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EXPRESSION OF LDL RECEPTOR-RELATED PROTEIN 5 (LRP5) AS A NOVEL MARKER FOR DISEASE PROGRESSION IN HIGH-GRADE OSTEOSARCOMA

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The Wingless-type (Wnt) family of proteins and its coreceptor LRP5 have recently been implicated in human skeletal development. Wnt pathway modulates cell fate and cell proliferation during embryonic development and carcinogenesis through activation of receptor-mediated signaling. Osteosarcoma (OS) is a bone-forming tumor of mesenchymal origin whose growth control has been linked to autocrine or paracrine stimulation by several growth factor families. We examined 4 OS cell lines for *WNT1, WNT4, WNT5A, WNT7A, WNT11, FZD1-10* and *LRP5* expression by reverse transcription polymerase chain reaction (RT-PCR). In addition, RT-PCR for *LRP5* expression was performed in 44 OS patient samples and the findings were correlated with clinical data. Expression profiling of *Wnts* and their receptors revealed the presence of several isoforms in OS cell lines. Overall, 22/44 (50%) of OS patient samples showed evidence of *LRP5* expression. Presence of *LRP5* correlated significantly with tumor metastasis ($p = 0.005$) and the chondroblastic subtype of OS ($p = 0.045$). In addition, patients whose tumors were positive for *LRP5* showed a trend toward decreased event-free survival ($p = 0.066$). No significant association was found between *LRP5* expression and age, gender, site of disease, site of metastasis or degree of chemotherapy-induced tumor necrosis. Sequencing of exon 3 of *LRP5* in 10 OS patient-derived cell cultures showed no activating mutation of *LRP5*. These results showed that expression of *LRP5* is a common event in OS and strongly suggest a role for LRP and Wnt signaling in the pathobiology and progression of this disease.
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Key words: low-density lipoprotein receptor-related protein; Wnt; osteosarcoma; metastasis; survival

Osteosarcoma (OS), the most common primary malignancy of bone, carries a 5-year survival rate near 60–70% in patients with no clinically detectable metastases at presentation.[1] Others will develop recurrent disease, with the lung being the most common site of distant recurrence. The most powerful predictor of outcome is the histologic response of the tumor to preoperative chemotherapy, graded according to the Huvos system.[2] However, one must wait until surgical resection to assess the histologic response of the tumor to preoperative chemotherapy, which can take 2–3 months. Clearly, there is a need to stratify patients early into low- and high-risk groups, according to their potential for disease progression. Several molecular markers, such as oncogene, tumor suppressor gene, matrix protease and growth factor families, are being investigated as prognostic indicators in this disease. Despite intensive efforts, however, molecular prognostic indicators for disease progression are still few in number.[3–7]

The *WNT* genes encode a family of highly conserved, secreted proteins, modulating cell fate and cell proliferation during embryonic development and carcinogenesis through activation of receptor-mediated signaling pathways.[8–10] Upon Wnt/frizzled interaction at the cell surface, the intracellular enzyme glycogen-synthase-kinase-3 (GSK3) is inactivated. GSK3 and the adenomatous polyposis coli (APC) tumor-suppressor are key factors that promote the degradation of cytoplasmic pool of β-catenin. Inhibition of GSK3 or mutations of APC leads to an accumulation of cytoplasmic β-catenin. Accumulated β-catenin can then translocate into the nucleus where it interacts with transcription factors LEF and T-cell factor (Tcf) to stimulate the expression of Wnt-responsive genes. Important targets of the Wnt-Fz-Tcf pathway include cell cycle regulators and oncogenes such as c-myc, matrilysin and cyclin D1.[11–13] Recent studies have uncovered additional members of this increasingly complex pathway.

