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Alterations in Cancer Stem Cell Marker CD44 Expression Predict Oncologic Outcome in Soft Tissue Sarcomas

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Abstract

Background—Cancer stem cells (CSCs) have been shown to resist chemotherapy and promote metastasis after cytotoxic therapies. We sought to determine if the expression of CSC markers (ALDH, CD44, and EGFR) predicted outcomes in soft tissue sarcoma (STS) patients.

Methods—We queried an institutional database of 23 STS patients and evaluated immunohistochemical expression of CSC markers ALDH, CD44, and EGFR. The Cancer Genome Atlas (TCGA) was also queried for STS clinical and genomic data. Disease-specific (DSS) and overall survival (OS) were assessed by univariate and Kaplan-Meier analysis.

Results—Of the 23 institutional patients, the majority was female, had high grade tumors, and had extremity tumors. With a median follow up of 27 months, 9 (39%) experienced distant recurrence, and 4 (17%) died of disease. Mean H-scores at diagnosis (±SEM) for CD44, ALDH1, and EGFR were 169±27, 77±15, and 144±23, respectively. On univariate analysis, there was a

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trend for increased CD44 score to predict both worse DSS and OS (HR = 1.01, 95% CI 1-1.02, P=0.056), while ALDH and EGFR scores did not. Analysis of 74 TCGA STS cases with complete clinical and genomic data revealed that CD44 copy number alterations (CNA) predicted worse DSS (9.89 months vs. 72.5 months, P=0.007) and a trend for worse OS (14.03 months vs. 38.6 months, P=0.12), while ALDH1 and EGFR CNA did not. Multivariate analysis of the combined datasets was consistent with worse DSS among patients with higher CD44 expression.

Conclusion—Institutional and national TCGA data show an association of elevated baseline CD44 expression with worse STS outcomes. Further study of CD44 as a possible novel STS biomarker appears indicated.

Keywords

Soft Tissue Sarcoma; Cancer Stem Cells; CD44; ALDH; EGFR; Oncologic Outcomes

Introduction

The cancer stem cell (CSC) hypothesis postulates that a sub-population of quiescent cells exist within tumors which are resistant to conventional cytotoxic/anti-proliferative therapies.[1–3] It is these CSCs which then seed tumor relapse, even in cases of apparent complete response to conventional cytotoxic treatments such as chemotherapy and radiotherapy (RT).[4–6] Cell surface markers (such as CD24, CD44, and CD133) as well as the intracellular enzyme aldehyde dehydrogenase (ALDH), have been used to identify CSC sub-populations in pre-clinical and clinical models, and CSC subpopulations have been identified in nearly all human malignancies.[7–9] Moreover, complex molecular and genetic analyses, including lineage tracing in stem-cell transfected reporter mice, have provided high-level/high impact evidence that CSCs exist and recapitulate key aspects of stem cell biology, including the capacity for self-renewal, differentiation, and homeostatic control.[4, 10–12] As a result of these studies in squamous cell carcinoma, intestinal adenomas, glioblastoma multiforme, and gastric cancer (among others), a growing body of work is focused on the clinical and biological importance of these cells, including efforts to preferentially target these cells to improve outcomes.[13, 14]

CSC markers – including the cell surface markers CD44 and epidermal growth factor receptor (EGFR) and ALDH – have been analyzed in diverse models.[1, 8, 15, 16] Despite observed variations in the expression of CSC markers across tumors and the models evaluated, these markers have been consistently identified as characteristic of stem-like cells in breast cancer, prostate cancer, and numerous other solid tumors, including soft tissue sarcomas (STS).[15, 17, 18] Furthermore, levels of $ALDH^{bright}$ cells as well as other CSC markers such as CD44 and EGFR have been observed to predict worse oncologic outcome across the spectrum of solid and hematological malignancies.[19–24] Additionally, overexpression of these markers has been correlated with the CSC phenotype as well as resistance to chemotherapy, RT, and epithelial-to-mesenchymal (EMT) transition.[1, 25]

