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The Prognostic Significance of Circulating Serum Amyloid A and CXC Chemokine Ligand 4 in Osteosarcoma

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Abstract

BACKGROUND—Osteosarcoma is the most common pediatric bone cancer. Despite advances in treatment regimens, the survival rate remains 60–70%. There is an urgent need to identify prognostic biomarkers, so that targeted therapies can be developed to improve the outcome.

PROCEDURE—Our lab has previously identified that circulating Serum Amyloid A (SAA) and CXC Chemokine Ligand 4 (CXCL4) are upregulated in patients with osteosarcoma. In this study, we tested if they could be used as prognostic biomarkers. We used ELISAs to measure their concentrations in serum samples (n = 233), and immunohistochemistry to examine expressions in

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Conflict of Interest

The authors disclosed no potential conflicts of interest.

primary tumors (n = 37). Prognostic significance of the serum concentrations and tumor expressions of the biomarkers was then evaluated.

RESULTS—Patients with “High SAA” and “Low CXCL4” circulating levels at diagnosis significantly correlated with a worse outcome (HR = 1.68, $p = 0.014$), which was independent of the metastatic status. These patients also exhibited a significantly higher rate of poor histological response to chemotherapy. Furthermore, low tumor expression of CXCL4 correlated with poor survival (HR = 3.57, $p = 0.005$).

CONCLUSIONS—Our results demonstrate that circulating SAA and CXCL4 may serve as prognostic biomarkers in osteosarcoma. Targeting CXCL4 has been reported, suggesting that it may be exploited as a therapeutic target in osteosarcoma.

Keywords

Osteosarcoma; circulating biomarkers; CXCL4; SAA; pediatric cancer

1. Introduction

Primary osteosarcoma (OS) is the most common bone cancer in children and young adults, and most commonly occurs in metaphyseal regions, i.e. growth plates of long bones, whereas OS metastases typically appear in lungs and distant bones [1]. Unfortunately, even with current treatment protocols, the 10-year overall survival rate for all patients is about 60–70% [2]. At the time of diagnosis, approximately 20% of OS patients present with metastatic disease, which is the poorest known prognostic factor [3]. In fact, the only clinically used prognostic factors are metastatic disease at diagnosis and response to pre-operative chemotherapy. Unfortunately, there are no known specific metastatic therapies other than surgical resection, and the histologic response is only measured following neoadjuvant chemotherapy; to date, these two prognostic factors have not translated into better patient outcomes [4]. Furthermore, many patients without detectable metastasis at diagnosis eventually develop metastatic lesions, indicating these patients had either undetectable micrometastases or primary tumors with high metastatic potential. We reason that identification of novel prognostic biomarkers to stratify patients and guide therapeutic options at diagnosis will have a high clinical significance and improve outcomes of children suffering from OS.

Serum Amyloid A (SAA) is a cytokine-induced acute-phase protein produced during tissue injury or inflammation, as well as in response to a variety of diseases including cancer [5]. SAA was proposed as a biomarker for lung cancer [6], and our group previously identified a plasma proteomic signature containing SAA that significantly discriminated OS from benign osteochondroma patients [7]. Similarly, CXC Chemokine Ligand 4 (CXCL4), also known as Platelet Factor 4, participates in inflammation and immune response [8]. We previously reported elevated CXCL4 levels in plasma and tumors of OS patients relative to controls [9], and also found that the “cell adhesion/chemokines and adhesion” pathway is associated with metastasis in OS [10]. In this study, we further examined the prognostic significance of circulating SAA and CXCL4 in a large cohort of OS patients. We showed that a combined model of the two serum proteins at the time of diagnosis correlated with outcomes. Lastly,

we showed that tumor expression of CXCL4, but not SAA, was associated with prognosis in osteosarcoma.