OS are tumors of mesenchymal origin whose growth control has been linked to autocrine or paracrine stimulation by several growth factor families.[4,14] However, experimental evidence for the involvement of the Wnt pathway in tumorigenesis of OS is scant. Initially, 2 mammalian genes encoding the Wnt-receptor Frizzled (Fz-1 and Fz-2) were first cloned from the rat osteosarcoma cell line UMR-106.[15] The presence of these receptors provides the first evidence that OS cells have the capacity to transduce either exogenous or endogenous Wnt signals. In human, APC and β-catenin have been demonstrated by immunohistochemical staining in both pre- and post-chemotherapy OS specimens.[16,17] Others have reported sporadic mutations of β-catenin and APC in a variety of bone and soft-tissue sarcomas.[18,19] Whether Wnt signaling plays a bystander or causative role in these diseases is still unknown. Clearly, the role for Wnt signaling in sarcomatous transformation needs to be better defined.

LDL receptor-related proteins (LRP) are cell surface molecules recently identified as Wnt coreceptors.[20,21] LRPs most likely form a complex with the Fz receptors on the cell membrane and together they transduce an intracellular Wnt signal. Mutations of LRP5 that affect Wnt signal transduction have been linked to diseases with significant skeletal manifestations.[22,23] In addition, gain-of-function mutation of LRP5 with resulting increase in bone formation was recently reported in humans.[24] Affected members of this kindred were found to have a mutation in which valine was

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substituted for glycine at codon 171 of LRP5. To explore the potential role of Wnt signaling in OS, a bone-forming malignancy, we focused on *LRP5*, given its important functions in human skeletal development. In addition, we also sought to determine the expression profile of *WNT1, WNT4, WNT5A, WNT7A, WNT11, FZD1-10* and *Frizzled* receptor isoforms in OS cell lines by a reverse transcription polymerase chain reaction (RT-PCR). We hypothesize that LRP5, being a mediator of cellular proliferation,[25] may serve as a potential marker for disease progression in human OS. In our study, we present evidence that expression of *LRP5* mRNA may serve as a candidate marker for disease progression with a significant linkage to the metastatic potential of human OS. In addition, expression analysis revealed the presence of multiple *WNT* and *Frizzled* isoforms in OS cells. Interestingly, activating mutation of *LRP5* as reported by Boyden *et al.*[24] was not detected in any of the samples examined. Taken together, these results suggest a role for Wnt signaling and specifically LRP5 in the pathobiology of human sarcomas.

MATERIAL AND METHODS

Cell lines and tumor samples

Human OS cell lines (U2OS, HOS, 143B and Saos-2) were obtained from the American Type Culture Collection (ATCC) and maintained in MEM-α that was supplemented with 20% fetal bovine serum (FBS) and antibiotics. Tissue samples from OS patients were collected with written, informed consent according to a protocol approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center (MSKCC). All samples were from biopsy or definitive resection specimens obtained from surgical procedures performed at MSKCC. Forty-four patients with the histologic diagnosis of high-grade OS were included in this study. The histologic response following neoadjuvant chemotherapy was determined according to the Huvos grading system as described previously[2] (Grade 1: 0–50% tumor necrosis; Grade 2: 51–90%; Grade 3: 91–99% and Grade 4: no viable tumor or 100% necrosis). Each case was categorized as either a good responder (grades 3 and 4) or a standard responder (grades 1 and 2). These samples represented various histologic subtypes and included both primary ($n = 34$) as well as metastatic tumors ($n = 10$).