Classically, validation of the CSC phenotype is predicated on functional assessment of stem-like tumor repopulation/tumor initiation behavior, including tumor xenograft formation in immunosuppressed mice, colony outgrowth in non-adherent cell culture conditions, or

spheroid formation.[1] In both pre-clinical models and primary soft tissue sarcoma (STS) specimens, we have previously shown that anti-proliferative therapies such as RT enrich for tumorigenic sarcoma CSCs, in particular ALDH^{bright} CSCs.[26–28] Although we were unable to directly evaluate for CSC behavior or function in archived STS specimens, in this study, we sought to determine if we could detect expression of CSC markers in clinical STS specimens and whether expression of these CSC markers predicted oncologic outcomes in patients with primary STS undergoing treatment with curative intent.

Methods and Materials

Evaluation of Archived Clinical Sarcoma Samples

This study was approved by the Institutional Review Board. Since it was considered no more than minimal risk, a waiver of consent was obtained. We then identified 23 patients from July 2011 to January 2015 from a prospectively-maintained cancer center database who underwent initial diagnostic biopsy at our institution followed by multi-modality therapy (including surgical resection) with curative intent and who had sufficient formalin-fixed, paraffin-embedded archived tumor tissue available for construction of a tissue microarray (TMA).

We then abstracted clinical, pathologic, and treatment data, including age, gender, tumor location, stage at presentation, histologic type, maximal tumor diameter, histologic grade, and tumor depth. Tumor size was analyzed as a continuous variable using maximal tumor dimension from initial pathological evaluation. Tumor sites included extremity (upper at or distal to the shoulder/axilla, and lower at or distal to the buttock/groin) and trunk. Retroperitoneal and visceral tumors were excluded. Histologic grade was classified using a three-tiered system (grade I through III) according to established criteria.[29]

Histologic diagnosis was assigned by the published criteria of the World Health Organization Classification of Tumors of Soft Tissue and Bone.[29] For purposes of statistical analysis, we limited our analysis to four histology categories, including liposarcoma, leiomyosarcoma, undifferentiated pleomorphic sarcoma (UPS), and “other” which represented a composite of synovial sarcoma, extraskeletal myxoid chondrosarcoma, solitary fibrous tumor, angiosarcoma, fibromyxoid sarcoma, clear cell sarcoma, epithelioid sarcoma, primitive neuroectodermal tumor, and sarcoma, NOS.

The date of recurrent disease was defined either by biopsy or by the radiologic detection of suspicious lesions when no biopsy was performed. Follow-up was counted from the date of diagnosis until the date of death or date of last follow-up. Patients who were free from recurrence or death were censored according to the date of their last follow-up. Distant-recurrence free (DRFS), disease-specific (DSS), and overall survival (OS) were calculated as described previously.[30, 31]

After antigen retrieval and blocking, TMA sections (4 μm) were immunostained using a commercially-available purified mouse anti-human ALDH1 antibody (BD Transduction Laboratories, San Jose, CA) as described previously (including SSO abstract, Boston, 2016).[26, 28] The specimens were also stained for CD44 and EGFR expression using a

monoclonal mouse anti-human CD44 (Clone DF1485, Dako North America, Carpinteria, CA) and a monoclonal mouse anti-human EGFR (Clone H11), respectively. A pathologist (M.C.) who was blinded to the clinical outcome reviewed the stained slides and scored each for the percentage and intensity of immunohistochemistry (IHC)-positive cells. An H-score was then calculated for each slide by multiplying the percentage of cells staining positive by the staining intensity.[32]

Querying the Cancer Genome Atlas Soft Tissue Sarcoma Data

Using the Data Matrix from The Cancer Genome Atlas (TCGA) website (https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm), we downloaded clinical outcome data from the TCGA provisional soft tissue sarcoma dataset. These data were downloaded on July 13, 2015. CSC biomarker expression data for CD44, ALDH1, and EGFR were downloaded from the Computational Biology Center at Memorial Sloan-Kettering cBioPortal website (http://www.cbioportal.org/). All cBioPortal data were downloaded on July 17, 2015.