2. Materials and Methods

Patient samples

This study analyzed 233 OS serum samples provided by Children's Oncology Group (COG) that were collected at diagnosis under three collection/treatment protocols (P9851, P9754 and AOST0121) from multiple institutions. The collection/treatment protocol did not significantly correlate with overall survival after correcting for metastatic status, and the date of sample collection (i.e. the length of time since sample collection) was not significantly associated with survival (published elsewhere). For clinical characteristics of all serum samples refer to Supplemental Table S1. Patients were 2 to 31 years old with a median follow-up for patients alive at last contact of 71 months (range: 2–117). Tissue microarrays from Novus Biologicals (Littleton, CO) and COG with survival information were used for tumor expression analysis of SAA and CXCL4, respectively.

Measure

CXCL4 concentrations were determined using the IMUCLONE Platelet Factor 4 ELISA kit (American Diagnostica, Stamford, CT), and SAA concentrations were determined using SAA ELISA Kit (Life Technologies Corp, Grand Island, NY). The experiments were conducted according to manufacturers' instructions. In addition, tumor expressions were measured by immunohistochemistry (IHC) with anti-SAA antibody (SAA, Biosource, Camarillo, CA) and anti-CXCL4 antibody (American Diagnostica, Stamford, Conn) as described previously [7, 9].

Statistical analysis

For circulating biomarkers, survival curves were assessed using Kaplan-Meier analysis, with median concentration serving as a cutoff for conversion into categorical "high" and "low" expression variables. 5-year survival rates and their 95% confidence intervals are included for all Kaplan-Meier curves. Significance was determined by log-rank test with respect to overall and event-free survival, with an event defined as either death or relapse, in univariate and multivariate Cox regression models. The multivariate model was used to correct for metastatic status at diagnosis. For the combined biomarker, patients with high SAA and low CXCL4 by median were combined into a "high-risk" group, whereas the remaining were considered "low-risk", and similar analysis as above were performed. Fisher's exact test was used to determine correlation of the model with response to chemotherapy, where the odds

ratio (OR) is calculated as $\frac{PG_l/(1 - PG_l)}{PG_h/(1 - PG_h)}$, where PG_l and PG_h represent the odds of poor response to chemotherapy for the low-risk and high-risk patients, respectively, testing the null-hypothesis that the OR = 1. P -value < 0.05 was considered to be significant for all statistical tests. For the IHC analysis, the pathologist (J.H.) scored the staining of a tissue microarray based on the proportion of tumor cells displaying positive staining in the core. Each sample was scored on a scale from 0 to 4 where a score of 0 = < 1%, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100% tumor cells stained. The median of the staining

score was used as a binary cutoff, dividing the samples into two groups, namely the “Low” and “High” expression groups. Tumor expression data were blindly scored and analyzed with respect to survival in the same manner as for circulating biomarkers described above.

3. Results

Combined SAA and CXCL4 biomarker is associated with overall survival

To evaluate the prognostic significance of the two previously identified OS biomarkers, we performed specific ELISA assays on a large patient cohort ($n = 233$) from the Children’s Oncology Group. We found that patients with serum concentrations of SAA higher or CXCL4 lower than the median concentration of the respective protein tended toward poorer overall survival, but neither reached statistical significance individually (Supplemental Figures S1A and S1B). Furthermore, serum concentrations of SAA and CXCL4 were poorly correlated to each other in the cohort ($R = -0.07$), suggesting that they may capture distinct prognostic information in the patients (Supplemental Figure S2). Thus, we tested the prognostic significance by combining the information from both SAA and CXCL4, where patients with both high levels of SAA and low levels of CXCL4 were categorized as “High-risk,” and all other patients as “Low-risk.” Analysis showed that the “High-risk” patients had significantly poorer overall survival than the “Low-risk” patients ($HR = 1.68$, $p = 0.014$; Figure 1B). The 5-year overall survival rates for “High-risk” and “Low-risk” patients were 47% (95% CI: 36–62%) and 64% (95% CI: 57–72%), respectively.

