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Decreased Local and Systemic Levels of sFRP3 protein in Osteosarcoma Patients

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Abstract

Osteosarcoma is a malignant bone tumor that occurs mainly in children and adolescents. Because Wnt signaling has been implicated in the pathogenesis of osteosarcoma, we have investigated the circulating and local levels of the Wnt antagonist protein, Secreted Frizzled Related Protein (sFRP)3, in osteosarcoma patients. Enzyme linked immunosorbent assay (ELISA) analysis of 67 osteosarcoma and age-matched non-diseased control sera showed that sFPR3 protein levels were significantly lower in osteosarcoma than in normal. Analysis of tumor and adjacent normal tissues (9 pairs) from osteosarcoma patients showed a decrease in sFRP3 expression in 5 out of 9 tumor samples compared to normal tissues. Furthermore, immunohistochemical analysis of tissue microarray revealed a significant decrease in sFRP3 levels in tumor compared to normal bone. RNA sequencing analysis in osteosarcoma cells shows suppression of sFRP3 and concomitant expression of multiple Wnt family members mediating canonical or non-canonical Wnt signaling. Taken together, our findings show that the systemic and local levels of sFRP3 protein are downregulated in osteosarcoma and sFRP3 levels could be explored further in the diagnosis and the care of osteosarcoma patients.

Keywords

Frzb; sFRP3; Wnt; Osteosarcoma

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1. Introduction

Osteosarcoma is a primary bone malignancy that affects predominantly children and young adults. The standard treatment for osteosarcoma involves a combination of surgery and chemotherapy (Arndt et al., 2012). Despite this treatment, one-third of the patients diagnosed with osteosarcoma will develop metastatic diseases (O'Reilly et al., 1996). Therefore, there is a critical need to define prognostic biomarkers with predictive potential for osteosarcoma progression.

The canonical and non-canonical Wnt signaling pathways play key roles in cell differentiation, survival, stem cell self-renewal and the homeostasis of many tissues (Logan and Nusse, 2004; Clevers, 2006; Monroe et al., 2012). The canonical pathway is mediated by β-catenin and activation of T-cell factor (TCF)/lymphoid enhancer fator-1(LEF) transcription pathways, while the non-canonical pathways involve planar-cell polarity (PCP-like pathway) and the Wnt/Ca2+ pathway. The Wnt pathway is a key regulator of bone formation and bone remodeling (Monroe et al., 2012). Activation of Wnt signaling has been implicated in many malignant diseases. A deregulated canonical Wnt pathway has been demonstrated in osteosarcoma and several other cancers (Morin et al., 1997; Fujie et al., 2001; Woo et al., 2001; Polakis, 2007; MacDonald et al., 2009). In addition, some reports show that non-canonical Wnt pathway is involved in certain malignancies (Jessen, 2009). Suppression of the Wnt pathway as a potential treatment approach has been explored in many tumors and Wnt antagonists have been studied for their anti-tumor effects (Suzuki et al., 2004; He et al., 2005; Guo et al., 2008; Chen et al., 2010; Saraswati et al., 2012). The Wnt antagonist sFRP3, also called Frizzled-related protein (FRZB), acts as a tumor suppressor in osteosarcoma and other cancers (Zi et al., 2005; Mandal et al., 2007; Guo et al., 2008). The sFRPs are secreted by osteocytes and inhibit the Wnt pathway as antagonistic decoy receptors. Because they share a high degree of similarity with the seven-transmembrane domain-spanning frizzled receptors, they are capable of sequestering agonistic Wnt glycoproteins thus preventing activation of Wnt signaling.

Our group and others have investigated examined pharmacological strategies (Benedikt et al., 2010; Maran et al., 2013; Yang et al., 2013; Bravo et al., 2017; Gustafson et al., 2017; Mamo et al., 2017) diagnostic markers (Pereira et al., 2009; Riester et al., 2017)and molecular mechanisms (van der Deen et al., 2012; van der Deen et al., 2013; Vega et al., 2017) linked to osteosarcoma formation and metastasis. These studies addressed the interplay between Wnt-signaling, transcriptional control and microRNA mediated regulation of gene expression in osteosarcoma. However, there are no reliable serum tumor markers for early diagnosis and prediction of metastasis. In this study, we assessed the potential of sFRP3/FRZB protein as a potential prognostic marker for osteosarcoma by investigating the levels of sFRP3 protein in normal and osteosarcoma tissue specimens.

2. Materials and Methods

Osteosarcoma sample collection

The serum samples were obtained through a study protocol approved by the institutional review board (IRB). To quantify the sFRP3 levels in human serum, we have analyzed

samples from 134 patients (67 osteosarcoma patients + 67 sex and age-matched, non-diseased controls) and clinical data from the medical records was correlated with experimental results. Baseline demographic and characteristics are shown in Table 1. Samples from thirty-nine male and twenty-eight females (Age range 8–75 years; Mean: 30 years; Median: 23 years) patients were analyzed, and six patients had low grade tumors, while sixty-one patients had high grade tumors (Table 1). Also, 45 patients had metastatic diseases and 22 had only local disease.