Using the TCGA barcodes, we then matched the TCGA clinical and genomic data for individual patients to create a database consisting of complex clinical outcome and genomic information for 74 STS patients. Additionally, Kaplan-Meier analyses for disease-free survival (DFS) and OS conducted by cBioPortal were downloaded and analyzed.

Statistical Considerations

Summary statistics were reported as mean ± standard error with median (range) where appropriate. Categorical variables were compared using a chi-squared test. Univariable Cox proportional hazards regression model was used to study the relationship between the event outcome variables (DRFS, DSS, and OS) and the predictor variables, ALDH1, CD44, and EGFR.[33] Multivariable Cox proportional hazards regression model was then used to study the relationship between the outcome variables and the three CSC markers.[33]

Results

Clinicopathologic Characteristics of Mono-Institutional and TCGA Cohorts

Of the 23 STS patients in the mono-institutional cohort, the median age was 53 years old, 16 (70%) were female, 18 (78%) were high grade, 12 (52%) were extremity, and 7 (30%) were retroperitoneal (Table 1). Four (18%) were myxofibrosarcomas, 4 (18%) were undifferentiated pleiomorphic sarcomas, and 8 (34%) were liposarcomas. Twenty-one tumors (91%) were deep-seated, and 18 patients (82%) underwent R0 resection. With a median follow up of 27 months, 9 (39%) experienced distant recurrence, four (17%) died of disease, and 8 (35%) were alive with disease (Table 1). At time of diagnosis/pre-treatment biopsy, the mean H-scores (±SEM) quantitating the expression of CSC markers CD44, ALDH1, and EGFR were 169±27, 77±15, and 144±23, respectively (Table 2). Representative photomicrographs of IHC staining for the various CSC markers are shown in Figure 1.

We also abstracted data on 74 STS patients in the TCGA database. Among these patients, 35 (47%) were female, and the median age at diagnosis was 61. Histologic sub-types included

36 (48%) leiomyosarcoma, 21 (28%) dedifferentiated liposarcoma, 8 (11%) undifferentiated pleiomorphic sarcoma, and 5 (7%) myxofibrosarcoma (Table 3). Among the TCGA cohort, 48 (65%) experienced a distant recurrence. At time of last documented follow-up, 27 (36%) died of disease and 28 (38%) were alive with disease. Using Z-scores to analyze changes in gene expression, the mean Z-scores (±SEM) for CD44, ALDH1, and EGFR in the STS TCGA dataset were −.035±.11, −.098±.12, and .132±.15, respectively (Table 4).

CD44 Expression Predicts Worse Oncologic Outcomes

Univariate analysis of the mono-institutional data revealed a trend for increased CD44 H-score to predict both worse DSS and OS (HR = 1.01, 95% CI 1.00 – 1.02, p=.056). In contrast, univariate analysis failed to show an association of H-score for either ALDH (HR = 0.98, 95% CI 0.95-1.01, p=.286) or EGFR (HR = 1.001, 95% CI 0.98-1.02, p=.876) with worse DSS or OS (Table 5).

As depicted in Figure 2, cBioPortal Kaplan-Meier analyses of TCGA STS data revealed that increased CD44 copy number alterations (CNA) predicted worse DFS (9.89 months vs. 72.5 months, p =.007). CNA for ALDH and EGFR was not associated with worse DFS or OS. In our institutional data, as shown in Figure 3, CD44 overexpression was associated with worse OS when comparing the sub-groups with the highest and lowest quartiles of expression (P=0.04), while there was no statistical difference in DSS or OS among patients when stratified by ALDH or EGFR expression. Multivariate analysis of pooled data from the monoinstitutional and TCGA cohorts, respectively, revealed that both CD44 high expression and STS type were significant predictors of worse DSS ($P < 0.05$).

