Original Article

Comparison of cachectic and non-cachectic sarcoma patients reveals an important role of Notch signaling in metastasis and myogenesis

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Abstract: Cancer-associated cachexia is a wasting syndrome that affects up to 50% of cancer patients. It is defined as unintentional weight loss ≥5% over 6 months and characterized by muscle atrophy, fatigue, and anorexia that are refractory to nutritional support. Sarcoma describes a diverse group of malignancies arising from the connective tissues. Sarcoma patients are uniquely susceptible to cancer-associated cachexia given its origins in the musculoskeletal system. Our previous research suggests that sarcoma cells may contribute to sarcoma-associated cachexia (SAC) via establishment of TNF-α-mediated inflammation and dysregulation of muscle homeostasis by abnormal Notch signaling. Here, we examine the role of the Notch pathway and pro-inflammatory cytokines in cells derived from cachectic and non-cachectic human sarcoma patients. We observed increased expression of Notch pathway genes in the cachexia group while no differences in pro-inflammatory cytokines were observed. Co-culture of muscle-derived stem cells (MDSCs) and sarcoma cells demonstrated the inhibition of MDSC maturation with both cachectic and non-cachectic patient cells, corresponding to elevated Pax7 and Notch pathway expression in MDSCs. Our findings suggest that there is no difference in inflammatory profile between cachexia and non-cachexia sarcoma samples. However, Cachectic sarcoma samples express increased Notch that mediates muscle wasting possibly through inhibition of myogenesis.

Keywords: Sarcoma, cachexia, metastasis, notch signaling, muscle differentiation

Introduction

Cachexia is a complex metabolic condition that is defined by unintentional weight loss due to the progressive loss of skeletal muscle and adipose tissue that is refractory to nutritional supplementation. Cachexia can occur in chronic disease states such as renal failure, sepsis, COPD, and HIV/AIDS, but is particularly devastating when associated with cancer [1]. Cancer-associated cachexia (CAC) is a prevalent, debilitating comorbidity of malignancy. CAC affects about 50% of cancer patients at the time of death [2]. It is correlated with diminished performance and the inability to perform activities of daily life. It is also correlated with advanced disease, increased treatment morbidity, and shorter progression-free survival. CAC creates an additional burden to caregivers due to the inability to reverse it with nutritional support alone. This problem remains a major issue in cancer treatment, and currently there are no management strategies that address this phenomenon [3, 4].

Sarcomas are mesenchymal connective tissue malignancies that arise from muscle, bone, cartilage, adipose, and other structural tissues. Sarcoma patients experience significant musculoskeletal impairment due to the intrinsic damage that sarcomas impart to the connective tissues involved in posture and ambulation.
as well as the effects of surgery and other aggressive treatments. Although sarcoma patients are uniquely susceptible to CAC, our understanding of this mechanism is virtually non-existent. Sarcoma-associated cachexia (SAC) may result from the systemic inflammation that accompanies chronic disease. The overproduction of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-1β, IL-6 and IL-8 could result in the dysregulation of muscle homeostasis and a catabolic state, potentially dysregulating muscle-derived stem cell (MDSC) differentiation [5-8].

Notch signaling plays a central role in stem cell quiescence and differentiation [9]. Previous work from the Musculoskeletal Oncology Laboratory (MOL) has shown that muscle atrophy in sarcoma-bearing mice is mediated by the Notch pathway and is rescued by Notch inhibition, suggesting that cachexia does not cause destruction or permanent loss of MDSC differentiation potential. This could offer the opportunity for pharmacological intervention, prevention, and the possible reversal of SAC in sarcoma patients [10].

In this study, we examined cytokine levels in the sarcomas and the role of Notch in SAC. We demonstrated increased Notch expression in cachectic patient-derived tumors and isolated primary cells. Our current work confirmed previous results of muscle atrophy in the setting of increased Pax7 and Notch 3 expression in a murine model of osteosarcoma, indicating the presence of SAC [10]. This confirmation suggests that these findings may be clinically relevant and warrant further research to understand the molecular differences between cachectic and non-cachectic sarcomas and the events that lead to SAC.