RNA isolation and RT-PCR

Fresh tumor samples were snap-frozen in RNAzol and stored at −80°C until processed. Total RNA was isolated from homogenized tumor tissue using the Ultraspec kit (Biotex, Houston, TX) according to the manufacturer's protocol. Messenger RNA was extracted from ATCC cell lines using a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia, Arlington Heights, IL). Reverse transcription of RNA was performed using the Reverse Transcription System kit (Promega, Madison, WI) with random primers. PCR amplifications of *LRP5* and *WNTs* were performed using the following primer pairs: *LRP5* - 5′-CCCGTCACAGG-TACATGTACT-3′(sense) and 5′-GAACGAGCCGTCCAGGTT-3′(antisense); *WNT1* - 5′-CACGACCTCGTCTAC TTCGAG-3′(sense) and 5′-ACAGACACTCGTGCAGTACGC-3′(antisense); *WNT4* - 5′-GCTGGAACTGCTCCACACTCG-3′(sense) and 5′-CCCGCATGTGTGTCAGGATGG-3′(antisense); *WNT5A* - 5′-ACTGCGTGCGCAATGAGAGC-3′(sense) and 5′-ACTACTTGC-ACACAAACTGG-3′(antisense); *WNT7A* - 5′-GTGCGTGCCAGC-CGCAACAA-3′(sense) and 5′-CTGGCCTGGGGAGCCGTCTT-3′ (antisense); *WNT11* - 5′-CGCCGCCTCCGC TCTCC-3′(sense) and 5′-AGCGCCAGCCACTTGATGCC-3′(antisense). These *WNT* members were selected given their involvement in skeletal development. PCR primers for *FZD1-10* were previously described.[26] The *LRP5* primer pair spanned 2 different exons of the *LRP5* gene. Nonreverse transcribed mRNAs isolated from the above cell lines were used as negative controls for PCR. *WNT* and *LRP5* PCR products were confirmed by DNA sequencing to ensure amplification of the correct target genes. Visualization of an ethidium bromide-stained PCR product of the correct size on 1% agarose gel was considered positive for expression; 0.5 μg of reverse-transcribed cDNA was used for each reaction and amplifications were performed on an Eppendorf Mastercycler Gradient thermocycler (Hamburg, Germany) as follows: 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min for 30 cycles. Amplification of *β-actin* controls using primers 5′-CCACTGGCATCGTGA-TGG-3′ (sense) and 5′-GCGGATGTCCACGTCACACT-3′ (antisense) was as follows: 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min for 25 cycles. Expression of *β-actin* was used as a positive control for the PCR reaction as well as a loading control. Samples with no detectable amplification of beta-actin were eliminated from this analysis.

Immunohistochemical analysis of β-catenin

Out of 44 patient samples used in this study, 26 paraffin-embedded specimens were available for immunohistochemical analysis. Four micrometer sections were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was performed using 10 mM sodium citrate (pH 6.0) in a water bath at 95°C for 15 min. Slides were incubated with a rabbit polyclonal anti-β-catenin antibody (Upstate Biotechnology, Waltham, MA) at 1:300 dilution for 12 hr at RT using a humidified chamber. Slides were then incubated with a biotinylated anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution for 1 hr. Staining was visualized with diaminobenzadine using the Vectastain Elite Kit (Vector Lab, Burlingame, CA) according to the manufacturer's instructions. Slides were counterstained with hematoxylin and photographed using a light microscope. A positive control (a section known to exhibit nuclear or cytoplasmic β-catenin staining) was used with each series of specimens. Negative control samples were exposed to rabbit IgG and stained as described. Samples were considered positive if cytoplasmic or nuclear staining was observed. Samples with isolated membrane staining or without staining were considered negative. Stained sections were independently examined by at least 2 investigators (one of whom is a musculoskeletal pathologist) who were blinded to the clinicopathologic data and *LRP5* status of these samples.

Genomic DNA isolation and sequencing

Ten surgical specimens were obtained from 10 different OS patients and primary cell cultures were made by mechanical disaggregation and standard collagenase digestion for 2 hr at 37°C. All isolated cells were cultured in RPMI 1640 supplemented with 10% FBS and antibiotics in a 5% CO_2-humidified incubator at 37°C. All studies were performed on cell cultures within 8 passages. Genomic DNA was extracted from these primary cultures using a Qiagen Dneasy Tissue kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. Radiolabeled sequencing reactions with the following primers were performed: 5′-GATCTGTGTTAGCTGCTTCTCTTG-3′ and 5′-GAACGAG-CCGTCCAGGTT-3′. Amplified PCR products were purified from 1% agarose gel. Sequencing reactions were performed on PCR products using a ThermoSequenase kit (USB, Cleveland, OH) according to the manufacturer's protocol, resolved by 7 M urea polyacrylamide gel electrophoresis, and visualized by autoradiography.

