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Research paper

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.

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-

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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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

2.1. 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.

2.2. 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).

2.3. 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) (Santa Cruz Biotechnology, Dallas, TX), anti-axin2 (1:50 dilution) (Aviva Systems Biology, San Diego, CA) and non-immune immunoglobulin (IgG) (1:200 dilution) (Santa Cruz Biotechnology). 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).

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)

2.4. 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).

2.5. 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).

2.6. 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. $P \leq 0.05$ was considered statistically significant. For tumor patients, a nonparametric Mann-Whitney test was used to analyze intragroup variations (Fig. 1).

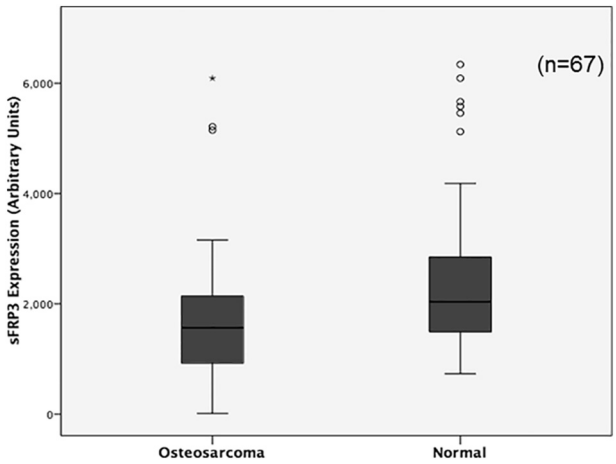

Fig. 1. Analysis of serum sFRP3 levels. Mean sFRP3 levels in the serum of normal and osteosarcoma patients were determined by ELISA. *$P \leq 0.05$ vs normal.