Osteosarcoma tissues and adjacent normal tissues were obtained by surgical resection through Mayo Clinic Institute Review Board (IRB)-approved protocol. Prior to use, the histological diagnosis of the tissues was confirmed by certified musculoskeletal pathologists at Mayo Clinic.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA analysis was performed to estimate sFRP3 levels as described in the manufacturer's protocol (Aviscera Bioscience, Santa Clara, CA).

Immunohistochemical staining of osteosarcoma arrays

Tissue microarrays representing malignant and normal bone were purchased from US Biomax, Inc (Rockville, MD) and analyzed by immunostaining, using anti-sFRP3 (1: 25 dilution), anti-axin2 (1: 50 dilution)and non-immune immunoglobulin (IgG) (1:200 dilution) (Santa Cruz Biotechnology, Dallas, TX). The anti-sFRP3- and anti-axin2-stained tissue arrays were normalized using IgG staining and the quantitation of signals was carried out using BIOQUANT OSTEO Image analysis system (Bioquant Image Analysis Corporation, Nashville, TN). The average densities for sFRP3 and Axin staining were calculated in normal and osteosarcoma tissues. The average density was determined by calculating the intensities of all significant pixels in the object and dividing that value by the number of pixels as described in the manufacturer's protocol (Bioquant Image Analysis Corporation).

Protein isolation and western blot hybridization

Cytoplasmic extracts were prepared by homogenizing the tissues in lysis buffer as described (Wimbauer et al., 2012). The protein concentration was determined by Bradford protein assay, and cytoplasmic extracts containing protein (60 μg) were analyzed by western blot hybridization as previously shown (Benedikt et al., 2010; Wimbauer et al., 2012) using anti-sFRP3 (1:2000 dilution) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5000 dilution) antibodies (Santa Cruz Biotechnology, Dallas, TX). The expression levels of proteins on the western blots were quantified using densitometer and Imagelab software (BioRad, Hercules, CA).

Cell Culture, RNA isolation and sequencing

Osteosarcoma cells (MG63, SAOS and U2OS) were cultured in DMEM/F12 media as described (Bravo et al., 2017). RNA isolation was carried out with the RNeasy mini kit (Qiagen, Germantown, MD) and subsequent RNA sequencing was performed using an established pipeline at the Mayo Clinic RNA sequencing Core facility as described previously (Dudakovic et al., 2014; Paradise et al., 2018)

Statistical Analysis

All values were expressed as means + standard error. Samples were analyzed using a Wilcoxon signed rank test for matched pairs to test the difference of the means, and the likelihood ratio was used to test difference in probabilities of sFRP3 differential regulation. P≤0.05 was considered statistically significant. For tumor patients, a nonparametric Mann-Whitney test was used to analyze intragroup variations.

3. Results

ELISA Analysis of sFRP3 levels in osteosarcoma patients

Serum sFRP3 protein levels were measured by ELISA in osteosarcoma patients (N=67) and age-matched controls (N=67). Our results show that patients with osteosarcoma have significantly decreased levels of sFRP3 compared to normal without the disease ($P \leq 0.005$) (Table.2). Quantitation of sFRP3 concentration revealed a mean concentration of 1715 ± 194 pg/ml and 2290 ± 165 pg/ml, in osteosarcoma and control sera, respectively (Table 2). The control group levels ranged from 731 to 6339 pg/mL with a median value of 1922 pg/ml and the values for osteosarcoma patients ranged from 13 to 6216 pg/mL with a median value of 1237 pg/ml (Table 2). Our analysis shows that sFRP3 protein was down regulated in 67.2% (45 patients), upregulated in 26.8% (18 patients) and remained unchanged in 6% (4 patients) of osteosarcoma patients (Fig.2).

sFRP3 regulation by age, gender, stage and grade in osteosarcoma

When stratified by age, two groups were considered based on the median value (23 years). The groups were: (1) adult ≥23; and (2) young adult/pediatric population <23 (Table. 3). Results showed that osteosarcoma patients had down-regulation in both age groups, but only a statistically significant difference (P≤0.023) was observed in the adult populations when compared to age-matched normal population (Table 3). Stratification of data based on gender points out significant decreases in female osteosarcoma patients but not in male patients (P<0.008), compared to age-matched normal population (Table 3). Patient disease characteristics gathered from our Mayo Clinic serum sample database were compared with differences in sFRP3 levels, and further divided into subcategories based on stage (metastasis vs non-metastasis) and tumor grade (low vs high grade). The results showed a down-regulation of sFRP3 levels in patients with metastatic disease (P≤0.0083) (Table 3), whereas in non-metastatic patients, no significant difference was observed (Table 3). Further a significant difference was noticed (P<0.01) in patients with high grade tumors but not in low grade osteosarcoma when compared to corresponding age-matched control specimens (P:0.006) (Table 3). Thus, this shows that in the osteosarcoma patients sFRP3 levels were significantly lower than in normal group. This difference was statistically significant in several subgroups including in patients older than 23 years, females, metastatic conditions, and high grade osteosarcomas.