Materials and methods

Patient samples

Patient samples were obtained for research purposes under the approved IRB PRO100-50461 at the University of Pittsburgh Medical Center (UPMC), Pittsburgh, PA. Patient sarcoma samples were grouped into cachexia or non-cachexia using total body weight (TBW) measurements collected from the electronic medical record (EMR) pre-surgery. Linear regression analyses were used to evaluate weight loss over a period of 6 months. Patients who maintained continued care and showed a TBW loss > 5% within this period were defined as cachectic. Patients who did not lose weight but maintained continued care were defined as non-cachectic. Patients who lost weight (1-5%) but did not meet the weight loss threshold for cachexia were labeled as pre-cachectic and excluded from this study.

Primary sarcoma cell populations

Using a human tumor dissociation kit (Cat#130-095-929, Miltenyi Biotec, Auburn, CA), tumors were dissociated and cultured under sterile conditions (37°C, 5% CO₂) until 80% confluence. Cells were then harvested and cryopreserved in 70% DMEM (#10-013-CV, Corning, Manassas, VA), 20% FBS (Cat#16000044, Gibco, Grand Island, NY), and 10% DMSO (4-X-5, ATCC, Manassas, VA).

Co-culture experimental design

The co-culture system contained Transwell® permeable supports (Cat# 353095, Corning, Pittston, PA) and the companion 24-well plate (Cat# 353504, Corning, Pittston, PA). MDSCs (1*10⁴ cells/well) were cultured in 24-well plates coated with collagen-I (Cat# A1048301, Gibco, Grand Island, NY) in DMEM enriched with 20% FBS, 1% Chick Embryo Extract (Cat# 100-163P, Gemini Bio-Products, West Sacramento, CA), and 1% penicillin and streptomycin (Cat#15140-122, Gibco, Grand Island, NY). Primary sarcoma cells (1*10⁴ cells/well) were cultured in transwells in DMEM enriched with 10% FBS and 1% penicillin and streptomycin. In the control group, MDSCs were cultured in both transwell and companion plates. After 2 days in co-culture, cell media from both the plate and the transwells were removed, washed once with 1X DPBS (Cat# 14190-144, Gibco, Grand Island, NY) and replaced with MyoTonic differentiation media for additional 4 days (Cat# MD-5555, Cook Myosite, Pittsburgh, PA).

Immunofluorescence (IF)

Myotubes were fluorescently labeled with fluorescent primary mouse myosin heavy chain antibody (anti-fMHC, Cat# M4276, Sigma-Aldrich, Saint Louis, MO) and secondary donkey anti-mouse IgG Alexa Fluor 594 (Cat# A21203,
Life Technologies, Eugene, OR). Nuclei were stained with DAPI (Cat#D1306, Thermo Fisher). Images were captured using an Olympus IX81-Motorized Inverted Microscope (Olympus, Center Valley, PA). Five distinct image fields were captured per well. Myotube formation was identified by positive immunofluorescence and quantified using ImageJ software (NIH, Bethesda, MD). Fusion index was calculated by the number of nuclei in myotubes with at least 2 nuclei divided by the total number of nuclei in a field.

**RNA extraction**

Tumor samples were homogenized and processed for RNA extraction using the Bullet Blender® homogenization protocol (Next Advance, Atkinson, NH). Frozen tumor was mechanically digested into small pieces and immediately transferred into Navy bead lysis kit (SKU: NAVYR5, Next Advance) with 1 ml Qiazol Lysis Reagent (Cat#79306, Qiagen). Samples were further homogenized using the bullet blender (Speed 12 and Time 3, 4°C) and repeated until uniform homogenization was achieved. Subsequent steps for RNA extraction from tumor samples were performed according to the Qiagen RNAeasy Mini kit protocol (Cat# 74106, Qiagen, Germantown, MD).