Next, we tested if the combined model was an independent prognostic factor from the known prognostic factor at diagnosis, i.e. metastasis at diagnosis (Figure 1A). The result showed that the combined model remained statistically significant after controlling for metastasis in a multivariate Cox Proportional Hazard model ($HR = 1.66$, $p = 0.017$). 5-year overall survival rates for “High-risk” and “Low-risk” groups were 58% (45–76%) and 70% (62–80%), respectively, in patients with no metastasis; and 28% (14–55%) and 51% (39–67%), respectively, in patients with metastasis at diagnosis (Figure 1C). We performed similar analyses with respect to event-free survival; however, the combined model revealed no significant correlations (Supplemental Figure S3).

Correlation of circulating CXCL4 with histologic response

Another commonly used prognostic factor is pre-operative response to chemotherapy or histologic response, which is measured at the time of surgery for local control. Data on histologic response were only available for 109 out of 233 (47%) cases in the patient cohort. Therefore, this subset of the patients was analyzed to determine the correlations between histologic responses and the risk groups defined by the biomarkers. Our results showed that the “High-risk” group was significantly associated with poor response to chemotherapy ($OR = 3.5$, $p = 0.019$, Figure 2A). The proportion of poor responders was much higher in the “High-Risk” group (75%) compared to the “Low-risk” group (46%). Interestingly, when analyzed individually, CXCL4 ($OR = 2.4$, $p = 0.033$, Figure 2B), but not SAA ($OR = 1.1$, $p = 0.85$), was significantly associated with poor response, suggesting that the combined biomarker correlation with chemotherapy response could be mainly attributed to CXCL4.

The “Low CXCL4” group had a higher proportion (65%) of poor responders than the “High CXCL4” group (43%).

Tumor expression of CXCL4

We have previously shown that CXCL4 is expressed in OS tissues using a tissue microarray from a commercial source. Even though 100% of patients had CXCL4 staining in at least 25% of their tumor cells, and 74% had staining in at least 75% of their tumor cells, the prognostic significance of CXCL4 expression has not been determined. To test this, we obtained the survival information of the OS cases and performed Kaplan-Meier analysis using the staining scores. We found that low proportion scores of CXCL4 expression significantly correlated with worse survival (HR = 0.28, $p = 0.005$, Figure 3A). 5-year overall survival rates were 25% (9–67%) and 71% (55–92%) in patients with “Low” and “High” CXCL4 staining, respectively. Tumor expression of CXCL4 remained to be a significant prognostic factor for overall survival after stratifying for metastasis at diagnosis (HR = 0.3, $p = 0.018$, Figure 3B). Furthermore, CXCL4 tumor expression was able to significantly predict survival in the patients with only localized disease at diagnosis (HR = 0.25, $p = 0.006$), indicating that it may be able identify high-risk patients in the population with no detectable metastases using imaging approaches. Statistically significant results were also found in event-free survival analysis (Supplemental Figure S4).

Our results from SAA tissue staining showed that SAA was expressed in the majority (> 50%) of tumor cells in 62% of the OS tumor cases. Tumor expression of SAA was not associated with overall survival in the analysis (Supplemental Figure S5); however, it showed a similar trend of correlation as in circulation (HR = 1.64, $p = 0.29$).

4. Discussion

Clinically, the only prognostic factor used at diagnosis for OS patients is detection of metastasis using imaging approaches, such as X-Ray, Chest CT scan and whole body bone scan. However, due to limitations of the imaging technologies, it is often difficult to detect microscopic or very small metastatic lesions. In fact, 30–50% of localized patients at diagnosis develop recurrent or metastatic disease during or after the treatment [11]. Furthermore, no risk-specific therapy is currently available for OS. Development of a non-invasive and molecular biomarker approach will likely improve the current risk-stratification and provide opportunities for risk-based therapy.

