-IgG antibody (#554001, BD Pharmingen; prediluted in a concentration of 0.59 μg/ml in Tris Buffer pH 7.3-7.7 with 1% BSA) and a CD31 antibody (JC70A, DAKO, dilution 1:300) and a CD31 antibody (JC70A, DAKO, dilution 1:300). Following PBS washing steps, nuclei were counterstained with DAPI. Likewise, to differentiate between tumor cell (CD163 negative) and macrophagocytic (CD163 positive) CD13 expression, an identical immunofluorescence colocalization analysis was performed, employing the same CD163 antibody as described above.

**HT1080 Human Tumor Cell Line Xenograft Model**

All procedures performed on animals were in agreement with German law (Tierversuchsgesetz §8, Abs. 2) and were approved by a specific project license [50.0835.1.0 (G35/2005), provided by Bezirksregierung Muenster, Germany]. CD-1 nude mice were purchased from Charles River Laboratories (Charles River Laboratories, Sulzfeld, Germany) and acclimated to our central animal experiment facility for at least 1 week before initiation of the experiments. Mice were maintained in individually ventilated cages (IVC) on a 12-hour:12-hour light:dark cycle in a low-stress environment (22°C, 50% humidity, low noise) and given food and water ad libitum.

The human fibrosarcoma cell line HT1080 was purchased from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco medium supplemented with 10% fetal calf serum (FCS) at 37°C, high humidity, and 5% CO2. Cell viability was evaluated by trypan blue dye exclusion test. Single tumor cell suspensions (5x10⁶ cells/100 μl) were injected subcutaneously (s.c.) into the right anterior flank of female CD-1 nude mice (9-12 weeks old). When tumor growth reached a size of approx. 1000 mm³, mice were randomly assigned to receive either tTF-NGR (1 mg/kg body weight) solved in saline or control saline systemically by subcutaneous (s.c.) application. Tumor size was evaluated using a standard caliper measuring tumor length and width in a blinded fashion and calculating the tumor volume as [length x width² x π/6]. For xenograft histology experiments, animals were sacrificed in deep CO2 anesthesia, and primary tumors were surgically removed. Tumor tissue was embedded in paraffin, sliced in 4-μm-thick sections, and stained with hematoxylin-eosin (H&E) and with both a monoclonal CD13 and a monoclonal CD31 antibody as described above.

**Flow Cytometry**

CD13 presence on HT1080 cells was analyzed by flow cytometry using the BD FACS Calibur flow cytometer [Becton-Dickinson (BD), San Jose, CA, USA] as described before [17]. Briefly, cells with

| Table 1. Characteristics of the Patients |
|----------------------------------------|
| **Total** | n = 97 |
| Age (years) | Median (range) | 51 (23-71) |
| **Gender** | | |
| Female | 41 | 42.2% |
| Male | 56 | 57.8% |
| **Disease site, n (%)** | | |
| Extremity | 77 | 79.4% |
| Trunk | 18 | 18.6% |
| Neck/head | 2 | 2.1% |
| **Histology, n (%)** | | |
| Undifferentiated sarcoma | 50 | 51.5% |
| Myxofibrosarcoma | 10 | 10.3% |
| Liposarcoma | 9 | 9.3% |
| Synovial sarcoma | 9 | 9.3% |
| Leiomysarcoma | 3 | 3.1% |
| Other | 16 | 16.5% |
| **Grading, n (%)** | | |
| G2, n (%) | 9 | 9.3% |
| G3, n (%) | 88 | 90.7% |
| **Tumor size, n (%)** | | |
| <5 cm, n (%) | 22 | 22.7% |
| ≥5-10 cm, n (%) | 40 | 41.2% |
| >10 cm, n (%) | 35 | 36.1% |
| **Final extent of resection, n (%)** | | |
| R0, n (%) | 94 | 96.9% |
| R1 or R2, n (%) | 3 | 3.1% |
| **Chemotherapy, n (%)** | | |
| 4 cycles | 85 | 87.6% |
| <4 cycles | 10 | 10.3% |
| **Missing** | 2 | 2.1% |
| **Staging** | | |
| I | 0 | 0% |
| II | 23 | 23.7% |
| III | 71 | 73.2% |
| IV | 3 | 3.1% |
| **Radiotherapy, n (%)** | | |
| Yes | 77 | 79.4% |
| No | 14 | 14.4% |
| **Missing** | 6 | 6.2% |

According to seventh UICC classification.
a density of 90% were harvested, washed twice with PBS, and blocked with human immunoglobulin G (IgG; 1 μg/1 × 10^5 cells). For direct staining of the cell surface protein, cells were incubated with the monoclonal mouse anti-CD13-phycocyanin (PE) antibody (ab69775, Abcam; 2 μl/1 × 10^5 cells) for 30 minutes at 4°C. After two washing steps with ice-cold PBS/10% FCS, cells were resuspended in 500 μl PBS/FCS and analyzed in the flow cytometer.