RNA extraction from primary sarcoma cells was performed using the Qiagen RNAeasy Mini kit protocol. RNA extraction from MDSCs was performed using SingleShot cell lysis kit (Cat #1725080, Biorad, Hercules, CA).

**RT-qPCR**

RT-qPCR for tumor samples and primary sarcoma cells was performed using BioRad iScript cDNA Synthesis Kit (Cat # 1708890, BioRad, Hercules, CA). For MDSCs, RT-qPCR was performed using the recommended iScript Advanced cDNA synthesis kit (Cat#1725038 BioRad, Hercules, CA). qPCR was performed for all samples and relative gene expression fold changes were calculated using the 2^ΔΔCt values. For tumors and primary cells, data were normalized to the geometric mean of three internal control genes (ALAS1, HMBS and POP4) and normalized to the non-cachexia group. Data for MDSCs were normalized to expression of GAPDH and normalized to control group.

**Enzyme-linked immunosorbent assay (ELISA)**

Cytokine levels (TNF-α, IL-1β, IL-6, IL-8) were measured in cell culture media of primary tumor cells cultured at 4*10^5 cells/ml in DMEM enriched with 10% FBS for 48 h from non-cachexia (n=7) and cachexia groups (n=9). Experiments were performed using the ELISA Max Deluxe standard protocol from Biolegend (San Diego, CA).

**Statistical analysis**

Statistical analyses were performed using the Mann-Whitney test for continuous variables and Fisher’s exact test for categorical data with α=0.05, *P < 0.05, **P < 0.01, ***P < 0.001. Graphs are presented as mean ± standard deviation (SD) (GraphPad Prism 7.0 software, San Diego, CA).

**Results**

**Patient characteristics**

A total of 22 patients were involved in this study, grouped into cachexia (n=12) and non-cachexia groups (n=10) based on percentage of weight changes. Patient characteristics including age, gender, weight change, metastasis and subtypes are shown in Table 1. There were no differences in age and sex. The cachexia group showed a significant weight change (-13.22 ± 5.56%) compared with the non-cachexia group (3.87 ± 6.68%). We observed a strong correlation between cachexia and tumor metastasis (P=0.0083) regardless of sarcoma histologic subtype. A total of ten sarcoma subtypes were involved in this study. Primary cell populations were successfully generated from nine samples in the cachexia group and seven samples in the non-cachexia group. RNA was extracted from eight tumors in the cachexia group and ten in the non-cachexia group.

**Pro-inflammatory cytokine levels do not differ between the cachexia and non-cachexia groups**

We examined the gene expression levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8) in patient tumor samples of cachexia and non-cachexia groups. There were no significant differences between TNF-α, IL-1β and IL-6 in
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Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Cachexia (n=12)</th>
<th>Non-cachexia (n=10)</th>
<th>p-value</th>
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</thead>
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<tr>
<td>Age, median (range)</td>
<td>50.5 (14, 72)</td>
<td>61 (42, 78)</td>
<td>0.17411</td>
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<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Male (%)</td>
<td>8 (67)</td>
<td>6 (60)</td>
<td>&gt; 0.99992</td>
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<tr>
<td>Female (%)</td>
<td>4 (33)</td>
<td>4 (40)</td>
<td></td>
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<tr>
<td>Weight change %</td>
<td>-13.22 ± 5.559</td>
<td>3.874 ± 6.677</td>
<td>&lt; 0.00013</td>
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<tr>
<td>Metastasis (%)</td>
<td>10 (83)</td>
<td>2 (20)</td>
<td>0.0083</td>
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<tr>
<td>Subtype</td>
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</tr>
<tr>
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<td>2</td>
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<td>Rhabdomyosarcoma</td>
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</tr>
<tr>
<td>Clear cell sarcoma</td>
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</tr>
<tr>
<td>High grade sarcoma</td>
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</table>


both groups. IL-8 expression was higher in the cachexia group (Figure 1A).

