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Identification of the cysteine-rich 61 (*CYR61*) gene variations in osteosarcoma patients

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Background/aim: Osteosarcoma requires an angiogenesis process. CYR61 is one of the extracellular signaling molecules that promote angiogenesis, tumor growth, and the malignancy of osteosarcoma. In the present study, we investigate the *CYR61* gene variations in osteosarcoma and their correlations with clinicopathological findings.

Materials and methods: We performed variation analysis of the *CYR61* gene in 58 patients with osteosarcoma. With an aim to ascertain the variety of variations in exons 2, 3, 4, and 5 of the *CYR61* gene in osteosarcoma, we did a PCR-SSCP followed by DNA sequencing.

Results: In osteosarcoma the *CYR61* gene variations found were 18.96% (11/58) in exon 2, 3.44% (2/58) in exon 3, 8.62% (5/58) in exon 4, and 15.51% (9/58) in exon 5. In our variation analysis, we detected one missense variation in exon 2 (Arg47Trp), one silent variation in exon 3 (Lys152Lys), one missense variation in exon 4 (Phe213Leu), and two missense variations in exon 5 (Gly315Arg and Asp339Asn). The overall *CYR61* variation frequency in exons 2, 3, 4, and 5 was determined to be 46.55% (27/58).

Conclusion: Our study specifies the role of *CYR61* gene variation in osteosarcoma. The result signifies that *CYR61* might be used as a prognostic/diagnostic marker in osteosarcoma patients.

Key words: Osteosarcoma, *CYR61*, PCR, SSCP, sequencing, gene variation

1. Introduction

Osteosarcoma, the most common primary malignant bone tumor, commonly arises in the metaphysis of long bones (1). Normally, osteosarcoma has a moderate rate of incidence, with 10 to 26 cases per million worldwide every year (2).

The 5-year survival in osteosarcoma in the first half of the 20th century was less than 20% (3). Amputation of the involved extremity aimed at compartmental resection was initially the mainstay of treatment (4). The primary target was to decrease the tumor load, although this does not prevent metastasis and pulmonary metastasis via hematogenous spread, which was the major cause of mortality in these patients. In a normal course, osteoblastic cells are responsible for the production of the bony matrix. The same occurs with these osteogenic cells in cancerous bones, the difference being in the maturation and strength of bone matrix thus produced, i.e. the bone matrix of a bone with osteosarcoma is not as strong as that of normal bones (5). Tumors, regardless of whether they are benign or malignant, can lead to pathological fractures of the involved bone by virtue of their potential to replace normal bony tissues, rendering them weak and prone to fracture. Osteosarcoma has five different histological types and their severity and prognosis varies accordingly. When diagnosed, it is usually in the late stages with high grades of severity, which makes salvage of the involved limbs difficult. Osteosarcomas originate from mesenchymal cells having osteoblastic features. Although the incidence of this bone tumor is low in the general population, unfortunately it is the most often diagnosed and lethal bone cancer in young adults (6). Osteosarcoma is a highly malignant osteoid-forming spindle cell sarcoma of the bone. Sometimes genetic variations may be linked with an increased risk of osteosarcoma (7).

The first discovered CCN protein, i.e. CYR61, also known as CNN1, was initially believed to be a classic growth factor. Later studies established that CCN1, instead of being a potent growth factor, also acted as a stimulator for some other growth factors, such as fibroblast growth factor and platelet-derived growth factor (8).

Though osteosarcoma can occur in any bone, the metaphysis of long bones is a common site of its occurrence

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(80%–90%), with a particular predilection for distal femoral metaphyses (35%), proximal tibial metaphyses (20%), and proximal humeral metaphyses (10%). In children, osteosarcoma accounts for 5% of all malignant bone tumors, with peak occurrence between the ages of 10 to 24 years (9). When symptomatic, it usually presents with localized pain, swelling, deformity, or a pathological fracture. The most common malignant bone tumors in children are osteosarcomas, which collectively make up 90% of pediatric bone tumors (10). The CYR61 proteins are composed of extracellular matrix-associated proteins that play crucial roles in skeletal development, wound healing, fibrosis, and cancer (11). The CYR61 proteins have four conserved cysteine-rich modular domains that trigger the processes of cell adhesion, proliferation, migration, differentiation, and survival via direct binding to specific integrin receptors and heparin sulfate proteoglycans (9). The effects of these processes are commonly regulated through signaling via integrins, vascular endothelial growth factor, bone morphogenetic protein, Wnt, and Notch pathways. In addition, current studies suggest that CYR61 proteins may affect progression of secondary metastatic bone tumors by moderating the bone microenvironment (11,12).