**Statistical Analysis**

Data collection was performed retrospectively. The study population was described by standard descriptive statistics. For categorical variables, absolute and relative frequencies are reported. Baseline information of the STS population is provided in Table 1. Survival time was computed from the date of histological diagnosis to death or censored at the date of last contact. For the overall survival analysis as depicted, we have not observed deaths with reasons other than disease-related. Thus, results represent also cause-specific survival. For investigation of the prognostic impact of CD13 protein expression in STS tissues, we have compared strong expression (score 3) with all other groups (scores 0-2), which divided the total cohort into two subcohorts of approximately the same size. Kaplan-Meier survival estimates were used for these two subcohorts.

![Figure 1](image-url)

**Figure 1.** Different intensity degrees of vascular/perivascular expression of CD13 in soft tissue sarcomas: D496.16, intensity score 0; D449.16, score 1; D933.16, score 2; D516.16, score 3 (original magnification 200×).
comparison, we used log-rank tests. Confidence intervals (at 95%) are based on the delta method and Greenwood variance. All survival analyses were performed with MATLAB (R2017b, The MathWorks, Natick, MA, USA). Additionally, multivariate Cox proportional hazard models (Table 3) were fitted using SPSS (SPSS Statistics, version released 2017, IBM SPSS Statistics for Mac, Version 25.0. Armonk, NY, USA). Inferential statistics and fitted models are intended to be exploratory (hypothesis generating), not confirmatory, and are interpreted accordingly. We used a significance level of .05 throughout. For the xenograft experiment, data were presented as means with SE. Statistical significances of differences between the tTF-NGR group and the control group were tested with two-tailed two-sample t tests for independent groups.

Results

Expression of CD13 in STS Tissues

Baseline characteristics of STS patients (n = 97) included in this study are summarized in Table 1 (for subgroup characterization see Materials and Methods). We have performed this retrospective analysis in a cohort of patients with locoregional STS including three cases with operable oligometastasis who were uniformly treated by wide surgical resection with following adjuvant chemo-radio-chemotherapy by a sandwich protocol. Detailed treatment protocol and overall therapeutic results of our complete patient group with local disease treated with this multimodal approach have been recently published [31]. Positive immunostaining (scores 1-3 according to the criteria described above) of tumor tissues with the CD13 antibody was found in endothelial cells and vessel-associated stroma cells in 92.8% (Figure 1, Table 2) and in STS tumor cells in 70.1% (Figure 2, Table 2). Comparison of CD13 expression pattern and expression levels revealed differences according to the histological subtypes of STS, with leiomyosarcoma and translocation-driven synovial sarcoma showing the lowest level of CD13 tumoral positivity (Table 2). Overall, strong staining (score 3) was found in 49.5% of the cases in tumor cells and in 48.5% in vascular cells (Table 2).

Using immunofluorescence staining with CD13 and CD31 antibodies for colocalization, we found CD13 expression in the tumor stroma to be mainly restricted to the vascular and perivascular cells (Figure 3, A and B). Using an antibody against CD163 for colocalization, some monocytes and macrophages inside the tumor stained positive for CD13, however, with CD163-negative tumor cells also being clearly CD13 positive (Figure 3C).

Prognostic Impact of CD13 Expression on Outcome

Next, we investigated the prognostic impact of CD13 protein expression in STS tissues. Univariate analysis showed a significant impact of CD13 staining on prognosis. Comparing strong expression (score 3) with all other groups (scores 0-2) divided the total cohort into two subcohorts of approximately the same size. Significant associations were found for both vascular/perivascular and tumor cell CD13 protein expression with RFS and OS (Figure 4). We have also performed this analysis without the histological entities showing comparatively low expression of CD13 (synovial sarcoma, leiomyosarcoma), and as can be seen in Figure 5, results are comparable.

Multivariate Cox proportional hazard models with patient age, tumor stage, and tumor site (extremity vs nonextremity) as additional clinical prognostic factors [2] showed that the independent impact of CD13 positivity on prognosis in tumor cells and in vascular/perivascular zones is significant (Table 3).