Based on our previous proteomic efforts, we developed a novel risk-stratification strategy for OS patients using serum concentrations of SAA and CXCL4 at diagnosis. SAA is an important clinical marker for acute phase response or inflammation and might play a role in the local inflammation of cancer [5, 12], correlate with tumor burden, and associate with reduced survival in different cancers [6, 12]. A previous study has also shown that SAA is expressed higher in relapsed OS patients [13]. Our result showed that high levels of serum SAA had a trend towards poor prognosis, suggesting that inflammation in OS patients may be associated with a worse outcome.

Previous studies have also correlated CXCL4 with cancer development and progression, and suggest that CXCL4 is a critical anti-tumor factor and its circulating levels are lowered in metastatic prostate cancer [14]. Similarly, lung CXCL4 levels are decreased in the pre-metastatic stage of melanoma and breast cancer animal models, and its levels negatively correlate with tumor stage and positively correlated with cancer patient survival [15]. Nevertheless, a recent study on 50 non-small cell lung cancer patients reported that circulating CXCL4 levels correlate with tumor vascularity, and increased levels in these patients associated with relapse and worse outcome [16]. Similarly, we have previously found that a high level of circulating CXCL4 correlates with poorer survival in a small cohort consisting of 33 OS patients [9]. Given that our previous result contradicted most of the literature, which suggests a correlation between high CXCL4 levels and better patient outcome, in this study we decided to employ a large multi-institutional cohort to better determine the prognostic role of circulating CXCL4 in OS. Our results showed that lower levels of CXCL4 trended towards a worse outcome in OS, but did not reach statistical significance.

Based on our results, we reason that the observed correlation-trend of low CXCL4 circulating levels with poor prognosis could be biologically relevant since CXCR3-B, the cognate receptor of CXCL4, has growth inhibitory function and a potent angiostatic effect [17–21]. Multiple studies also suggest that CXCL4 platelet levels are elevated in early tumor growth, and then fall during tumor progression [22]. In addition, platelet proteomics in a phase 1 clinical trial of VEGFR inhibitor in breast cancer revealed that CXCL4 is significantly higher in pre-treatment samples compared to healthy controls, but its level decreases during subsequent anti-angiogenic treatment cycles [23].

Similar to SAA, CXCL4 is also considered a positive acute-phase reactant, indicating its level can increase during pro-inflammatory conditions. For example, the release of pro-inflammatory cytokines, such as CXCL4, by activated platelets plays an important role in the activation of monocytes during acute vascular injury or chronic disease [24]. Hence, the prognostic values of SAA and CXCL4 in this study may be associated to their roles in tumor burden or inflammation. In the current study, we found that lower CXCL4 levels trended toward a poor outcome, which argues against its prognostic correlation being solely attributed to its role as an inflammation marker.

Nonetheless, the roles of SAA and CXCL4 in the inflammatory processes of OS warrant further investigation. Cancer-related inflammation is considered a hallmark of tumorigenesis, metastasis, angiogenesis, evasion of host immunity, and reduced response to chemotherapeutic agents [25]. Multiple studies also indicate that systemic inflammatory response markers such as C-reactive protein (CRP), albumin, platelet and neutrophil count predict cancer survival in solid tumors [26]. Hence, measures of systemic inflammatory response have been proposed to be a better risk-stratification approach of cancer patients [26]. Consistent with this notion, we have previously reported that higher circulating levels of CXCL10 and IFNG, which are acute phase reactants, predict poor survival in OS patients at diagnosis [27]. Combining the prognostic information of these circulating biomarkers may generate a novel and improved risk stratification method for predicting outcomes in OS patients.