Discussion

Evidence suggests that CSCs are a small group of typically quiescent cells that compromise a minority of cells within the overall tumor bulk.[1, 16] CSCs resist conventional cytotoxic cancer therapies and are able to repopulate tumors after apparent complete response to chemotherapy and/or RT.[4, 5, 34] Furthermore, CSCs seem to be capable of self-renewal and differentiation,[5, 17, 35, 36] which further indicates that they may be responsible for seeding relapse after the bulk tumor has been cyto-reduced by therapies which target proliferating cancer cells/clones.[4]

Previous studies have focused on classifying the behavior and phenotype of CSCs, and attention has been focused on demonstrating that the expression of cell-surface markers like CD44 and the level of activity of the intracellular enzyme ALDH consistently predict the CSC phenotype. CD44 is a cell-surface glycoprotein involved with cell-cell signaling, cell adhesion/migration, and malignant tumor initiation. CD44 also interacts multivalently with hyaluronan resulting in signal pathway activation of tyrosine kinases such as ErbB2, EGFR, TGF-β, and beta-catenin/Wnt, and all of these growth/transcription factors have been implicated in key oncogenesis pathways.[37] Multiple studies have linked CD44 expression with the CSC phenotype and by extension tumor progression and prognosis in multiple malignancies, although data are limited for CD44 in STS.[14, 38–40] In fact, to our knowledge, this is the first study to show an association of CD44 expression with oncologic outcome in STS.

The Cancer Genome Atlas (TCGA) was established by the National Cancer Institute and National Human Genome Research Institute to describe and catalogue the landscape of genetic mutations in human cancers.[41] This collaborative project uses hybrid-capture sequencing technology to sequence the entire genomes of available tumors, including at least 6,000 candidate genes and microRNA sequences. In addition, limited (but annotated) clinical data are available for these specimens in order to provide information on important oncologic outcomes, including survival. Data from TCGA has been the foundation of high impact papers on the genomic characterization of high grade gliomas, breast tumors, colorectal carcinoma, and lung cancer, among others.[42–46]

Overall, ALDH expression has been more closely associated with worse outcomes than other CSC markers in many human cancers, including STS.[7, 17, 19, 23, 47, 48] However, in this study, we demonstrate that CD44, but not ALDH1 or EGFR, predicts worse oncologic outcomes in STS. This is, to our knowledge, one of the first studies investigating *in vivo* the relationship between these known and validated CSC biomarkers and oncologic outcomes for this cancer type. We also believe that this is one of the first studies comparing an institutional TMA comprised of clinical and genomic data for STS to a similarly complex dataset for STS derived from national TCGA data.

One of the strengths of our study is the similar relationship observed between CSC biomarkers and the outcome variables DSS and OS seen in the UC Davis and the TCGA data. This parallelism suggests that CD44 may be a reliable predictor of worse oncologic outcomes in STS and could therefore be a target for novel STS therapies. Additionally, the similarities between the institutional data and the TCGA data highlight the usefulness of this national database in increasing sample size and providing a valuable check for institutionally-derived scientific results.

Despite the fact that we show a statistically meaningful relationship between CD44 and worse oncologic outcomes in STS, we must acknowledge several limitations of our study. The sample size of the institutional dataset was small at only 23 patients. Although we observed similar trends when comparing our institutional results with the TCGA data for the CSC biomarkers in question, there remains a risk that established predictors of survival (such as histologic grade and tumor size) confounded the results. In addition, the pooling of data from the two distinct cohorts to perform multivariate analysis may introduce error or bias into results because of the distinct measurements of CD44 expression between cohorts (immunohistochemical scoring in the monoinstitutional cohort versus gene expression profiling of transcript levels in the TCGA cohort). This is an important caveat for future studies. In addition, it is important to note that CD44 has pleotropic cellular functions, some of which are poorly characterized, and it may have functions independent of CSCs. Since we did not independently validate the CSC behavior of CD44 (or the other markers of interest) in this study, it is possible that the associations observed are unrelated to stem-like behavior of CD44-expressing STS cells.

Finally, although the majority of STS subtypes share a common mesenchymal origin, STS is a heterogeneous group of malignancies. Since we did not study the predictive relationship of CD44, ALDH1, or EGFR for each STS subtype, it is possible that the expression of these

biomarkers and the effect of altered baseline expressions on oncologic outcomes may vary with STS subtype. Finally, we did not measure changes in CSC expression following treatment, and enrichment in CSCs after upfront therapy (such as cytotoxic chemotherapy or radiotherapy) may also provide a biologically relevant assessment of response or resistance to therapy.