Due to the rather small sample size, further subgroup analysis for the different histologies included into the STS group was not meaningful and therefore not performed.

Expression of CD13 and Systemic Therapeutic Activity of tTF-NGR in the HT1080 Human Sarcoma Cell Line Xenograft Model

For preclinical experiments establishing therapeutic CD13 targeting of STS, we performed flow cytometry of the human HT1080 fibrosarcoma cell line. In contrast to the human small cell lung cancer cell line HTB119, which was tested as a negative control, HT1080 cells stained strongly positive for CD13 (Figure 6A). In addition, immunohistochemistry revealed strong staining for CD13 in HT1080 xenograft sections (Figure 6B).

The next set of experiments investigated the therapeutic activity of systemically given tTF-NGR against subcutaneous HT1080 xenograft growth. In the experimental example shown in Figure 6C, tTF-NGR or saline (control) was applied subcutaneously distant from the tumor daily for 6 days. Tumor growth was observed for the
tTF-NGR as well as the saline control group and showed significant regression of tumor size as long as treatment was given. Additionally to the data shown in Figure 6, in a total of 39 experiments using the HT1080 xenograft model with \( N = 5 \) mice per group, different routes of application (intravenous or intraperitoneal besides the subcutaneous route), dose and time schedules of tTF-NGR versus saline controls after tumor inoculation were tested to describe the therapeutic window of tTF-NGR. These experiments have been published before ([20,22,25] tTF-NGR Investigators Brochure on file). HT1080 xenograft size in nude mice treated with tTF-NGR was significantly reduced in comparison to the saline control groups in 31 out of those 39 different experiments, with negative results in dose-response experiments using single tTF-NGR doses \( \leq 0.75 \) mg/kg body weight. We have established the best therapeutic window using slow intravenous injection of tTF-NGR with therapeutically active doses of approx. 1 mg/kg body weight given 3-6 times, whereas lethal dose for 10\% of the treated animals \( (LD_{10}) \) was found at single injections of 5 mg/kg body weight. However, long-term or complete remissions were rare when using tTF-NGR alone, and tumors often showed regrowth after treatment was stopped ([20]; see also Figure 6C). The therapeutic effect, however, was durable when combination of a cytotoxic such as doxorubicin was used together with tTF-NGR in a sequence allowing the vascular occlusion by tTF-NGR to entrap high doxorubicin concentrations inside the tumor for extended time periods [25].

These observations clearly demonstrate that human STSs are therapeutically targetable by CD13-directed tTF-NGR.

**Discussion**

Despite improving multidisciplinary diagnostics and therapy, the prognosis of advanced STS is still poor with median survival times not much longer than 1 year upon start of first-line systemic treatment [32] and few choices for second-line therapy [2–5]. Besides tumor cellular targets, the tumor vasculature of STS has been proven to express suitable targets for therapeutic agents [4,6]. However, there is an unmet need for further investigation of new targets and therapeutic agents in this group of fatal diseases.

We have developed a group of fusion proteins targeting tissue factor to the tumor vasculature with the result of tumor vessel infarction and occlusion, subsequently leading to consecutive growth inhibition of tumor xenografts [19–25]. The lead protein, tTF-NGR (HIS\(_{tag}\)tTF\(_{1-218}\)-GNGRAHA), is currently being investigated in a clinical phase I study in patients with advanced solid tumors or lymphomas beyond standard therapies (NCT02902237). Its main binding site is aminopeptidase N (APN, CD13) [10].
A transmembrane enzyme, is present in a variety of tissues and cells (endothelial, epithelial, fibroblasts, leukocytes) and carries different functions including degradation of extracellular tissue for an invasive cell [12]. However, CD13 protein expression is also present in some normal tissues [33]. This is however of minor importance for the therapeutic approach discussed here since normal tissue CD13 expression may only lead to tTF-NGR toxicity in a coagulation-competent area, such as blood vessels in contrast to, e.g., small bile ducts. Furthermore, the molecule is upregulated on endothelial cells in tumors and tissues that undergo angiogenesis and is essential for capillary tube formation [12,34]. CD13 is often used for tumor imaging [35–45]. Some NGR peptides bind in particular to a CD13 isoform preferentially present in tumor vascular cells as shown by binding assays using radiolabeled NGR peptide-linked tumor necrosis factor (NGR-hTNF) [11]. The antitumor activity of tTF-NGR in vivo is not restricted by tumor histology, as the main target is the tumor vasculature. Even in tumor entities such as small cell lung cancer, in which the tumor cells rarely express CD13 whereas vascular cells stain positive, significant therapeutic effects of systemically administered tTF-NGR can be shown [29]. However, sarcoma xenografts are among the most sensitive tumor entities for tTF-NGR induced vascular thrombosis and infarction as shown in this report and previously [20].