Statistical analysis

Fisher's exact test or χ^2 analysis was performed for categorical variables (gender, site, histologic subtype, histologic response to chemotherapy, presence or absence of metastasis and metastatic site). Survival analysis using the Kaplan-Meier method was performed on samples whose clinical outcome data were available. The log-rank test was used to compare between patients with or without mRNA expression with respect to their event-free survival. Event-free survival (EFS) was defined as the interval from diagnosis to relapse, death or last follow-up. Three patients with metastatic diseases at presentation were not included in our analysis for EFS. Student's t-test was used to compare the means for age. $p < 0.05$ was considered statistically significant. All data was

TABLE I – EXPRESSION OF *LRP5* AND DIFFERENT *WNT* AND *FZ* ISOFORMS IN HUMAN OS CELL LINES[1]

	U2OS	HOS	143B	Saos-2
LRP5	+	+	+	+
Wnt-1	–	+	–	+
Wnt-4	–	+	+	+
Wnt-5a	+	+	+	+
Wnt-7a	+	+	+	+
Wnt-11	–	–	–	–
fz-1	+	+	+	+
fz-2	+	+	+	+
fz-3	–	–	+	–
fz-4	+	+	+	+
fz-5	+	+	+	+
fz-6	–	–	–	–
fz-7	+	+	+	+
fz-8	+	+	+	–
fz-9	+	+	+	+
fz-10	–	–	–	–

[1]Results shown are based on RT-PCR analysis with mRNA isolated from the above cell lines (U2OS, HOS, 143B, and Saos-2). +, Positive expression by visualization of an ethidium bromide-stained PCR product of the correct size. –, No detectable expression. β-actin was used as a positive loading control.

entered and analyzed using a contemporary statistical package (SPSS 10.0, Chicago, IL).

RESULTS

Evaluation of WNT, FZD, and LRP5 expression in OS by RT-PCR

Table I summarizes the expression profiles of *WNT, FZD* and *LRP5* in OS cell lines. Of the *WNT*-related genes, highly transforming *WNT1* was expressed by 2 out of 4 cell lines. Three out of 4 cell lines expressed *WNT4*, while all cell lines expressed weakly transforming *WNT5A* and *7a*. None of these cell lines showed any detectable expression of *WNT11*. Of the receptors, *FZD1*, *FZD2*, *FZD4*, *FZD5*, *FZD9* and *LRP5* were expressed by all, while *FZD8* was expressed by 3 out of 4 cell lines. *FZD3* was only expressed by 143B. These results suggest an autocrine or paracrine mode of proliferation for OS cells *via* the Wnt signaling pathway.

Forty-four patient-derived cDNA samples were available for analysis. These OS samples represent a variety of histologic subtypes and contain both primary as well as metastatic lesions (Table II). PCR results from several representative OS cases are shown in Figure 1. Presence of a single 260 basepair (bp) band on an ethidium bromide-stained agarose gel was considered positive for *LRP5* expression. In 22 out of 44 patients (50%), *LRP5* expression was considered positive by RT-PCR (Table II). These results suggest that the expression of *LRP5* is a relatively common event in human OS. Interestingly, during our attempt to optimize PCR conditions using cell line-derived cDNAs, we were able to show expression of Dickkopf-3/Reduced Expression in Immortalized Cells (*DKK-3/REIC*), an antagonist of LRP and Wnt, in 2 OS cell lines–U2OS and HOS. We then performed a pilot study to examine the expression of this putative tumor suppressor in human OS samples. None of the thirty samples examined showed any detectable expression of *DKK-3/REIC* by RT-PCR (data not shown). This finding suggests that in OS cells, selective down-regulation of this tumor suppressor gene might be a common event.