To examine whether primary sarcoma cells exhibit a similar cytokine profile, we measured TNF-α, IL-1β, IL-6 and IL-8 gene expression levels in the primary cell populations. There were no differences in TNF-α, IL-1β, IL-6 and IL-8 gene expression levels between the cachexia and the non-cachexia groups consistent with the primary tumor samples (Figure 1B). To further assess whether pro-inflammatory cytokines mediate cachexia, TNF-α, IL-1β, IL-6 and IL-8 protein levels were measured in cell culture media from primary sarcoma cells after 2d culture. Again, there were no differences in IL-1β and IL-6 between the two groups. IL-8 was increased in the non-cachexia group. TNF-α was below the detection range (Figure 1C).

Elevated Notch receptor expression is observed in the tumors and primary cell populations of the cachexia group

Dysregulation of Notch signaling is associated with higher tumor grading and risk of metastasis [11, 12]. There is a strong correlation in our patient samples between cachexia and tumor metastasis (Table 1, P=0.0083). To assess the role of Notch signaling in SAC, gene expressions of DLL1, JAG1, Notch1, Notch3 and Hes1 were measured in tumor and primary sarcoma cells. Notch1 and Notch3 were increased in the cachexia group in both tumor and primary sarcoma cells. There were no differences in DLL1 and Hes1 (Figure 2).

Cachectic and non-cachectic primary sarcoma cells inhibit muscle differentiation

One mechanism of muscle wasting in cachexia is the dysregulation of muscle stem cell differentiation and muscle repair. To assess if there are differences in the ability to inhibit muscle differentiation, a co-culture system that allows the study of sarcoma paracrine influences on muscle stem cell differentiation was performed using either cachectic or non-cachectic primary sarcoma cells (Figure 3A). There was no difference in the degree of inhibition of myotube formation in MDSCs co-cultured with either cachectic or non-cachectic sarcoma cells (Figure 3A). There was no difference in the degree of inhibition of myotube formation in MDSCs co-cultured with either cachectic or non-cachectic sarcoma cells. Quantitatively, this was measured by fusion index (FI), showing that both cachectic and non-cachectic groups inhibit muscle differentiation compared to the control group (Figure 3B, 3C).

Inhibition of muscle differentiation was associated with increased Pax7 and Notch in MDSCs

We measured the expression of muscle differentiation biomarkers and the Notch pathway in MDSCs in the co-culture system. Gene expression levels of Pax7 were increased in MDSCs...
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Figure 1. There is no significant difference in the cytokine profile between cachectic and non-cachectic sarcoma groups in general. A. Gene expression levels of pro-inflammatory cytokines in tumor. IL-8 gene expression level is increased in cachectic sarcoma tumors compared to non-cachectic sarcoma tumors. qPCR was performed on tumors from cachectic (n=8) and non-cachectic patients (n=10). Experiment was performed at least twice. Each tumor sample was tested in triplicate. Mann-Whitney test, *P < 0.05. B. Gene expression levels of pro-inflammatory factors in primary cell culture. There is no difference in gene expression levels of TNF-a, IL-1b, IL-6, and IL-8 between primary cell culture of cachectic (n=9) and non-cachectic (n=7) sarcoma groups. Patient tumor samples were homogenized and cultured for isolation of primary sarcoma cells. Experiment was performed once. Each patient sample was tested in triplicate. Mann-Whitney test, *P < 0.05. C. Assessing cytokine release from tumor samples in primary cell culture. Using ELISA, protein levels of IL-1b, TNF-a, IL-6 and IL-8 were measured in cell culture media after 48 h. IL-8 expression level was decreased in cachectic (n=9) compared to non-cachectic (n=7) sarcoma groups in primary cell culture, Mann-Whitney test, *P < 0.05.

cocultured with both cachectic and non-cachectic primary sarcoma cells compared to the control group. Increased MyoD1 and MYH1 gene expression levels were observed in
MDSCs co-cultured with non-cachexia primary sarcoma cells compared to the cachexia and control groups (Figure 4A-C). These markers, differentially elevated during specific stages of myotube formation, indicate a dominant self-renewal phenomenon and delayed differentiation in the cachexia and non-cachexia groups given the observation of abundant f-MHC protein levels (RFP) and low Pax7 levels in the control group compared to the sarcoma groups.