Analysis of sFRP3 expression by immunohistochemistry

Tissue microarrays containing osteosarcoma and normal tissues were analyzed for sFRP3 expression. Immunostaining, followed by quantitation of signals, showed decreased staining

in tumor compared to normal tissues (Fig. 3A and 3B). In order to further verify our findings and determine whether downstream Wnt signal is regulated, we have analyzed the expression of axin2 using the tissue microarrays. Our results show that axin2 expression is increased in osteosarcoma tissues compared to the normal bone tissues (Fig.4A and 4B).

Measurement of sFRP3 protein levels by western blot analysis

To further verify above findings, cytoplasmic extracts from osteosarcoma and adjacent normal tissues from patients were analyzed by western blot analysis. Figures 5A and 5B show representative blots from tissues and quantitation of signals from 9 sets of tissues, respectively. The results showed that the osteosarcoma tissue specimens had decreased sFRP3 levels compared to the control samples in 5 out of 9 sets. The sFRP3 protein levels were upregulated in 2 specimens and remained unchanged in 2 specimens (Fig. 5B).

Analysis of sFRP3 and Wnt mRNA levels in osteosarcoma cell lines

To further investigate the impact of sFRP3 downregulation, we examined the gene expression profiles and patterns of Wnt family genes using RNA sequencing in osteosarcoma cells. Our analysis reveal that sFRP3 expression is very low or at undetectable levels in 5 different cell osteosarcoma cell lines (143B, U2OS, MG63, KHOS and SAOS2). In contrast, a number of Wnt family members (e.g., Wnt2B, Wnt3, Wnt4, Wnt5 A, Wnt5b, Wnt6, Wnt7A, Wnt7B, Wnt9A, Wnt10A, Wnt10B and Wnt11) are robustly expressed to different degrees depending on the cell type. Importantly, the results show that Wnt5A and Wnt5B are most consistently expressed in all osteosarcoma cell types examined.

4. Discussion

We show that the sFRP3 proteins levels are significantly decreased in osteosarcoma patients. Using various techniques (ELISA, immunohistochemistry and western blot analysis), we have demonstrated that both systemic and local levels of sFRP3 are decreased in osteosarcoma patients compared to normal. Thus, this study corroborates our previous results on mRNA levels in osteosarcoma (Mandal et al., 2007) indicating that monitoring sFRP3 expression levels could be a valuable approach in the care of osteosarcoma patients.

Establishing a valid diagnostic marker in osteosarcoma can serve many purposes: a) help improve the prognosis for osteosarcoma by early detection; and b) provide molecular targets for developing novel therapies. The markers reported represent an extensive mixture of compounds including carbohydrates, glycoproteins, polyamines, proteins and immunoglobulins. Preclinical and clinical studies have revealed that a few serum proteins are associated with osteosarcoma. Various in vitro, in vivo and patient tissue investigations have identified that the expression of MMP-2, MMP-9 (Foukas et al., 2002), uPA (Clark et al., 2008), CXCR4(Laverdiere et al., 2005), Survivin (Osaka et al., 2006), Ezrin (Park et al., 2006) and RUNX2 (Pereira et al., 2009; van der Deen et al., 2013)are upregulated, and the expression of P53 (Park et al., 2001; Pereira et al., 2009)and Rb (Wadayama et al., 1994; Pereira et al., 2009)are down regulated in osteosarcoma. Our findings show that sFRP3 is down regulated in 67% of the cases studied indicating that sFRP3 may be useful in both diagnosis and monitoring of osteosarcoma.

Stratification of serum data showed a significant decrease in sFRP3 levels in adult patients over 23 years. Also, the current study, revealed a significant decrease in sFRP3 protein expression in females. Earlier reports show that the incidence of osteosarcoma occurs in males more frequently than in females (Gatta et al., 2002; Jessen, 2009). These investigations point out that osteosarcoma could occur in females due to the earlier onset of growth spurt. However, it remains to be determined whether earlier growth spurt directly contributes to down regulation of sFRP3 expression and activation of Wnt signaling that lead to the development of osteosarcoma.