Hence, our combined biomarker strategy suggests that the “High-risk” OS patients may share common properties of angiogenesis and increased inflammation as indicated by “Low” CXCL4 and “High” SAA, respectively. A potential issue of this study is that the combined circulating CXCL4 and SAA biomarkers significantly stratified patients for overall survival, but not for event-free survival. A confounding factor that might produce different results between overall survival and event-free survival analyses is subsequent therapies given following disease relapse [28]. In particular, relapsed osteosarcoma treatment includes several modalities such as surgery and different salvage chemotherapy regimens [29], which may prolong overall survival, but not event-free survival. In this study, three collection/therapeutic protocols were used to collect the blood samples, in which the patients were not treated in an identical manner. Therefore, event-free survival may not be the most appropriate end point in this study, and caution is encouraged to interpret the lack of significance in the event-free survival analysis. Given that overall survival is the gold-standard end-point for survival analysis [28], a biomarker model that predicts overall survival is a valuable tool that warrants further validation.

In this study, we also found that all tumor samples exhibited staining for CXCL4 in at least 25% of tumor cells, whereas patients with staining in at least 75% of their tumor cells had substantially better survival than those with lower staining proportion. However, it is unclear whether OS tumor CXCL4 is derived directly from the tumor cells or other cells in the microenvironment. Our IHC staining results showed that CXCL4 was mainly localized in and around the tumor cells, but the relative contributions of the observed CXCL4 from platelets and the tumor cells cannot be adequately determined by IHC alone. In fact, previous studies suggest that CXCL4 is released locally by platelets in the tumor microenvironment as a feedback response to pro-angiogenic factors [22]. Interestingly, higher levels of CXCL4 derived from tumor-infiltrating platelets can be sustained in the tumor, while insignificant changes are observed in the circulation [30]. Also, circulating CXCL4 can be sequestered by platelets, rendering its level in the serum more variable [30]. This may explain our findings that lower circulating CXCL4 levels were only trending towards a poor outcome, but lower tumor levels significantly correlated with both overall survival and event-free survival, even though the samples size of the tumor study was much smaller than that in the circulating biomarker study. A further study will be needed to investigate if the CXCL4 staining observed in the tumor samples is derived from platelets and/or tumor cells.

Our results are also supported by the competition-based framework proposed by Kareva et al [31] to explain angiogenesis during wound healing and tumor growth. In the model, platelet-associated factors in charge of regulating angiogenesis are released in a specific order from platelet clots corresponding to their affinities to glycosaminoglycans (GAG) in extracellular matrix. Low-affinity VEGF is released at first as an angiogenesis stimulator, followed by medium-affinity growth factors, such as PDGF-B, and subsequently by high-affinity inhibitors, such as CXCL4. Therefore, as the concentrations of angiogenesis stimulators peak, the levels of inhibitors, like CXCL4, increase to bring the angiogenesis process back to a quiescent state [31].

Multiple studies have shown increased levels of low- and medium-affinity platelet factors in the tumor microenvironment [32]. In addition, expression of heparan sulfate is increased in the tumor [33], and its cleavage by heparanase results in extracellular matrix remodeling, which facilitates tumor cell invasion. The competition-based model proposes that increased levels of low-affinity growth factors outcompetes binding sites in heparin sulfate, leading to inability of inhibitors to bind and, hence, increased angiogenesis. In addition, angiogenesis inhibitors, such as CXCL4, take a longer time to be released from platelet clots. This model corroborates our observation that lower tumor CXCL4 levels may result in less inhibition of angiogenesis, which lead to a poor prognosis in OS patients.

Notably, in this study low percentage of tumor expression predicted poor overall and event-free survival in patients presenting with no detectable metastasis at diagnosis. Therefore, the biomarker strategy is able to identify “High-risk” patients within the non-metastatic population, which would otherwise be considered low-risk given the absence of detectable metastasis. This finding may complement the existing imaging approaches used in clinics for detecting high-risk patients or combine with other biomarkers, such as p27 autoantibody, to further improve the prognostic prediction [34].