CYR61 is a product of an immediate and early gene that induces cell migration, mediates cell adhesion, enhances growth factor-induced DNA synthesis in fibroblasts and endothelial cells, stimulates chemotaxis of fibroblasts and endothelial cells, and increases the chondrogenesis in mesenchymal cells (13–15). The expression of CYR61 protein enhances neovascularization and tumor formation of human tumor cells in immunodeficient mice significantly (16–18).

Overall, a better understanding of *CYR61* gene protein-regulated pathways in osteoblast/osteoclast can improve our knowledge of the pathogenesis of the disease and can potentially aid in developing CYR61 protein-based diagnostic marker and therapeutic strategies for the management of osteosarcoma. Therefore, study of the CYR61 protein in patients with osteosarcoma is being considered as a promising biomarker for future research work.

2. Materials and methods

2.1. Specimen collection

Fifty-eight osteosarcoma specimens were collected from the Department of Orthopedic Surgery, King George's Medical University, Lucknow, Uttar Pradesh, India. In such cases, fine-needle aspiration biopsy yielded positive cytology and based on these results, the tissue was diagnosed as osteosarcoma. The 58 cases were diagnosed as osteosarcoma using quantitative, qualitative, and histological grade according to Enneking et al. (19) and Bickels et al. (20). This study was carried out from May 2011 to March 2015. The study was approved by the institutional ethics committee. Before enrollment in the study, each subject's written informed consent was obtained in response to a fully written and verbal explanation of the nature of the study.

2.2. DNA isolation

Samples were collected from 58 biopsy tissues diagnosed as osteosarcoma based on categorized and grading as per the internationally accepted standard. The genomic DNA was extracted by a Fermentas DNA extraction kit (Germany) and stored at –80 °C.

2.3. Genomic DNA PCR amplification

Primers for the *CYR61* gene were designed using GENE TOOL software. PCR was performed in a gradient thermocycler (ABI, USA) using thin-walled 0.2-mL PCR tubes. The final volume of the PCR reaction mixture was 25 µL, containing 10–40 ng of genomic DNA, 1 pmol of forward and reverse primers, and 2X master mix (ABI, USA) at a concentration of 1X. Amplification was carried out using different primers for different exons (Table 1) and different PCR programs (Table 2) for different exonic regions of the *CYR61* gene. A further 5 µL of the amplified product was checked on 2% agarose gel with ethidium bromide staining.

Table 1. PCR primers of the *CYR61* gene.

Exon	Forward/reverse primers	Amplicon size (bp)
2	F 5'-GCGCTCTCCACCTGCCCC-3' R 5'-GCTCTGAAGGGGATCTGCAGA-3'	213
3	F 5'-CAGTCAGAGGGCAGACCCTGT-3' R 5'-AGCTCACTGAAGCGGCTCCCT-3'	354
4	F 5'-TTAGGAATGGAGCCTCGC-3' R 5'-GTGTACAGCAGCCTGAAA-3'	207
5	F 5'-AAGGGCAAGAAATGCAGCAAG-3' R 5'-ACATTCACAAATTTAGGGAC-3'	300

Table 2. PCR programs of different exons of the *CYR61* gene.

Exon	Step 1 (denaturation)	Step 2 (annealing)			Cycles	Step 3 (extension)
2	95 °C→ 7 min	95 °C→ 1 min	55 °C→ 50 s	72 °C→ 1 min	35	72 °C→ 5 min
3	94 °C→ 8 min	94 °C→ 1 min	56 °C→ 30 s	72 °C→ 1 min	40	72 °C→ 7 min
4	95 °C→ 5 min	95 °C→ 1 min	56 °C→ 1 min	72 °C→ 1 min	35	72 °C→ 8 min
5	95 °C→ 10 min	95 °C→ 1 min	58 °C→ 45 s	72 °C→ 1 min	41	72 °C→ 5 min