Conclusion

In summary, analysis of both institutional IHC and national TCGA data shows a significant effect of elevated baseline CD44 expression, but not ALDH1 or EGFR, on worse oncologic outcomes in STS. These data indicate that CD44 may be a novel prognostic marker of oncologic outcome in STS. Consequently, further study of CD44 targeting in STS appears indicated.

Acknowledgments

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Synopsis

Using both an institutional database and The Cancer Genome Atlas, we analyzed the association of cancer stem cell marker expression (ALDH, CD44, and EGFR) with oncologic outcome in primary STS. We found baseline CD44 expression to predict worse survival in both datasets.

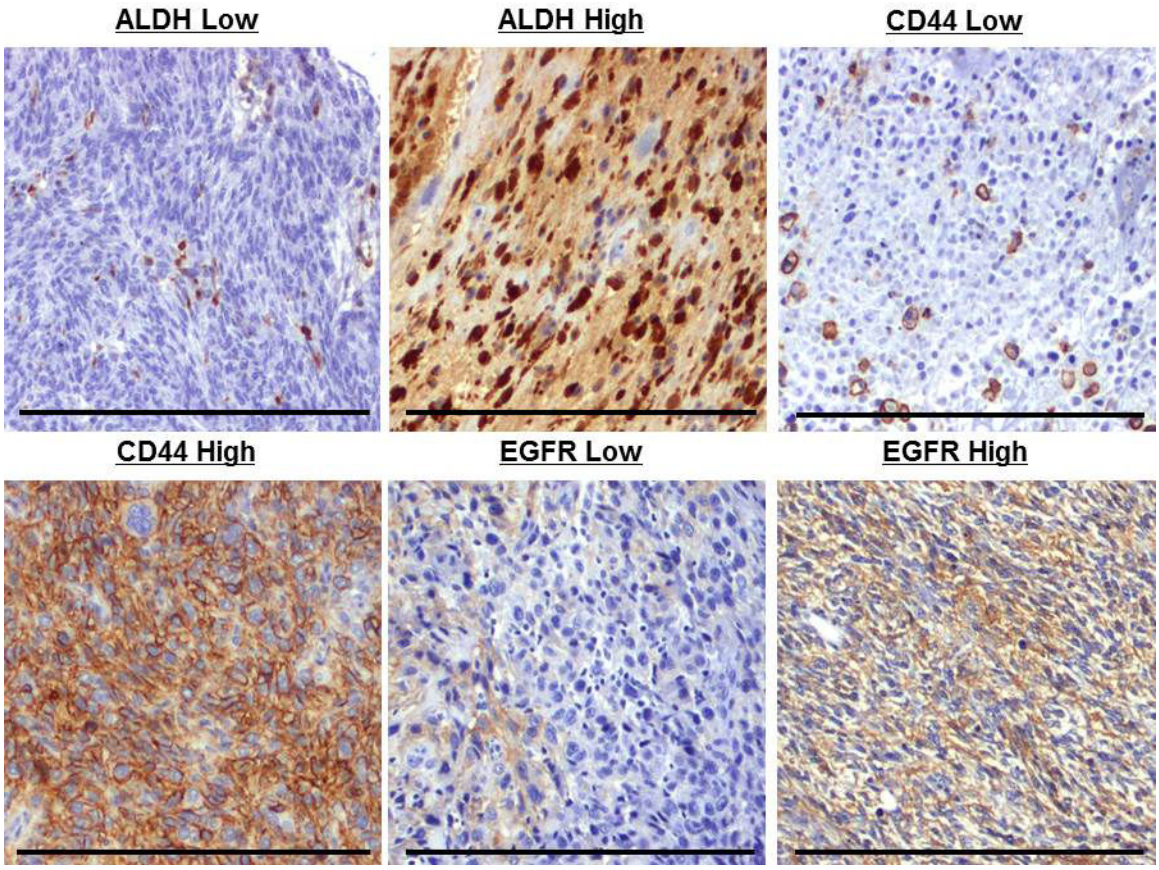

Figure 1. Immunohistochemical Evaluation of Archived Clinical Sarcoma Samples

A tissue microarray (TMA) was created of 23 STS patients using formalin-fixed, paraffin-embedded clinical specimens. After antigen retrieval and blocking, TMA sections (4 μm) were immunostained using commercially-available antibodies for ALDH1, CD44, and EGFR. The stained slides were reviewed in a blinded fashion in order to calculate an H-score by multiplying the percentage of cells staining positive and the staining intensity. Scale bar = 300 μM