Furthermore, our results show a statistically significant decrease of sFRP3 protein levels in high grade osteosarcomas compared to low and moderate grade osteosarcomas. It is histologically challenging to diagnose low grade osteosarcomas and often will be mistaken for fibrous dysplasia or myositis ossificans. The diagnosis can be facilitated with the use of MDM2 and cyclin-dependent kinase 4 (CDK4) genes which have been identified as candidate markers for low grade osteosarcomas (Zi et al., 2005). Also, co-expression has been specifically associated with low grade tumors that progressed to high-grade osteosarcomas (Yoshida et al., 2012). It is unclear whether similar correlation exists between sFRP3 down regulation and progression to high grade osteosarcomas in low grade tumors. In addition, our data suggest that sFRP3 down regulation is significant in metastatic diseases compared to local diseases. Upregulation of the genes uPA, CXCR4 and Ezrin during osteosarcoma invasion and metastasis have been demonstrated before. Verifying our finding in an animal model would help establish the regulation of sFRP3 in metastasis, could lead to a better understanding of the role of sFRP3 in tumor induction and progression.

In this report, the results from serum studies have been corroborated by immunohistochemistry and quantitation of tissue microarray analysis from 11 osteosarcoma patients. Significant downregulation of sFRP3 protein was observed in osteosarcoma patients compared to normal without the disease. Also, these results have been further confirmed by higher Axin2 levels in osteosarcoma compared to normal indicating increased Wnt signaling in osteosarcoma. In addition, analysis of osteosarcoma tissues and adjacent normal tissues from patients showed a downregulation of sFRP3 in 5 out of 9 osteosarcoma patients (1.5 to 24 fold). This observation supports the serum results, but warrants further studies in a larger cohort of osteosarcoma patients. These results in tissues and serum samples which show downregulation of sFRP3 in majority of patients and upregulation in certain cases support the possibility of a context-dependent dual role for sFRP3 protein in osteosarcoma as observed in certain malignancies (Mii and Taira, 2011; Surana et al., 2014). We have previously demonstrated that sFRP3 transcription is suppressed in osteosarcoma patients (Mandal et al., 2007). Current results further extend these observations and show that sFRP3 protein level is decreased in osteosarcoma patients. Our recent report shows that sFRP3 expression is increased in osteosarcoma cells treated with anti-tumor drugs, and reveals that sFRP3 contributes to tumor suppression (Bravo et al., 2017).

Down regulation of sFRP3 protein levels and its potential function as a tumor suppressor has been studied in cancers other than osteosarcoma. It exhibits anti-tumor activity and reverses epithelial to mesenchymal transition in prostate cancer cells (Zi et al., 2005). Methylation and loss of sFRP3 enhances cell migration and invasion in melanoma (Ekstrom et al., 2011).

Other evidences point out epigenetic silencing and tumor suppressor roles for sFRP3 in medulloblastoma (Kongkham et al., 2010), as well as in colorectal cancer (Qi et al., 2006). The level of sFRP3 protein was found to be high in normal kidney, low in primary renal cancer tissues and high in metastatic renal cancer tissues. The changes in sFRP3 expression levels suggest that this protein may function as a tumor suppressor and oncogene during renal cancer progression.

RNA-seq analysis of osteosarcoma cells reveals that sFRP3 is not expressed at appreciable levels, while there are a number of mRNAs encoding Wnt proteins that are robustly expressed in at least five different osteosarcoma cell lines. The expression of these Wnt proteins provides a paracrine microenvironment for osteosarcoma cells that could be blocked by sFRP3. The mRNA expression profile for all Wnt members indicates that both canonical and non-canonical Wnt signaling pathways may be supported in each of the different osteosarcoma cell types we analyzed. We find that Wnt5A, which activates non-canonical Wnt signaling, is expressed in all cell types examined. The latter is notable because the non-canonical Wnt pathway, controls the planar cell polarity pathway in proliferating osteoblasts in relation to mechanical stimulation (Galea et al., 2013; Galea et al., 2015)The RNA-seq data suggest that sFRP3 may antagonize Wnt signaling through both canonical and non-canonical pathways to control osteosarcoma progression.

Our investigation has limitations and needs to be verified using a larger cohort of serum samples, as well as with specimens covering the primary tumor and adjacent tissues. However, our findings offer valuable indications that local and systemic levels of sFRP3 are downregulated in osteosarcoma and could be useful in monitoring the progression in osteosarcoma. From a mechanistic perspective, the absence of sFRP3/FRZB expression in osteosarcoma cells and elevated expression of both canonical and non-canonical Wnt members suggests that serum levels of sFRP3/FRZB may not only be diagnostic of disease, but also further point towards the importance of Wnt paracrine regulatory events that control proliferation during the disease progression in osteosarcoma.

5. Conclusion

Our results show that systemic and local levels of sFRP3 are decreased in osteosarcoma patients and estimation of circulating sFRP3 protein levels could be explored further in the diagnosis and treatment of osteosarcoma.