2.4. Variation screening of *CYR61* exons by SSCP

Single-strand conformational polymorphism (SSCP) analysis was performed according to Orita et al. (21,22) with minor modifications. Samples were denatured at 95 °C for 5 min with denaturing dye and immediately transferred to ice. Next, 25 µL of amplified PCR product was loaded along with 25 µL of denaturing dye on 8% polyacrylamide gel. The gel was run in precooled 1X TBE buffer. The gel tank was placed in a cold room at 4 °C and run for 8–10 h at 130 V. DNA on the gel was stained after electrophoresis with silver stain. Electrophoresis mobility shifts in single-stranded or double-stranded DNA products from patients were detected by comparison with DNA products from normal controls run in adjacent lanes (Figure 1).

2.5. *CYR61* variation analysis by sequencing

Amplified fragments of all samples were characterized by automated sequencing. The PCR product of each sample was first purified and then submitted in a quantity of 25 µL with 10 pmol of appropriate primer. The sequencing was performed by automated direct DNA sequencing technique, which incorporates fluorescently labeled di-deoxy-nucleotides during cycle sequencing and separates the resulting products by capillary electrophoresis for detection on an ABI 3730XL DNA Analyzer (Applied Biosystems, USA). Multiple alignment and sequence analysis were done using BLAST, BioEdit, FinchTV, and Auto Assembler Software (Applied Biosystems, USA). Variations were reconfirmed by sequencing amplicons in both directions and in independent second samples.

3. Results

A total of 58 cases were diagnosed as osteosarcoma according to Enneking et al. (19) and Bickels et al. (20). The mean age of these patients was 20 ± 3.90 years, ranging from 10 years to 30 years. Four missense variations were detected in 25 osteosarcoma cases in exons 2, 4, and 5 and one silent variation was detected in two osteosarcoma cases in exon 3 (Figures 2–5). One missense variation was detected in exon 2 (Arg47Trp), one silent variation in exon 3 (Lys152Lys), one missense variation in exon 4 (Phe213Leu), and two missense variations in exon 5 (Gly315Arg and Asp339Asn). We observed the *CYR61* gene missense variation rates in grade G1 (33.33% in a total of 10/30 cases), grade G2 (43.75% in a total of 7/16 cases), and grade G1/G2 (66.67% in a total of 8/12 cases). *CYR61* gene silent variations were found in grade G1 (6.67% in a total of 2/30 cases). Details of the clinical data and missense and silent variations in exons 2, 3, 4, and 5 are shown in Table 3. Out of 58 osteosarcoma cases, 39 were found to have variations by a shift in DNA position on SSCP-PAGE with respect to DNA from healthy donors (Figure 1). These results, after comparison to previously reported findings, are shown in Table 4. The *CYR61* gene missense variation in exon 3 for codon Lys152Lys, exon 4 for codon Phe213Leu, and exon 5 for codons Gly315Arg and Asp339Asn are described here for the first time.

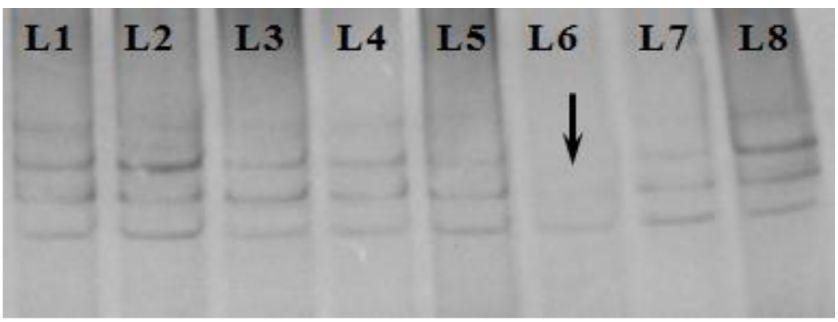

Figure 1. SSCP-PAGE showing electrophoresis mobility shift bands on native PAGE. Control: L1; no shift bands in L2–L5, L7, and L8; and shift bands in L6 (arrow).