Notch signaling plays a central role in muscle stem cell self-renewal. Increased expression of DLL1, JAG1 and Notch3 in cachexia and non-cachexia groups further suggest delayed transition from MDSC to mature myocyte (Figure 4D-H).

Discussion

Sarcoma patients are uniquely susceptible to cachexia given their mesenchymal origins. However, little is known about SAC because of the rarity of sarcoma and the impact of cachexia on prognosis. To our knowledge, this is the first study to evaluate SAC biology with patient-derived sarcoma tissues and cells. In our cohort, we compared several factors among individuals diagnosed with sarcoma (Table 1). Our preliminary analyses showed a strong correlation between SAC and metastasis. We therefore sought to examine banked primary tumor samples from patients categorized as cachectic or non-cachectic for changes that might underlie the enhanced dysregulation of muscle stem cell renewal and integrity associated with cachexia.

Pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8) modulate the sequelae associated with chronic systemic inflammation and have been suggested as clinical biomarkers for cachexia. Elevated levels of these cytokines have been reported in CAC patients [8, 13] while...
other studies showed no differences between cachectic and non-cachectic patient groups [14, 15]. As none of these studies focused on SAC, we therefore measured the gene and protein levels of these cytokines in tumors and primary sarcoma cell samples. Our gene expression analyses showed no differences in pro-inflammatory cytokine levels between the two
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Figure 4. Increased expression levels in Pax7 and Notch pathway are observed in MDSCs co-cultured with primary sarcoma tumor cells. A-C. Stages of muscle differentiation. Pax7 mRNA levels were increased in MDSCs co-cultured with primary sarcoma cells from both cachexia (n=8) and non-cachexia (n=6) groups compared to the control group. The control and cachexia groups (n=8) show lower MyoD1 and MYH1 expression levels compared to the non-cachexia group (n=6). D-H. Increased Notch signaling is associated with muscle differentiation inhibition. DLL1, JAG1 and Notch3 mRNA levels are upregulated in MDSCs co-cultured with either cachexia (n=8) and non-cachexia (n=6) primary tumor cells. All treatment groups were performed in triplicate. mRNA levels were measured using qPCR. Mann-Whitney test, P < 0.05 (GraphPad Prism 7.0).

Notch signaling plays a central role in stem cell development and pluripotency via self-renewal and the inhibition of differentiation. Constitutive activation of Notch signaling is associated with tumorigenesis and has been associated with sarcoma development [16-18]. Notch overexpression has been observed in many cancer and sarcoma types including rhabdomyosarcoma [11, 19, 20]. Our previous work reported elevated Notch signaling in a highly metastatic murine osteosarcoma cell line compared with its weakly metastatic isogenic cell line [21]. This background information formed the basis of our examination of whether there are any differences in Notch expression between the cachexia and non-cachexia groups. We observed increased Notch1 and Notch3 expression in both tumor and primary cell samples from the cachexia group compared with the non-cachexia group (Figure 2A, 2B) indicating a correlation between SAC, increased Notch signaling, and metastatic potential. JAG1, a major Notch ligand, was elevated in both tumor and primary cell samples but only achieved significance in the primary cell cohort (Figure 2A, 2B). No differences were observed in the expression of DLL1, another Notch ligand and Hes1, a downstream effector of Notch that functions as a transcription factor. Considering the upregulation of Notch1 and Notch3 in the cachexia group, this suggests differences in other downstream factors regulated by the Notch pathway. Upregulation of Notch is also consistent with increased metastasis in SAC patients. Our data suggest that upregulation of the Notch signaling pathway is associated with SAC.
Cachexia is a result of chronic systemic illness that leads to muscle wasting that cannot be reversed by nutritional supplementation. Traditionally, this phenomenon was thought to be caused by increased protein degradation without adequate compensatory protein synthesis [22]. However, this study and recently published work support the central role of stem cell differentiation in the maintenance of muscle mass [23]. Specifically, we show that factors released from sarcoma cells inhibit stem cell differentiation as a possible mechanism of cachexia. Alternatively, while we did not test this, it is possible that paracrine signals from the sarcoma cells may diminish the stem cell pool in muscles. In our comparison of the ability of cachectic and non-cachectic sarcoma cells to inhibit muscle differentiation, we demonstrated that the cachexia group exhibits lower biomarker expression of muscle differentiation suggestive of stronger inhibition. Additionally, upregulation of Pax7 in MDSCs co-cultured with cachectic or non-cachectic primary sarcoma cells indicates increased proliferation and self-renewal of MDSCs. Expression of MyoD1 and MYH1, markers for muscle differentiation were increased in MDSCs co-cultured with non-cachexia primary sarcoma cells while the levels of these markers remained low in the cachexia and control groups. f-MHC quantification by IF showed higher myotube formation in the control group but not the cachexia group. We interpret these findings to mean that the MDSCs from the control group were further differentiated whereas the MDSCs from both sarcoma groups were differentiating at a significantly low rate due to paracrine influences from sarcoma cells. Increased levels of MyoD1 and MYH1 in the MDSCs co-cultured with the non-cachectic primary sarcoma cells reflect an intermediate rate of muscle stem cell differentiation between the cachexia and control groups, with the MDSCs in the control group showing a faster rate of differentiation and MDSCs in the cachexia group exhibiting a stronger inhibition of myotube formation (Figure 5).