LRP5 expression correlated with metastatic disease in OS

Patient clinicopathologic characteristics are summarized in Table II. *LRP5* status of each patient sample was correlated with the following clinical variables: age, gender, site, presence or absence of distant metastasis, histologic subtype and histologic response to preoperative chemotherapy (Huvos grade). There was a significant correlation between expression of *LRP5* and the presence of metastatic disease (p = 0.005). *LRP5* expression has a significant

TABLE II – RELATIONSHIP BETWEEN *LRP5* EXPRESSION AND CLINICOPATHOLOGIC DATA

Features	Total	LRP5$^+$ (%)	LRP5$^-$ (%)	p
Total	44	22 (50)	22 (50)	
Age (years)				
Range	3–77	4–77	3–74	
Mean	23.1	21.7	24.3	0.65[2]
Gender				
Male	24 (54.5)	10 (48)	14 (61)	
Female	20 (45.5)	11 (52)	9 (39)	0.55
Site				
Extremity	39 (88)	18 (86)	21 (92)	
Pelvis	3 (7)	2 (9.5)	1 (4)	
Other	2 (5)	1 (4.5)	1 (4)	0.79
Metastasis				
Absent	18 (41)	4 (18)	14 (64)	
Present	26 (59)	18 (82)	8 (36)	0.005
Histological subtype				
Chondroblastic	13 (30)	10 (48)	5 (23)	
Osteoblastic	9 (21)	4 (19)	3 (14)	
Other	21 (49)	7 (33)	14 (64)	0.045
Chemotherapy response[1]				
Good	11 (37)	7 (47)	4 (27)	
Standard	19 (63)	8 (53)	11 (73)	0.45
Follow-up (months)				
Range	5–89	5–89	10–55	
Median	36	37	35	

[1]Chemotherapy response was determined by the Huvos grading system only in available materials from definitive surgery (n = 30).–[2]Student's t-test.

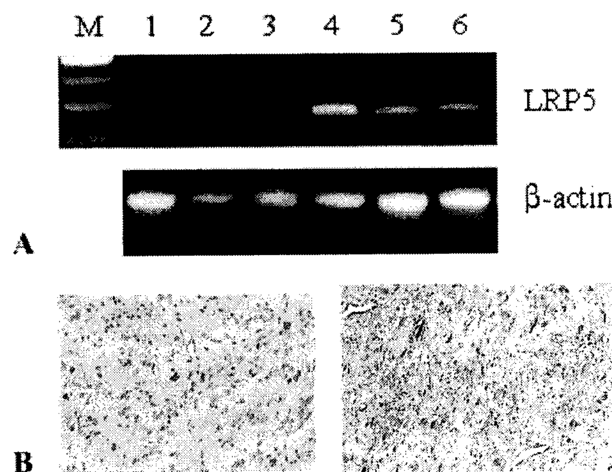

FIGURE 1 – (*a*) A representative panel of 6 different OS patient samples (Lanes 1–6) showing LRP5 expression by RT-PCR. Samples 2, 4, 5 and 6 are positive for LRP5, while samples 1 and 3 show no detectable expression. β-actin was used as a positive loading control. M = 100 bp ladder molecular marker. (*b*) Representative immunohistochemical analysis of β-catenin expression in OS tissue showing a negatively (left) and positively (right) stained samples. Samples were considered positive if cytoplasmic or nuclear staining was observed. Slides were counterstained with hematoxylin and photographed at ×100 magnification.

association with the chondroblastic subtype of OS (p = 0.045). However, tumor histologic subtype also correlated significantly with metastatic disease by χ^2 analysis (p = 0.009), suggesting that the association of *LRP5* with the OS subtype may be related to tumor metastasis. Further analysis revealed a significant association (p = 0.014) between *LRP5* expression in primary OS cases and the eventual development of distant metastases (n = 34)

TABLE III – RELATIONSHIP BETWEEN *LRP5* EXPRESSION IN PRIMARY OS CASES (n = 34) AND THE OCCURRENCE OF DISTANT METASTASIS

	LRP5+	LRP5−	*p*
Metastasis			
Present (n = 16)	11	5	
Absent (n = 18)	4	14	0.014
Metastatic site[1]			
Lung (n = 22)	16	6	
Bone (n = 7)	6	1	
Other (n = 6)	2	4	0.1

[1]Each patient may have more than one metastatic sites.