Codon	45	46	47	48	49	50	150	51	52	53	54	213	14	15	16	17	314	15	16	17	18	338	39	40	41	42	43
Wild Type	L	V	R	D	G	C	L	V	K	V	T	F	G	M	E	P	C	G	S	C	V	E	D	G	E	T	F
11 Cases	L	V	W	D	G	C	L	V	K	V	T	F	G	M	E	P	C	G	S	C	V	E	D	G	E	T	F
07 Cases	L	V	R	D	G	C	L	V	K	V	T	F	G	M	E	P	C	R	S	C	V	E	D	G	E	T	F
05 Cases	L	V	R	D	G	C	L	V	K	V	T	L	G	M	E	P	C	G	S	C	V	E	D	G	E	T	F
02 Cases	L	V	R	D	G	C	L	V	K	V	T	F	G	M	E	P	C	G	S	C	V	E	N	G	E	T	F
02 Cases	L	V	R	D	G	C	L	V	K	V	T	F	G	M	E	P	C	G	S	C	V	E	D	G	E	T	F

Figure 2. Amino acid sequences of exons 2, 3, 4, and 5 of the *CYR61* gene. The wild-type sequence is shown above the cases. Missense and silent variations are shown in colored squares.

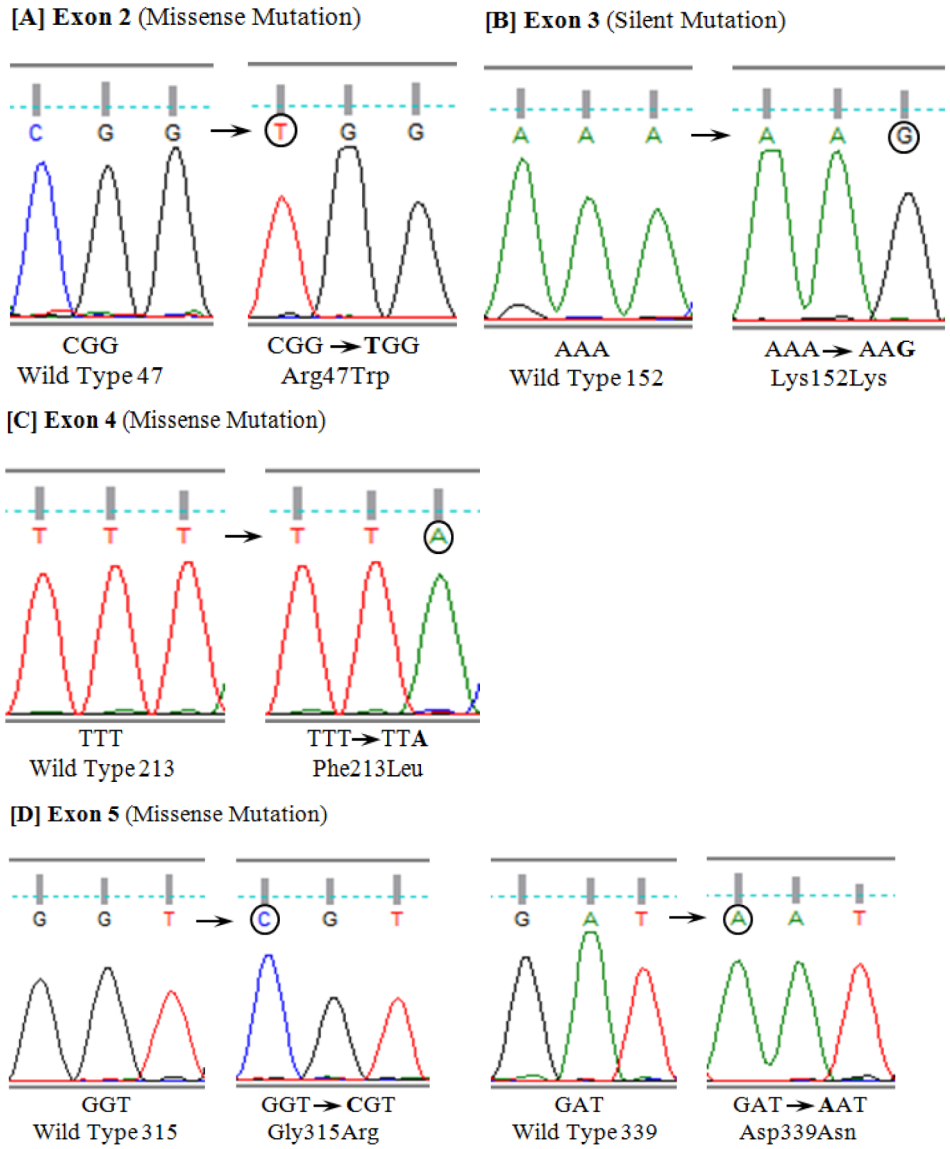