Mechtersheimer et al. [46] and later Aoki and colleagues [26] showed in the first small series that some mesenchymal neoplasias show tumoral expression of the CD13 protein. However, there is neither any report specifically quantifying differential expression of CD13 in STS cells versus sarcoma vascular endothelial cells or perivascular cells in cohorts of homogeneously treated patients with

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**Figure 3.** Immunofluorescence stainings. Immunofluorescence stainings showing vascular/perivascular CD13 positivity (A) and partial CD13 expression by the tumor cells (B). (C) CD163/CD13 immunofluorescence documents co-expression of CD13 on CD163-positive intralosomal macrophages along with clearly discernible expression of CD13 by non-CD163 positive tumor cells (original magnification 600×).
We show here that, in STS, the CD13 protein is expressed in the vast majority of the samples tested both by tumor cells and by vascular and perivascular cells. The latter anatomical distribution was confirmed by co-staining with CD31 in immunofluorescence analyses. CD31 (platelet endothelial cell adhesion molecule 1, PECAM-1) is among other markers widely used for evaluation of angiogenesis in human solid tumors [47].

In our STS patient cohort, univariate survival analysis did yield a significant prognostic impact of CD13 expression for both endothelial and perivascular cells and for tumor cells. Higher staining intensities correlated with better RFS and OS. When adjusting for clinical risk factors [2] such as patient age, tumor stage, and tumor site in multivariable analyses, the prognostic significance of CD13 was maintained. As this analysis is retrospective, it is hypothesis-generating and the finding should be verified in a prospective study. However, CD13 may be interpreted as a marker for angiogenic vasculature. In previous investigations, we have established microvessel density (MVD) and vascular endothelial growth factor (VEGF) as prognostic markers for better clinical outcome in chemotherapy-treated patient groups with osteosarcoma and Ewing’s sarcoma, respectively [48,49]. This pattern might reflect the relative chemo- and radiosensitivity of sarcomas, as a proangiogenic phenotype in small metastases might improve early chemotherapeutic intervention by a more efficient delivery of cytotoxic drugs. The observation that antiangiogenic therapy can decrease delivery of cytotoxic drugs into tumors also argues in this direction [50]. However, since a negative prognostic impact of an angiogenic phenotype in STS has also been described [51,52] and some authors could not find a prognostic impact of proangiogenic features in STS [52], it seems to be important to restrict the interpretation of our observation to the group of patients uniformly treated with an intensive adjuvant chemo-radio-chemotherapy “sandwich” protocol after wide resection of the primary tumor. Finally, the authors envisage that the prognostic impact of CD13 as shown may be of limited importance since the results are only established for the heterogeneous group of STS without including detailed information on single histologic subtypes. On the other hand, STS is a rare group of malignant diseases, and this is the largest group with uniform treatment, of which we could obtain a complete IHC and survival data set. Further subtype analysis was not performed since it would not yield reliable data due to the small numbers of patients.

Although being a marker without obvious driver characteristics for the tumors, CD13 could have potential as a target for tTF-NGR induced tumor vascular infarction in STS and may thus serve as a biomarker for patient selection in further studies with this compound. Upon treatment with tTF-NGR, CD-1 nude mice with HT1080 SCLC xenotransplants revealed a significant reduction of tumor growth in comparison to the saline control group. HT1080 cells were found to be positive for CD13 at an expression intensity of 3 when compared to the cohort of human STS tissues. As a CD13-negative

![Figure 4](image-url). Prognostic impact of CD13 expression in a group of 97 STS patients. Colored areas represent 95% confidence intervals. A and C represent survival curves in correlation with tumor cell CD13 staining; B and D represent survival curves in correlation with vascular/perivascular cell CD13 staining.
Figure 5. Prognostic impact of CD13 expression in a subgroup of STS patients without synovial sarcoma and leiomyosarcoma. For further details, see Figure 4.