(Table III). In these primary cases, there is no significant association between the histologic subtype and the development of metastases (p = 0.14), suggesting that *LRP5* status correlated with metastatic disease independent of the histologic subtype. Interestingly, in patients where both biopsy and definitive resection samples were available for analysis, *LRP5* expression was not altered after neoadjuvant chemotherapy (data not shown). Evidently, chemotherapy has little or no effect on suppressing this receptor of the Wnt pathway. There was no significant correlation between *LRP5* expression and histologic necrosis following induction chemotherapy in this group of patients (p = 0.45). In addition, there was no clear association between *LRP5* expression and gender, age, site of disease or site of metastasis (p > 0.1). However, given a large preponderance of extremity OS cases in this cohort of patients (Table II), statistical analysis for site of disease may be of limited power.

LRP5 expression correlated with β-catenin protein expression by immunohistochemistry

Twenty-six patient samples were available for β-catenin immunohistochemical analysis. For each specimen, a histologic diagnosis of OS was confirmed by a musculoskeletal pathologist. Samples were designated as positive if staining was detected in the cytoplasm and/or nucleus. Isolated membrane or no detectable staining was considered negative. Overall, 16 samples showed nuclear or cytoplasmic staining pattern. The remaining 10 samples showed no detectable staining for β-catenin. No isolated membrane staining was observed in any specimen, although membrane signal can be detected in combination with nuclear/cytoplasmic staining. *LRP5* expression by RT-PCR was observed in 14 out of 16 positively stained samples. In contrast, no *LRP5* mRNA was detected in 10 samples with negative β-catenin immunostaining. In 2 remaining specimens, β-catenin staining was detected in the cytoplasm despite negative expression of *LRP5*, suggesting that β-catenin may accumulate in this tumor by a different receptor mechanism. Overall, there is a significant association between *LRP5* status and β-catenin accumulation by Fisher's exact test (P < 0.001).

Correlation of LRP5 expression with clinical outcome in OS patients

We performed survival analysis on 41 patients for whom clinical outcome data were available. None of these patients presented with metastatic disease at initial diagnosis. As shown in Figure 2, univariate analysis revealed a trend toward a lower probability of event-free survival for *LRP5*-positive patients (p = 0.066). No statistical significance was found for patient overall survival (data not shown). Given its association with the development of distant metastasis, *LRP5* expression may be predictive of more aggressive disease and may serve as a marker for disease progression in human OS.

Sequencing analysis of exon 3 of LRP5 revealed no activating mutation

To explore the potential role of LRP5 activating mutation in OS, we isolated genomic DNA from 10 patient-derived cell cultures and performed DNA sequencing analysis of exon 3. Exon 3 was

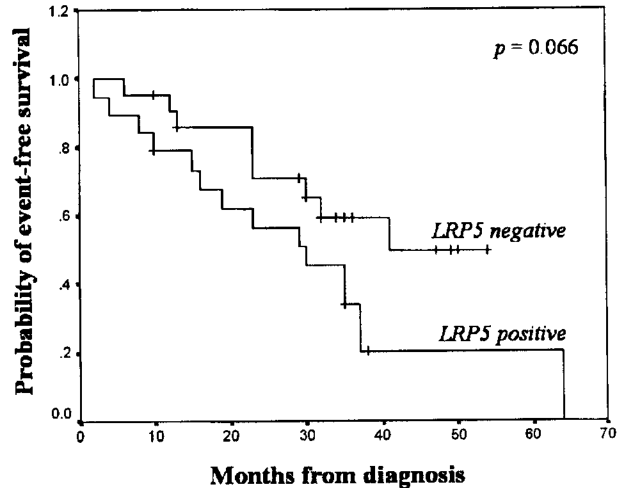

FIGURE 2 – Kaplan-Meier curve of event-free survival for OS patients in the presence or absence of LRP5 expression. Patients with positive LRP5 expression showed a trend toward lower survival probability (p = 0.066).