Figure 3. Nucleotide sequence chromatograms of *CYR61* gene missense and silent variations: A) exon 2 nucleotides C→T, resulting in the amino acid substitution Arg47Trp; B) exon 3 nucleotides A→G, resulting in the amino acid substitution Lys152Lys; C) exon 4 nucleotides T→A, resulting in the amino acid substitution Phe213Leu; D) exon 5 nucleotides G→C, G→A, resulting in the amino acid substitutions Gly315Arg and Asp339Asn.

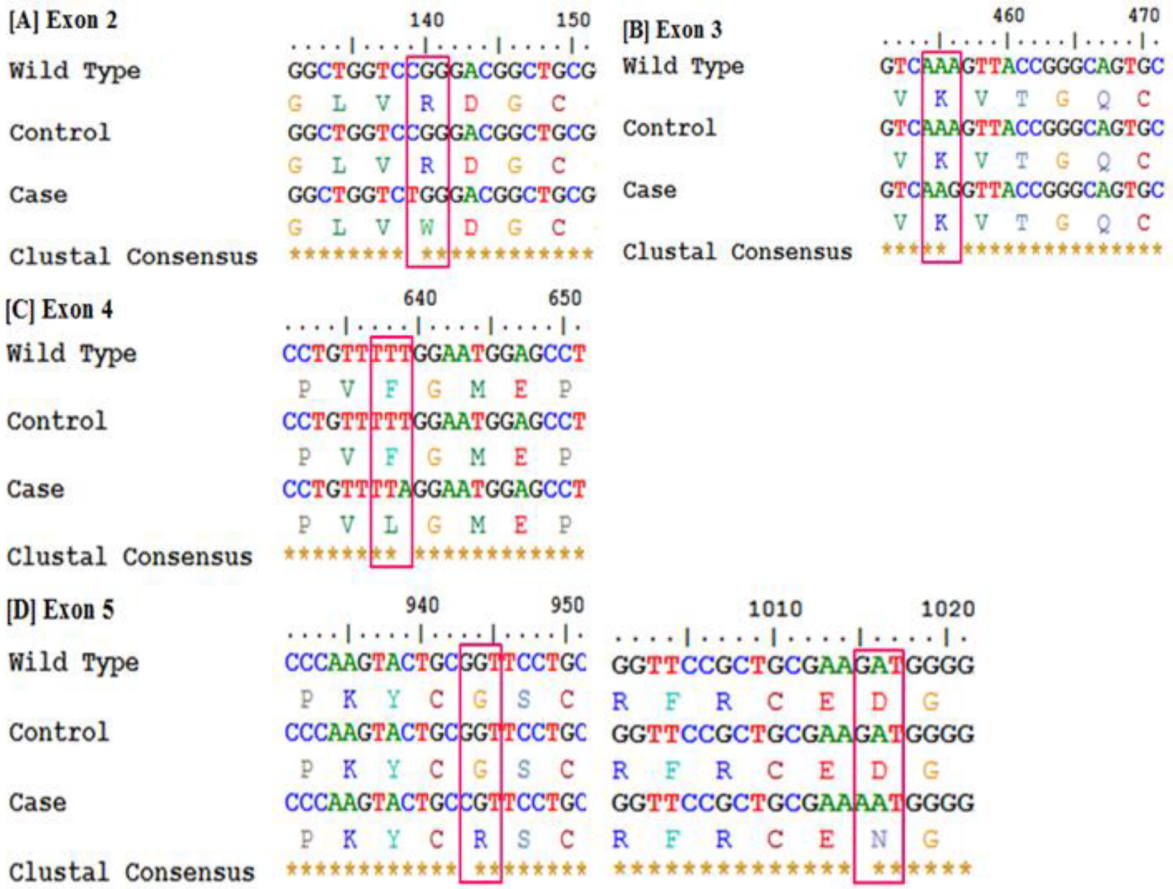

Figure 4. Variation analysis of the *CYR61* gene showing changes of nucleotides and amino acids: A) exon 2 missense variation codon Arg47Trp (CGG→TGG); B) exon 3 silent variation codon Lys152Lys (AAA→AAG); C) exon 4 missense variation codon Phe213Leu (TTT→TTA); D) exon 5 missense variation codon Gly315Arg (GGT→CGT) and codon Asp339Asn (GAT→AAT).