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Abbreviations List

sFRP3	Secreted Frizzled Related Protein 3
ELISA	Enzyme linked immunosorbent assay

IgG	immunoglobulin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
CDK4	cyclin-dependent kinase 4

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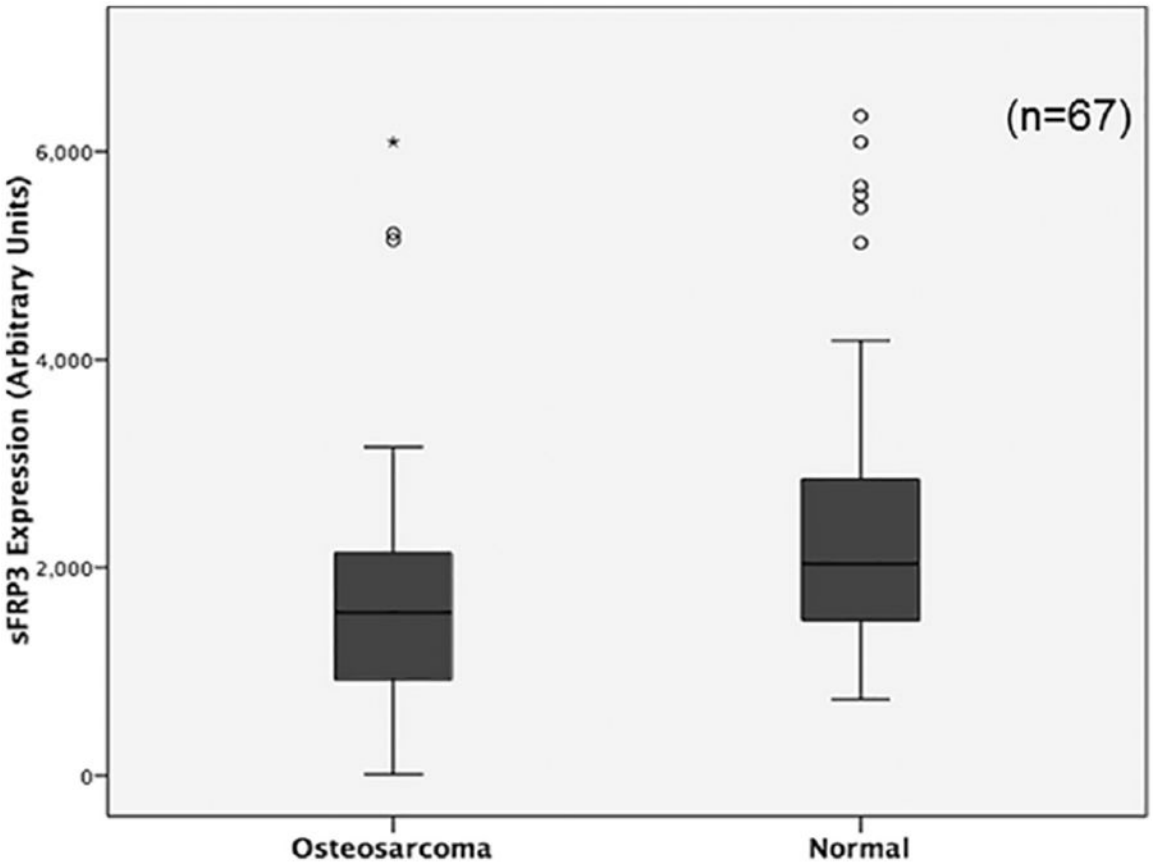

Fig.1.

Analysis of serum sFRP3 levels. Mean sFRP3 levels in the serum of normal and osteosarcoma patients were determined by ELISA. *P≤0.05 *vs* normal