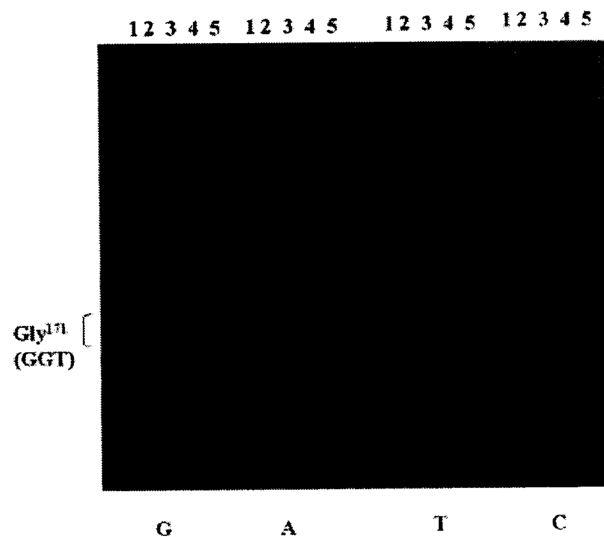

FIGURE 3 – DNA sequencing analyses of exon 3 of LRP5 gene in human OS. Genomic DNA isolated from patient-derived primary cell cultures were utilized for DNA sequencing. Representative panel of DNA sequences from 5 different patients are shown. All samples are from high-grade OS tissues. Site of the codon (GGT = Gly^{171}) previously reported to contain a missense mutation resulting in the amino acid glycine being changed to valine is indicated. Each lane represents one type of base (A, C, G or T) for all 5 patient samples (1–5). This method of sample loading was chosen to facilitate screening for a single base-substituting mutation.

chosen for this analysis because it can harbor an activating missense mutation (Gly^{171} to Val^{171}), resulting in increased osteoblastic proliferation.[24] Genomic DNA was isolated from primary cultures and amplified using oligonucleotide primers flanking the region of the reported mutation. Figure 3 illustrates the genomic DNA sequence of 5 representative patient samples, showing no detectable genetic alterations in the coding sequence of *LRP5* at this site. These results suggest that this particular activating mu-

tation is unlikely to be the cause of cellular proliferation seen in OS.

DISCUSSION

The elucidation of signaling components of the Wnt pathway has influenced our understanding of the pathogenesis of cancer. Activation of this pathway has been linked to an increasing number of human malignancies, exemplified by loss-of-function mutations of the APC tumor-suppressor and gain-of-function mutations of β-catenin in colon cancer and melanomas.[27] However, data on the involvement of this pathway in human sarcomas are still very limited.

Studies in model organisms have established a canonical signaling pathway where Wnt ligand binds to its receptor Frizzled on the cell surface to trigger an intracellular cascade of events that lead to cellular proliferation and in some instances tumorigenesis. Recently, the LDL receptor-related family of proteins (LRP) has been shown to participate in this pathway as coreceptors for Wnt.[20,21] In particular, the interaction between LRP5 and Wnt has been implicated in a variety of human diseases with significant perturbation in skeletal tissue.[22,24] In our study, we present the first clinical evidence for the involvement of LRP5 in a primary bone sarcoma and for its association with metastatic potential of OS. Our results suggest that LRP5 might be involved in OS disease progression as reflected in the tendency for tumors that express this receptor to metastasize.