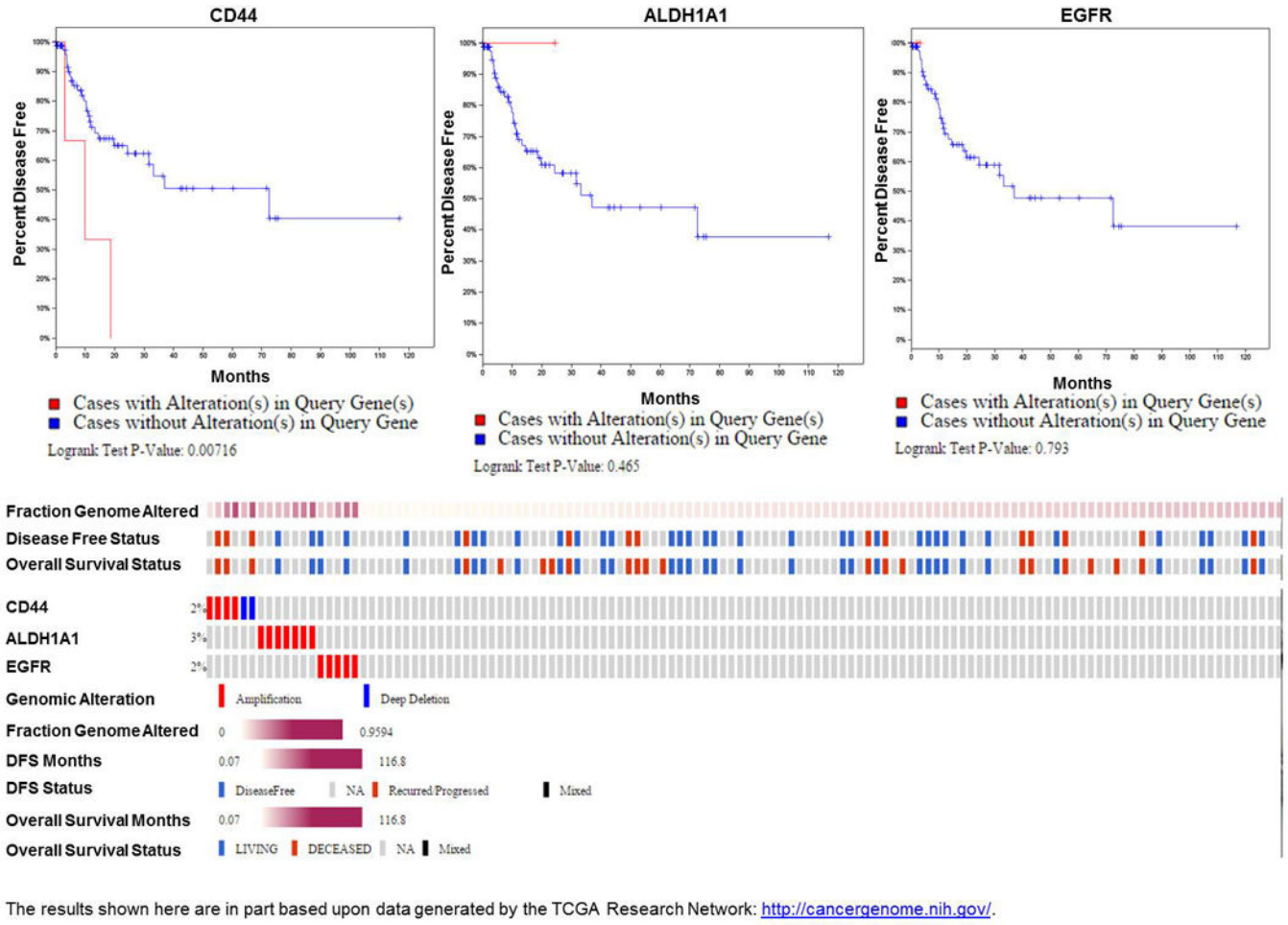

Figure 2. Copy Number Alterations and Disease-Free Survival in TCGA STS Patients

Genomic data from the publicly available TCGA database was analyzed for CSC marker expression and disease-specific survival. CD44 copy number alterations predicted worse DSS (9.89 months vs. 72.5 months, P=0.007) and a trend for worse OS (14.03 months vs. 38.6 months, P=0.12), while ALDH1 and EGFR did not.