Prior to this study, the contribution of Notch signaling in SAC was poorly understood. Our current findings support the previous work reporting that increased Pax7 and Notch signaling mediate muscle atrophy in our mouse model of osteosarcoma [10]. Additionally, we show that there are no differences in the expression of DLL1, JAG1, Notch1, Notch3 and Hes1 in the MDSCs co-cultured with primary sarcoma cells. Taken together, these observations suggest that while the inflammatory mechanism of cachectic and non-cachectic sarcoma cells on stem cell renewal and differentiation may be similar, cachectic sarcoma samples express increased Notch signaling that mediates muscle wasting possibly through inhibition of myogenesis. Notch1 is responsible for maintaining proliferation of MDSCs and inhibiting differentiation, while Notch3 regulates self-renewal of

Figure 5. Summary of Notch signaling in association with myogenesis in cachexia. In normal myogenic process, Pax7 is expressed during proliferation and self-renewal and reduced when MDSCs are committed to differentiation and express MyoD. Mature myotubes express MHC. Notch signaling is decreased in this process. Cachexia sarcomas exhibit higher expression of Notch signaling and stronger inhibition of muscle differentiation which maintain the main population in a stem cell state. Non-cachexia sarcomas exhibit relatively low expression of Notch signaling and a portion of MDSCs start to commit differentiation and maturation.
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MDSCs by preventing proliferation and encouraging quiescence [24, 25]. Our results suggest that Notch1 and Notch3 play an important role in sarcoma-mediated inhibition of muscle differentiation. Additionally, the role of Notch in tumor de-differentiation may underlie the increased incidence of metastasis in the SAC group.

Our study has several limitations. First, the sample size is relatively small. As sarcomas are extremely rare diseases this is largely unavoidable, but a limitation none the less. Second, a large number (n=10) of sarcoma histologic subtypes were evaluated, and this was a heterogeneous group. This may be viewed as a strength or weakness, but certainly a high level of heterogeneity within an already small sample size lends itself to difficulties in data interpretation.

This study is the first to use clinical sarcoma specimens combined with clinical histories to subtype patients into cachexia and non-cachexia groups. In summary, this subtyping appears to suggest that cachexia-causing sarcomas are associated with increased Notch signaling and a higher incidence of metastasis. Further investigation is required to determine what undetermined factors might contribute to sarcoma cell-induced repression of MDSC maturation, and if Notch inhibition is an effective strategy against SAC. Finally, the possible relationship between SAC and sarcoma metastasis must be thoroughly explored.

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Disclosure of conflict of interest

None.

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