

Original Research Article

## Transcriptomic and Proteomic Expression Analysis of Cysteine Rich Angiogenic Inducer 61 in Normal and Diaphyseal Tibial Fractures Patients: A Case Control Study

Manish Yadav<sup>1\*</sup>, Ajai Singh<sup>2\*</sup>, Sabir Ali<sup>3\*</sup>, Abbas Ali Mahdi<sup>2\*\*</sup>

<sup>1</sup>Senior Research Assistant, <sup>2</sup>Professor, <sup>3</sup>Ph.D. Scholar

\*Department of Orthopaedic Surgery, \*\*Department of Biochemistry, King George's Medical University, Lucknow, Uttar Pradesh, India- 226003.

Corresponding Author: Ajai Singh

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### ABSTRACT

Angiogenesis is the process of the formation of new blood vessels from pre-existing vasculature. Angiogenesis is a prerequisite for fracture repair. Cysteine Rich Angiogenic Inducer 61 (CYR61) is one of the markers responsible for angiogenesis as well as chondrogenesis. In the present case-control study, we aimed to analyse the transcriptomic and proteomic expression of CYR61 in normal (control) and patients with diaphyseal tibial fractures. A total 129 cases with simple, fresh traumatic diaphyseal fractures of both bones of the leg managed conservatively and 129 healthy controls enrolled. Samplings of blood were taken at 4<sup>th</sup> day of fracture in cases and in controls. The CYR61 mRNA and protein expressions were quantified by using qRT-PCR and western blotting assay technique. In this study, we found significant difference in expression level of both CYR61 mRNA and protein between cases and controls. Simultaneously, as the difference in expression obtained in early phase of fracture healing, we conclude that the CYR61 might be used as a potential biomarker to predict the fracture healing outcomes early.

**Keywords:** CYR61; Angiogenesis; Angiogenic Marker; Diaphyseal tibial fractures, Biomarker.

### INTRODUCTION

Amongst long bones, tibia is one of the commonest bones that are prone to fracture involving relatively higher incidence of impaired healing (2-10%). [1-3] Angiogenesis leads to the formation of new blood vessels and plays a vital role during intramembranous bone formation and endochondral ossification while fracture healing and remodelling. [4] A sufficient amount of blood supply to the fracture site is needed for the reconstitution of bone tissue, whereas insufficiency of this may leads to impaired bone healing. [5]

The Cysteine Rich Angiogenic Inducer 61 (CYR61) gene is key indicative molecule of angiogenesis process. Previous

studies reveals CYR61 to be works as an extracellular signaling molecule in human bone. [6-8] Hadjiargyrou et al. [9] and Jasmin et al. [10] primarily identified CYR61 to be up-regulated during fracture healing. Both of them suggested CYR61 to be an important regulator of fracture healing and may play a vital role in cartilage and bone formation. In another study, it was also observed that the genotype of CYR61 affects the mRNA expression and acts as a risk factor that could synergistically increase the susceptibility of a patient to develop fracture nonunion. [11] Wong et al. [12] and O'Brien et al. [13] suggested crucial role of CYR61 in chondrogenesis process. Simultaneously, they also observed

important role of CYR61 in normal growth, differentiation, or morphogenesis of the cartilaginous skeleton of the embryo. [12,13]

In the present case-control study, we aimed to analyze the transcriptomic and proteomic expression of CYR61 in normal (control) and diaphyseal tibial fractures patients.

## MATERIALS AND METHODS

This is a case-control study carried out between 2011 to 2016 at our institutional trauma center. A total of 129 patients of both sexes of aged between 18 and 40 years with simple, fresh (less than 03 days) traumatic diaphyseal fractures of both bones leg managed conservatively were included as cases, however normal healthy age-sex matched individual without any fracture/trauma enrolled as controls (n=129). Exclusion criteria of cases included age of less than 18 and more than 45 years; osteoporotic fractures; polytrauma; pathological fractures; compound or infected fractures; alcoholic; smoker; immune-compromised; single tibial fracture with intact fibula; uncontrolled diabetes; bile duct obstruction; chronic inflammatory bowel disease; patients managed surgically; patients coming after 03rd post-fracture days; malnourished; and prolonged use of anabolic steroids, thiazides, diuretics, hormonal therapy, non-steroidal anti-inflammatories, calcium, fluorides, and immunosuppressive drugs. After obtaining ethical clearance (Ref. Code: 55 E.C.M. IIB/P6) from institutional ethical review committee and informed consent, demographic data of all enrolled patients were collected.