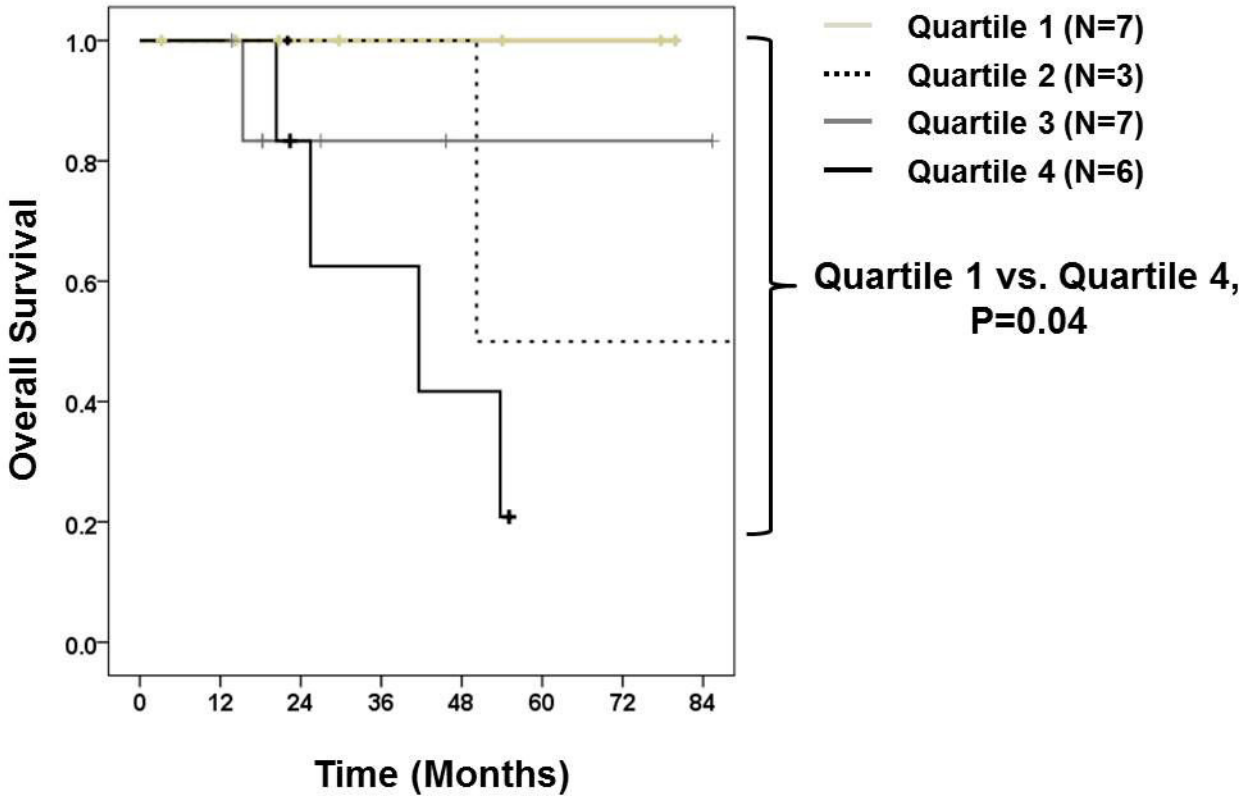

Figure 3. Kaplan-Meier Curve Depicting Overall Survival of Mono-Institutional STS Patients Stratified by CD44 Expression

Patients were divided into quartiles based on statistical distribution of immunohistochemical H-score. Kaplan-Meier analysis was performed. Log rank test with pairwise over strata for quartile 1 vs. quartile 4 is shown.