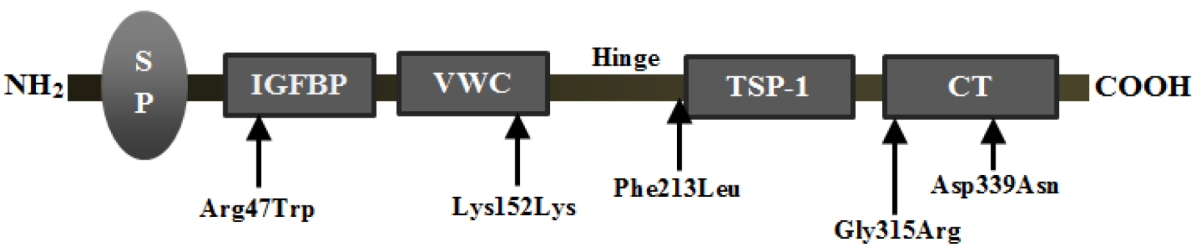

Figure 5. Location of *CYR61* gene variations describes our results: missense and silent variations in exons 2, 3, 4, and 5 and the distinctive molecular domains encompass functional structures, e.g., NH_2-terminal, signaling peptide (SP), insulin-like growth factor binding protein domain (IGFBP), von Willebrand factor domain (VWC), thrombospondin-homology domain (TSP-1), cysteine knot domain (CT), and COOH terminal, correspondingly.

3.1. Analysis of variations in exons 2, 3, 4, and 5

In 58 osteosarcoma cases, 25 samples showed a shift in position in native SSCP-PAGE in exon 2. These were directly sequenced by an automated sequencer. Eleven missense variations were detected in 11 osteosarcoma patients (Figures 2–4; Table 3).

Out of 58 osteosarcoma cases, 10 samples displayed a shift in position in native SSCP-PAGE in exon 3. These were directly sequenced by an automated sequencer. Two silent variations were detected in two osteosarcoma patients (Figures 2–4; Table 3).

In 58 osteosarcoma cases, 13 samples showed a shift in position in native SSCP-PAGE in exon 4. These were directly sequenced by an automated sequencer. Five missense variations were detected in five osteosarcoma patients (Figures 2–4; Table 3).

Table 3. Details of the clinical data and *CYR61* gene variations in osteosarcoma cases.

No. of cases	Grade	Stage	Codon exon 2	Codon exon 3	Codon exon 4	Codon exon 5
10	G1	IA, IB	Arg47Trp		Phe213Leu	Gly315Arg
07	G2	IIA, IIB	Arg47Trp		Phe213Leu	Gly315Arg Asp339Asn
08	G1/G2	III	Arg47Trp		Phe213Leu	Gly315Arg Asp339Asn
02	G1	IA, IB		Lys152Lys		

IA & IIA: Intracompartmental.
IB & IIB: Extracompartmental.
III: Either grade with metastases.

Table 4. Variations detected in our study of exons 2, 3, 4, and 5 in the *CYR61* gene and their allele frequency.

Exons	Variations in aa	Variations in codons	Variation's ID or references	Allele frequency
Exon 2	Arg47Trp	CGG → TGG	[23]	0.517
Exon 3	Lys152Lys	AAA → AAG	Not reported	0.275
Exon 4	Phe213Leu	TTT → TTA	rs755958207	0.543
Exon 5	Gly315Arg	GGT → CGT	rs532164537	0.560
Exon 5	Asp339Asn	GAT → AAT	rs375944529	0.448

Out of 58 osteosarcoma cases, 23 samples showed a shift in position in native SSCP-PAGE in exon 5. These were directly sequenced by an automated sequencer. Nine missense variations were detected in nine osteosarcoma patients (Figures 2–4; Table 3).