The single sampling was carried out by taking peripheral blood (morning samples) at 04<sup>th</sup> day of post-fracture in cases and controls. The total CYR61 mRNA and serum protein from the whole blood was isolated as per standard protocol using Trizol and centrifugation method respectively. The CYR61 mRNA expression was done by qRT-PCR analysis as per standard protocol using primers and probe as follows: CYR61; Forward Primer-

TGGAGTTATATTCACAGGGTCTG;  
Reverse Primer-  
GCAGCTCAACGAGGACTG; Probe-  
CGCCGAAGTTGCATTCCAGCC (IDT,  
Prime Time Standard qPCR Assay, FAM-  
TAMRA) and was normalized by  
housekeeping gene, glyceraldehyde-3-  
phosphate- dehydrogenase (GAPDH). The  
normalized amount of targets was then  
compared using the comparative Ct-method.  
The CYR61 protein expression was done by  
western blotting assay using CYR61  
primary antibody (1:100, CYR61 (H-78)  
rabbit polyclonal IgG, SC-13100), followed  
by corresponding horseradish peroxidase-  
conjugated secondary antibodies (1.5 h,  
1:5,000, Goat anti-rabbit IgG-HRP, SC-  
2004) and normalized with GAPDH (SC-  
25778) as per standard protocol.

### Statistical Analysis

Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA) for Windows program (15.0 versions). The continuous variables were evaluated by mean ( $\pm$ standard deviation) or range value when required. For comparison of the means between the two groups, analysis by Student's *t*-test with 95% confidence interval, Mann-Whitney U-test, and Pearson correlation coefficient was used. A *P* < 0.05 or 0.001 was regarded as significant.

## RESULTS

In the present study, total 129 cases and 129 controls were included as per inclusion-exclusion criteria. Table 1 describes the baseline characteristics of cases and controls, which do not show any statistically significant difference.

In cases, the mean fold CYR61 mRNA and protein expressions were  $2.31 \pm 0.56$  and  $0.38 \pm 0.21$  respectively. In controls, the mean fold CYR61 mRNA and protein expressions were  $2.02 \pm 0.27$  and  $0.27 \pm 0.18$  respectively. In cases, both the CYR61 mRNA and protein are higher in expression as compare to controls and shows a significant statistical difference [Fig-1(a), (b) & (c)].

However, while comparing of expression between CYR61 mRNA and protein in cases and controls, insignificant correlation were observed [Table-2; Fig-2 (a) & (b)].

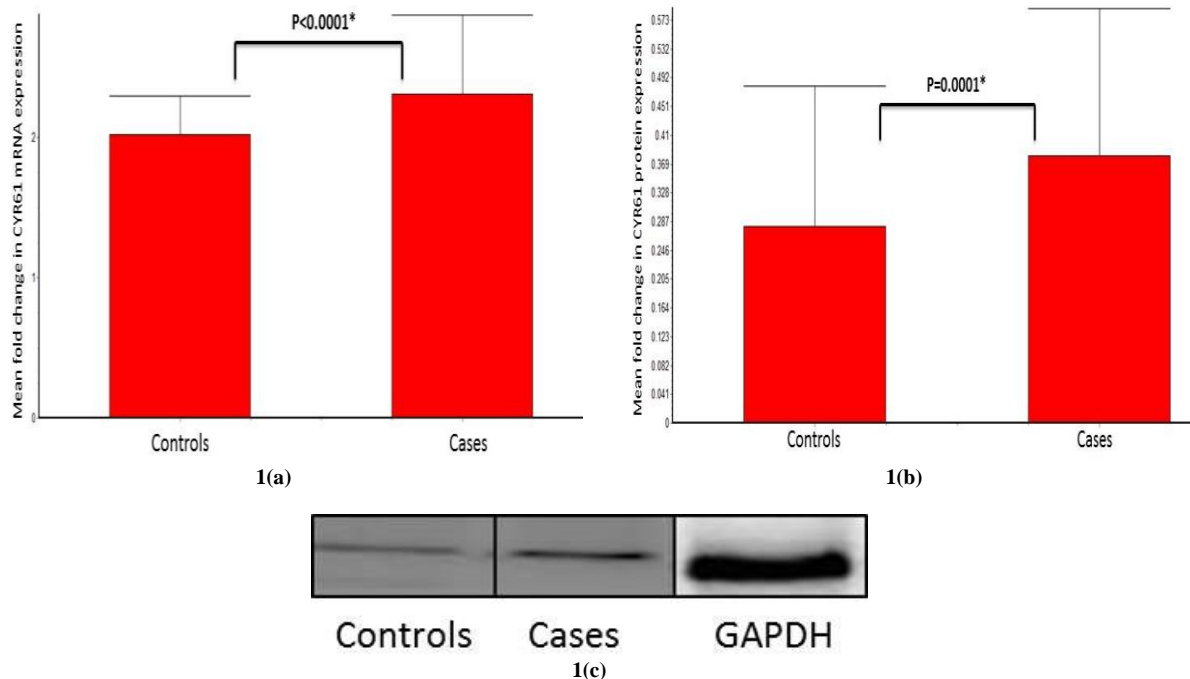
**Table1: Comparison of baseline characteristics between Cases and Controls.**