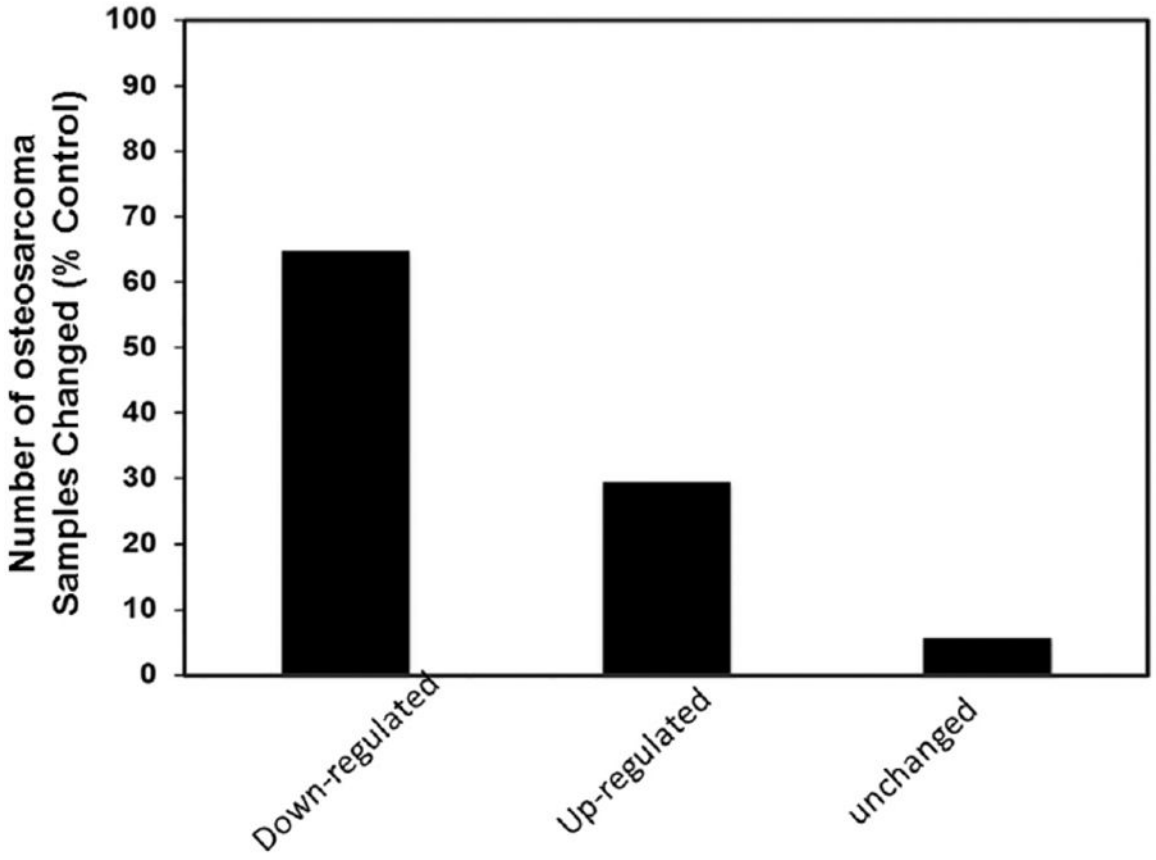

Fig.2.

Percent change in sFRP3 levels in osteosarcoma patients. Serum sFRP3 levels determined by ELISA were compared to age-matched normal samples.

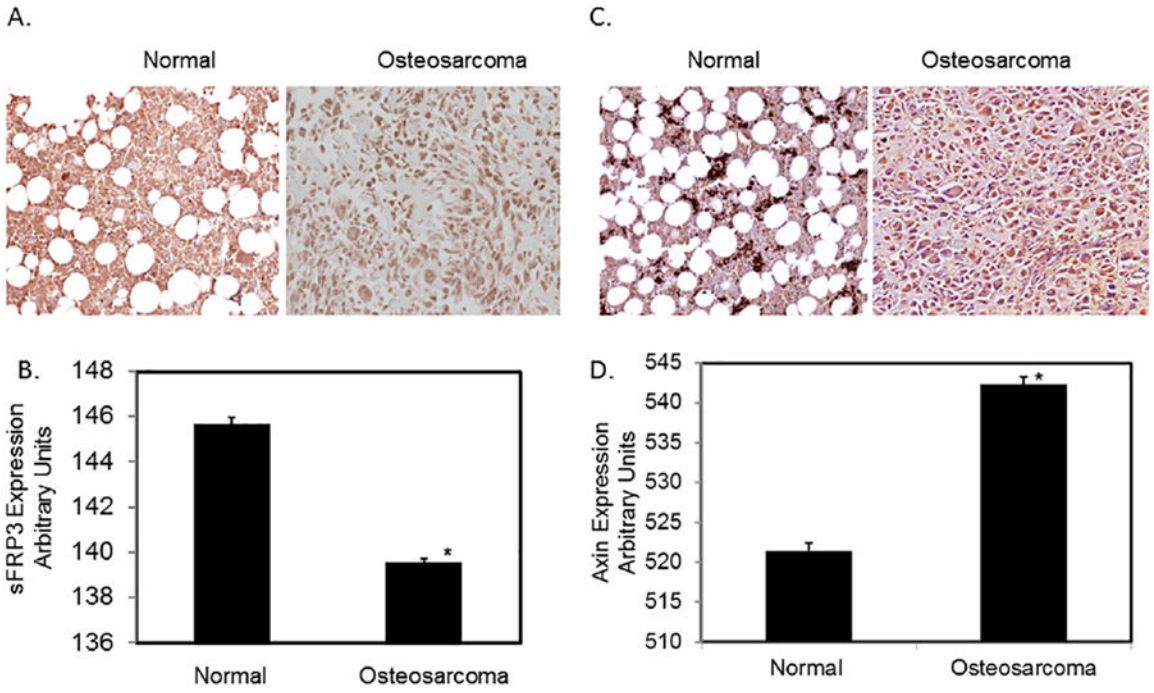

Fig.3.

Tissue microarray analysis of osteosarcoma and normal bone samples.