The fact that low circulating CXCL4 levels were associated with poor chemotherapy response suggests that “Low CXCL4” patients may benefit from targeted treatment with CXCL4 agonists alongside the current standard chemotherapy. In fact, recombinant human CXCL4 has been previously developed for anti-tumor treatment and was well tolerated in Phase I trials for metastatic colorectal cancer [35]. Phase II trials indicate good anti-tumor responses in Kaposi sarcoma [36]. Furthermore, CXCL4-derived molecules exhibiting stronger anti-angiogenic properties and better delivery systems have recently been developed for higher tumor-specificity [37]. Together, CXCL4 may serve as a prognostic biomarker and potential therapeutic target in future clinical trials for OS.

In contrast to CXCL4, tumor expression of SAA showed the same trend as circulating expression, but it did not significantly correlate with outcome in OS patients. These results are consistent with the notion that SAA is a non-specific marker of inflammation and mainly produced by the liver [5]. Therefore, serum levels of SAA may not be heavily influenced by the expression from tumor or tumor microenvironment.

In conclusion, this study reports on the prognostic significance of circulating SAA and CXCL4, as well as tumor expression of CXCL4, in OS. Given the retrospective nature of this study and the use of different collection/treatment protocols, the prognostic value of SAA and CXCL4 will need to be validated in a prospective biomarker study. We believe our findings have high potential clinical impact as combined circulating SAA and CXCL4 may serve as a non-invasive prognostic biomarker. In addition, the role of CXCL4 in the tumor microenvironment as an anti-angiogenic agent is an active area of research. Our results showed that CXCL4 tumor expression can significantly risk-stratify OS patients, which is consistent with reports in other solid tumors. Thus, CXCL4 is a candidate for future clinical trials of novel targeted therapy, wherein recombinant CXCL4 or its derivatives can be used to treat patients with low endogenous levels of the chemokine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

COG	Children's Oncology Group
CXCL4	CXC Chemokine Ligand 4
IHC	Immunohistochemistry
OR	Odds ratio
OS	Osteosarcoma
SAA	Serum Amyloid A
ELISA	Enzyme-linked immunosorbent assay

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Figure 1. Kaplan–Meier analysis of overall survival in OS serum samples

(A) Patient survival with respect to metastatic status at diagnosis. (B) Survival of “High-risk” cohort consisting of patients with “High SAA” and “Low CXCL4 levels”, compared to “Low-risk” cohort consisting of all others. (C) “High” and “Low-risk” patients stratified by metastatic status at diagnosis. P values correspond to the log rank-test p-value. “Met” and “Non-Met” refer to patients with and without metastatic disease at initial diagnosis, respectively. “High” and “Low” levels refer to circulating levels either higher or lower than the median concentration for each biomarker. All 5-year survival rates are described in Results.

A Response to Chemotherapy

Response	SAA High & CXCL4 Low		Totals
	No	Yes	
Good	46	6	52
Poor	39	18	57
Totals	85	24	109

Odds Ratio = 3.5, $p = 0.019$

B Response to Chemotherapy

Response	CXCL4 Low		Totals
	No	Yes	
Good	35	17	52
Poor	26	31	57
Totals	61	48	109

Odds Ratio = 2.4, $p = 0.033$

Figure 2. Correlations between the two biomarkers and response to chemotherapy

(A) A stacked bar graph shows a significant association between the combined “High SAA/Low CXCL4” biomarker and histologic response to chemotherapy in all osteosarcoma patients. The “High-risk” group was significantly enriched with poor responders. (B) A stacked bar graph shows a significant association between “Low CXCL4” and poor chemotherapy response.

Figure 3. Kaplan–Meier analysis of CXCL4 tumor expression

(A) Low tumor CXCL4 expression significantly correlated with poor overall survival in osteosarcoma patients. (B) Tumor CXCL4 expression significantly correlated with overall survival after stratification by metastatic status. “CXCL4 –” refers to low expression (i.e. percentage of stained cells < median), and “CXCL4 +” refers to high expression (i.e. percentage of stained cells > median). “Met” and “Non-Met” refer to patients with and without metastatic disease at initial diagnosis, respectively. (C) Representative immunohistochemistry results of high CXCL4 expression on the OS tissue array are shown.