Table 3. Multivariable Regression Analyses *

| Variables in the Model | HR   | 95% CI     | P Value |
|------------------------|------|-----------|---------|
| **CD13 on tumor cells** |      |           |         |
| Overall survival       |      |           |         |
| Age at diagnosis: continuous | 0.98 | 0.94-1.02 | .36     |
| Tumor site: noneextremity vs extremity | 3.02 | 1.07-8.53 | .037    |
| Stage:                 |      |           |         |
| II vs IV               | 0.16 | 0.01-1.83 | .14     |
| III vs IV              | 0.68 | 0.09-5.37 | .71     |
| CD13: 3 vs 0-2         | 0.28 | 0.10-0.80 | .017    |
| Relapse-free survival  |      |           |         |
| Age at diagnosis: continuous | 1.00 | 0.98-1.03 | .98     |
| Tumor site: noneextremity vs extremity | 1.53 | 0.72-3.26 | .27     |
| Stage:                 |      |           |         |
| II vs IV               | 0.09 | 0.02-0.37 | .001    |
| III vs IV              | 0.15 | 0.04-0.54 | .004    |
| CD13: 3 vs 0-2         | 0.50 | 0.27-0.94 | .030    |
| **CD13 on vascular/perivascular cells** |      |           |         |
| Overall survival       |      |           |         |
| Age at diagnosis: continuous | 0.99 | 0.95-1.03 | .60     |
| Tumor site: noneextremity vs extremity | 2.48 | 0.89-6.93 | .083    |
| Stage:                 |      |           |         |
| II vs IV               | 0.08 | 0.01-1.01 | .051    |
| III vs IV              | 0.31 | 0.04-2.61 | .28     |
| CD13: 3 vs 0-2         | 0.26 | 0.09-0.80 | .019    |
| Relapse-free survival  |      |           |         |
| Age at diagnosis: continuous | 1.00 | 0.98-1.03 | .85     |
| Tumor site: noneextremity vs extremity | 1.50 | 0.71-3.19 | .29     |
| Stage:                 |      |           |         |
| II vs IV               | 0.06 | 0.01-0.24 | <.001   |
| III vs IV              | 0.09 | 0.02-0.34 | <.001   |
| CD13: 3 vs 0-2         | 0.64 | 0.35-1.19 | .16     |

Hazard ratios (HR) greater or less than 1.0 indicate an increased or decreased risk, respectively, of an event for the first category listed.

* Tumor stage according to UICC 2010 definitions.
STS xenograft model was not available, we refer to our published data on small cell lung cancer, which showed that tumors with CD13 positivity only in the tumor vascular cells but CD13 negative on the tumor cells can also be effectively treated with tTF-NGR [29].

With reference to the mechanism of action of tTF-NGR, we have reported on tumor vascular thrombosis with intratumoral blood pooling, vascular disruption, decrease of vascular volume fraction in contrast-enhanced magnetic resonance imaging, and resulting tumor infarction with necrosis before [19–25]. In addition, biodistribution studies were performed showing tTF-NGR accumulation in the tumor rim [23]. Fluorescence Reflectance Imaging using Alexa-stained fibrinogen visualized downstream targets of tTF-NGR action through the TF:FVIIa:FX complex within the coagulation cascade within the tumor, and vascular anatomy became visible by fluorescent fibrin upon treatment with tTF-NGR [21,23].

Due to the frequent expression of CD13 by tumor endothelial cells, vessel-associated stroma cells, or tumor cells and due to the in vivo sensitivity of STS xenografts towards the therapeutic activity of tTF-NGR, patients suffering from CD13-positive sarcomas might benefit from tTF-NGR.

Recently, two NGR-peptide targeted molecules entered clinical trials in oncology. tTF-NGR is in clinical phase I (NCT02902237), and NGR-hTNF has already entered phase III [53]. Data based on phase II [54] and recent phase III studies (NCT01098266) have been submitted for approval in malignant pleural mesothelioma. Beside NGR peptides, CD13 is targeted by a variety of different compounds [55], with bestatin increasing the overall survival of patients with stage I squamous cell lung cancer when given after surgery in a randomized trial [56]. This strengthens the potential of CD13 as a vascular target for antitumor therapies, and further systematic investigation of the value of CD13 as a therapeutic target in STS is recommended.

**Authors’ Contributions**

T. K., W. E. B., W. H., and C. Schl. designed the study; all authors provided methodology and performed experiments; A. B. contributed experimental work in immunohistology and immunofluorescence histology as part of her MD thesis; M. G., A. B., L. A., T. K., C. Schw. analyzed data and performed statistics; T. K., W. E. B., W. H., C. Schl. wrote the manuscript. All authors revised and approved the manuscript.

**Conflict of Interest**

W. E. B. and R. M. share a patent on vascular targeting with tissue factor factor constructs. The other authors declared no competing financial interests.
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