TABLE 1

Clinicopathologic Characteristics and Vital Status of Soft Tissue Sarcoma Patients in Institutional Cohort

Characteristic		Number (N=23)	%
Gender	Male	7	30
	Female	16	70
Age at Diagnosis, median (range)		53 (22 – 85)	
Site	Extremity	12	52
	Trunk	4	18
	Retroperitoneal	7	30
Histology	Liposarcoma *	8	34
	Myxofibrosarcoma	4	18
	Undifferentiated Pleiomorphic	4	18
	Other **	7	30
Primary Tumor Size	≤ 5 cm	5	21
	5-10 cm	3	13
	> 10 cm	15	65
Grade	Low	4	18
	Intermediate	1	4
	High	18	78
Depth	Deep	21	91
	Superficial	2	9
Margin Status	R0	18	82
	R1	4	18
Status at Last Follow-Up	No evidence of disease	11	48
	Alive with disease	8	35
	Died of disease	4	17

* Includes 5 dedifferentiated liposarcoma, 2 myxoid/round cell liposarcoma, 1 pleomorphic liposarcoma.

** Includes 1 alveolar soft parts sarcoma, 1 extraskeletal mesenchymal chondrosarcoma, 1 leiomyosarcoma, 1 malignant peripheral nerve sheath tumor, 1 extraskeletal osteosarcoma, 1 synovial sarcoma, and 1 primitive neuro-ectodermal tumor.

TABLE 2

Cancer Stem Cell (CSC) Marker Expression and Oncologic Outcome among Soft Tissue Sarcoma Patients in the UC Davis Tissue Microarray

CSC Marker	H-Score
CD44 Expression, mean (±SEM)	169 (±27)
ALDH1 Expression, mean (±SEM)	77 (±15)
EGFR Expression, mean (±SEM)	144 (±23)

TABLE 3

Clinicopathologic Characteristics of TCGA Soft Tissue Sarcoma Cohort

Characteristic		Number (N=74)	%
Gender	Male	39	53
	Female	35	47
Age at Diagnosis	Median (range)	61 (27 – 87)	
Histology	Leiomyosarcoma	36	48
	Dedifferentiated Liposarcoma	21	28
	Undifferentiated Pleiomorphic Sarcoma	8	11
	Myxofibrosarcoma	5	7
	Malignant Peripheral Nerve Sheath Tumor	2	3
	Synovial	1	1
	Unknown	1	1

TABLE 4

Cancer Stem Cell Markers and Oncologic Outcome among TCGA Soft Tissue Sarcoma Patients

Characteristic			%
CD44 Expression	Mean Z-score (±SEM)	−.035 (±.11)	
ALDH1 Expression	Mean Z-score (±SEM)	−.098 (±.12)	
EGFR Expression	Mean Z-score (±SEM)	.132 (±.15)	
Distant Recurrence Free Survival (months)	Median (range)	17.6 (.3 – 126)	
Disease Specific Survival (months)	Median (range)	27.6 (0 -145)	
Overall Survival (months)	Median (range)	27.6 (0 -145)	
Status at Last Follow-Up	No evidence of disease	17	23
	Alive with disease	28	38
	Dead of disease	27	36
	Not Available	3	4

Table 5

Univariable Analysis of Cancer Stem Marker Expression and Disease-Specific Survival *

Marker	HR (95%CI)	P value
ALDH1	0.98 (0.95 – 1.01)	0.29
CD44	1.01 (1.00 – 1.03)	0.056
EGFR	1.00 (0.98 – 1.02)	0.88

* Since all deaths were related to STS, results for disease-specific survival and overall survival were identical. Because of limited sample size (N=23), results of Cox proportional hazards multivariate analysis for DSS and OS were considered unreliable and are, therefore, not reported.