The involvement of a Wnt receptor in a human malignancy is not without precedent. Up-regulation of the *frizzled*-class (*FZD*) receptors has been linked to a variety of human cancers, including gastric and colorectal cancer.[28,29] Expression level of *FZD7* was much higher in the gastric cancer cell lines and in primary gastric cancer than in normal gastric mucosa, implicating increased activation of Wnt signaling in gastric carcinogenesis.[28] In OS, a similar comparison between normal osteoblasts and sarcomatous cells is difficult to perform due to difficulty in isolating RNA from skeletal tissue and in maintaining normal osteoblasts in primary culture. As a result, we are currently limited in our ability to compare levels of expression of *LRP5* between normal bone and OS tissue obtained from the same cohort of patients.

Down-regulation of tumor suppressor genes confers a growth advantage for a malignancy. Our preliminary data showed that *DKK-3/REIC*, a known Wnt/LRP inhibitor and putative tumor suppressor, was present in 2 OS cell lines but not detectable in any patient samples. This discordance in expression pattern between OS cell lines and patient samples may be related to a difference in methylation status, whereby the *Dkk-3/REIC* gene promoter is more heavily methylated in tumor samples than in OS cell lines. Further investigation is necessary to test this hypothesis. However, one must be cautious in interpreting this as evidence for down-regulation of a tumor suppressor gene since comparison of expression levels between normal bone and OS tissue could not be performed. Nevertheless, our data is consistent with findings of other groups that show down-regulation of this gene in a variety of human malignancies including lung, esophageal and hepatocellular carcinomas.[30,31]

Theoretically, LRP5 when present at a sufficient level may significantly activate the Wnt/Fz/Tcf pathway to up-regulate downstream genes. Among several target genes of this pathway are the metalloproteinases (MMPs).[32] The MMPs have long been implicated in cancer metastasis. Therefore, it is conceivable that LRP5 may regulate tumor metastasis *via* these MMPs. Interestingly, our data from human cell lines confirmed that OS cells indeed express a variety of *WNT* ligand as well as *FZD* and *LRP5* receptor mRNAs. Coexpression of a growth factor and its receptor suggests their role in autocrine or paracrine growth mechanisms. One can envision that LRP5, when present in OS cells, may interact with 1 or multiple Frizzleds to transduce Wnt signal, causing over-proliferation of these cells. Although our study does not provide the molecular evidence for the pro-invasive ability of LRP5, our clinical findings should prompt more in-depth investigations to elucidate the mechanisms by which LRP5 confers a higher metastatic potential for these tumors.

Half of our cases showed detectable *LRP5* expression by RT-PCR. A recent study of β-catenin expression by immunohistochemistry has shown that approximately 70% of OS samples displayed deregulation of this oncoprotein.[17] Since β-catenin is a direct target of the Wnt/Fz/LRP interaction, it is conceivable that the expression of LRP or other upstream components of this pathway induces this abnormal accumulation of β-catenin. However, Haydon *et al.*[17] did not find any association between β-catenin accumulation and survival data in their small group of patients. In our study, there is a significant association between *LRP5* mRNA expression and cytoplasmic or nuclear β-catenin accumulation in OS clinical samples, suggesting that Wnt signaling may contribute to the progression of this disease. It is possible that the number of LRP5-positive cases may vary if detected by immunohistochemistry, depending on the quality of the tumor tissues as well as the immunoreactivity of the antibody. The advantage of our approach is in the sensitivity of RT-PCR to detect very small quantity of RNA and thus represents a relatively accurate assessment of whether a particular transcript is expressed.

In summary, we examined a series of 44 osteosarcomas for the expression of *LDL receptor-related protein 5*. Our results strongly implicate this Wnt coreceptor as a novel marker of disease progression in human OS. Furthermore, our data demonstrate that OS cells express multiple other receptors and ligands of the Wnt pathway. As such, the contribution of Wnt signaling to the pathobiology of OS may be of significance and certainly warrants further investigations. Studies of specific inhibitors of this pathway may also reveal new molecular targets for therapy.

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