Tissue arrays were analyzed by immunostaining with anti-sFRP3 and anti-axin2 antibodies and quantitated using digital image software analysis as described in Methods. A & C) Representative images of Normal bone and osteosarcoma samples. B & D) Quantitation of immunostaining signals through Bioquant Image analysis. *P< 0.05 *vs* normal.

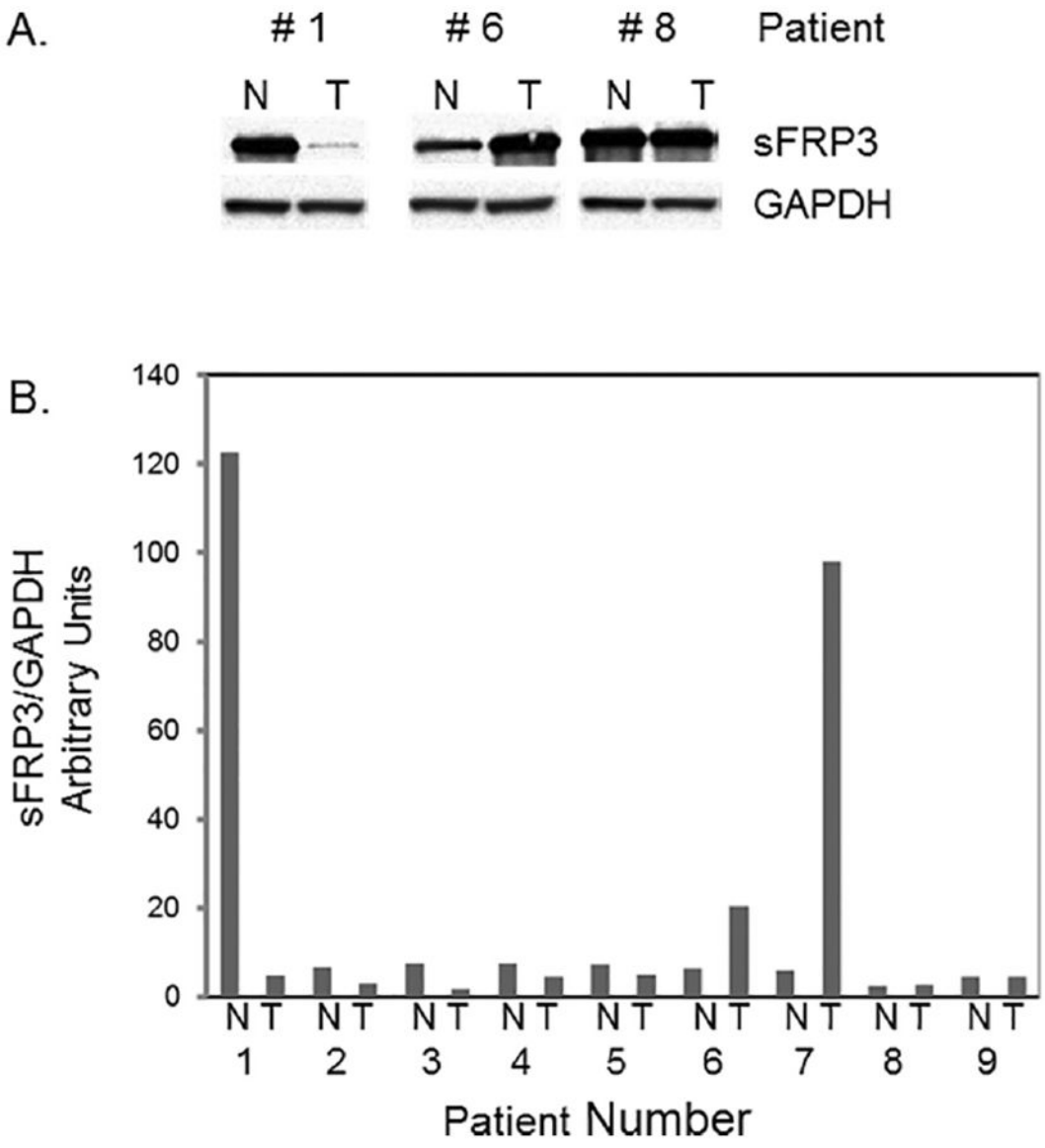

Fig.4.

sFRP3 levels of tissue samples analyzed by western blot. Cytoplasmic extracts prepared from tumor and adjacent normal tissues from patients (9 sets) were analyzed using anti-sFRP3 and anti-GAPDH antibodies and quantitated by densitometry as described in Methods. T,tumor; N, normal. A) Representative blots; B) Quantitation of densitometry signals