For these findings, where we found variations around the protein, it was important to address where in the protein these variations were located in order to determine the possible implications of these variations in protein function. Therefore, we analyzed the protein sequence using BioEdit and Pyre2 software. The Arg47Trp variation for exon 2 is located on the insulin-like growth factor binding protein domain (IGFBP), the Lys152Lys silent variation for exon 3 is located on the von Willebrand factor domain (VWC), the Phe213Leu variation for exon 4 is located on the thrombospondin-homology domain (TSP-1), and the Gly315Arg and Asp339Asn variations for exon 5 are located on the cysteine knot domain (CT) in osteosarcoma cases.

4. Discussion

To our knowledge, this study is the first in northern India to report variations of the *CYR61* gene in osteosarcoma patients. Osteosarcoma is the most common primary bone tumor. As it is a very fast-growing tumor with metastasis in early stages, it becomes difficult to diagnose it in initial stages. In almost all cases, micrometastasis in the pulmonary system has already taken place by the time a clinical diagnosis is made. This tumor is resistant to a number of chemotherapeutic agents, which is responsible for the treatment failure and high mortality rate of patients.

Previous molecular studies revealed several variations in different types of patients from different ethnic groups. Variations in exons 2, 3, and 4 of the *CYR61* gene are less frequently detected than in exon 5 in different types of patients (23–25). However, during our variation analysis, besides the detection of one missense variation in exon 2 (Arg47Trp), there was one silent variation in exon 3 (Lys152Lys), one missense variation in exon 4 (Phe213Leu), and two missense variations in exon 5 (Gly315Arg and

Asp339Asn). The overall *CYR61* variation frequency in exons 2, 3, 4, and 5 was determined to be 46.55% (27/58). With these observations, our study supports the role of *CYR61* gene variation in osteosarcoma, and we concluded that by virtue of its behavior in osteosarcoma patients, this gene is justified for use as a prognostic/diagnostic marker. We also recommend the *CYR61* gene as a new drug target for the treatment of osteosarcoma, though further multicenter studies are needed to validate this.

As the *CYR61* gene is located in 1p22.3 on chromosome 1, which has numerous translocations, it is reported to be the most common region of chromosome involvement in human cancers (26), especially in osteosarcoma cells, which have a high percentage of polysomy in chromosome 1 (27).

Wittig et al. (28) suggested *CYR61* as a candidate gene responsible for drug resistance in melanoma. Overexpression of *CYR61* was observed to increase the potential of resistance to chemotherapeutic drugs in ovarian and breast cancer cells (29,30). Later on Fromigue et al. (31) observed the antitumoral effect of the treatments targeting the *CYR61* gene, which also enhances the potential of chemotherapy in bone tumors.

Fromigue et al. (31) also noticed the expression level of *CYR61*, which is significantly higher in primary osteosarcoma than in normal bone tissue. The *CYR61* expression positively correlates with tumor invasiveness in human osteosarcoma and attenuation of *CYR61* reduces cancer cell progression. Fromigue et al. also showed that the overexpression of the *CYR61* gene increases the tumor behavior in osteosarcoma by acting on pulmonary metastatic foci, both in number and size.

High expression levels of *CYR61* were reported in malignant melanomas, rhabdomyosarcomas, colon adenocarcinomas, and bladder papillomas (21,32). According to Xie et al., *CYR61* also exhibited high levels in malignant gliomas, which enhanced the tumorigenicity (33). Overexpression of *CYR61* was recently identified in peritoneal metastases from human pancreatic cancer (34). We also found a positive association of the *CYR61* gene with osteosarcoma, which raises speculation that *CYR61* might play distinct roles in osteosarcoma with different stages.

In conclusion, using a translational approach, we identified the missense variations (F213L and G315R) in osteosarcoma patients with linkage to *CYR61*, an important factor in carcinogenesis and its subsequent progression. The present study supports an important role of the *CYR61* gene in human osteosarcoma and we recommend further multicenter studies with large sample sizes to confirm the roles of these variations in osteosarcoma patients and to establish an association. This gene can also provide a molecular target that will open the fields for a novel and effective therapeutic strategy to reduce the mortality and morbidity associated with the disease. The results signify that the *CYR61* gene might be used as a prognostic/diagnostic marker in osteosarcoma patients.

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