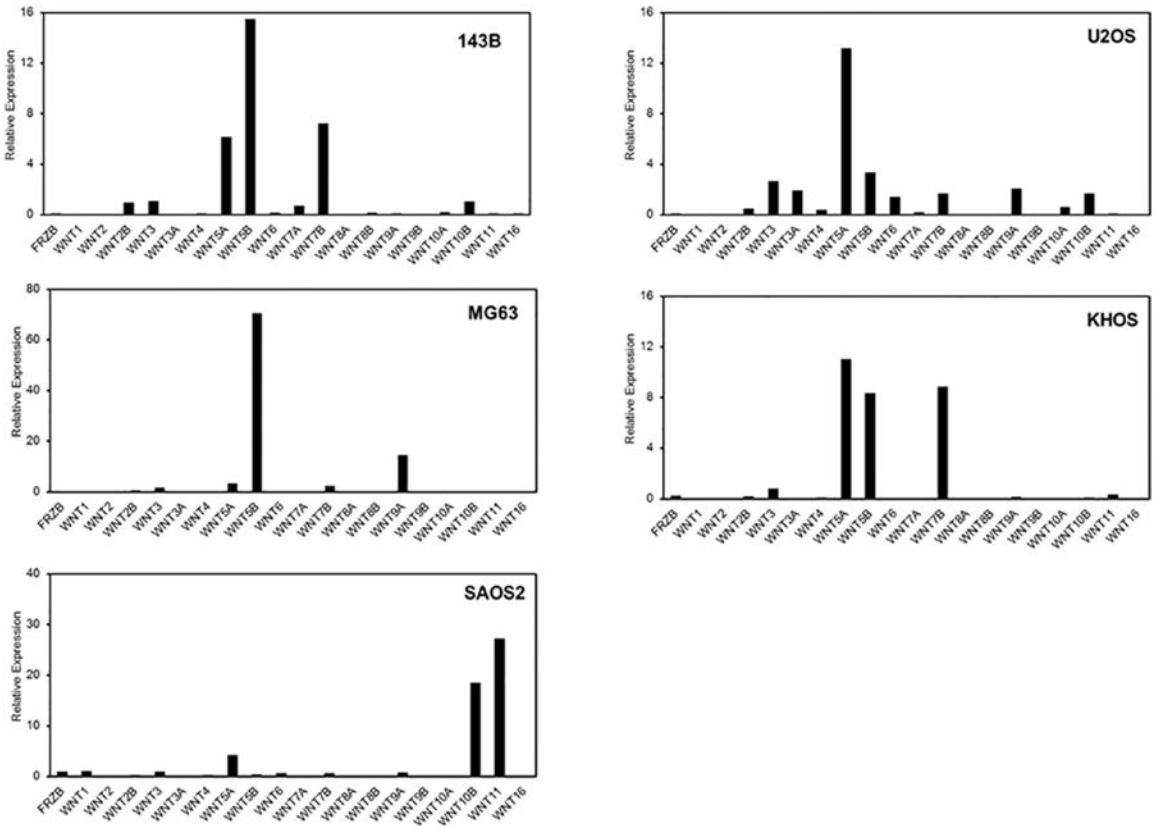

Fig.5.

sFRP3 and Wnt mRNA levels in osteosarcoma cells. Total RNA isolated from 143B, U2OS, MG63, KHOS and SAOS2 cells were analyzed by RNA sequencing as described in Methods.

Table 1.

Details on tissue samples

Patient Information	Number (%)
Number of patients	
Normal	67
Osteosarcoma	67
Age	
Mean	30
Median	21
Range	8–75
Gender	
Female	28 **(42)**
Male	39 **(58)**
Grade	
Low grade	6 **(9)**
High Grade	61 **(91)**
Metastatic status	
Metastatic	45 **(67)**
Non-metastatic	22 **(33)**

Table 2.

Mean serum sFRP3 levels for normal and osteosarcoma samples.

Variables of SFRP3 levels	Osteosarcoma patients (pg/ml)	Control patients (pg/ml)	P value
Mean ± SE	1715 ± 194	2290 ± 165	0.005 *
Median	1237	1922	
Range	13–6216	730–6339	

* significant in tumor compared to age matched control

Table 3.

Mean serum sFRP3 levels for normal and osteosarcoma patients in different subgroups

Variables of SFRP3 levels	Osteosarcoma patients Mean±SE (pg/ml)	Control patients mean±SE (pg/ml)	P value (Wilcoxon test)
Age			
≤23 age (N: **35/67**)	**1646±292**	**1981±156**	>0.05
>23 age (N: **32/67**)	**1792±255**	**2629±291**	0.007 *
Gender			
Female **(N: 28/67)**	**1790±295**	**2556±281**	0.008 *
Male **(N: 39/67)**	**1662±260**	**2099±196**	>0.05
Stage			
Metastatic **(45/67)**	**1825±248**	**2525±214**	0.008 *
Non-metastatic **(22/67)**	**1492±303**	**1810±219**	>0.05
Grade			
Low & moderate grade **(6/67)**	**1937±730**	**2452±790**	>0.05
High grade **(61/67)**	**1694±202**	**2274±166**	0.003 *

* Significant in tumor compared to age matched control