Characteristics	Controls(n=129)	Cases (n=129)	Significance of difference
Mean age ±SD (range) in years	29.71±8.35(18-40)	31.67±7.81 (18-40)	P=0.0526 <sup>†</sup>
<b>Gender</b>			
Male	104 (86.59%)	112 (79.44%)	P=0.2376 <sup>‡</sup>
Female	25 (13.4%)	17 (5.61%)	
<b>Side of fracture</b>			
Left	-	72 (46.73%)	
Right	-	57 (38.31%)	
<b>Mode of injury</b>			
Fall from height	-	37 (22.42%)	
Road Traffic Accident	-	63 (42.99%)	
Simple fall	-	29 (19.62%)	
Slip on ground	-	0 (0)	

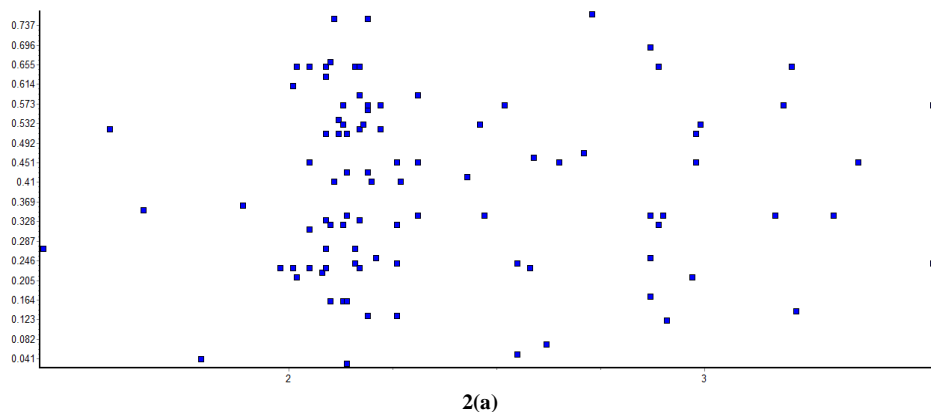
<sup>†</sup> Unpaired t test (Two tailed), <sup>‡</sup> Chi square test

**Table 2: Correlation between CYR61 mRNA and protein expression level in (a) Cases and (b) Control**

CYR61 mRNA versus Protein expressions	Spearman r	95% Confidence Interval	P (two-tailed)
Controls	-0.0084	-0.1861 to 0.1697	0.9244
Cases	-0.0119	-0.1894 to 0.1664	0.4158



**Figure 1: Mean CYR61 (a) mRNA; (b) protein expression and (c) western blot band in Cases and Controls.**



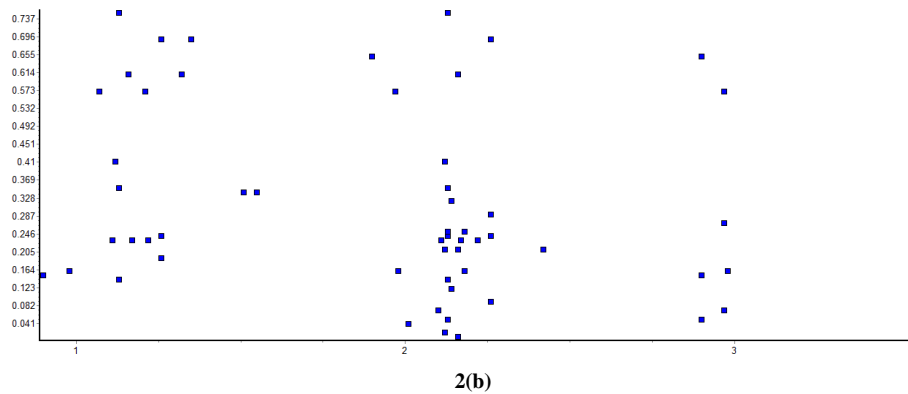


Figure 2: Correlation between CYR61 mRNA and protein expression level in (a) Cases and (b) Controls.

## DISCUSSION

In the present study, the basic aim was to analyze the transcriptomic and proteomic expression of CYR61 in controls and diaphyseal tibial fractures patients. Our research hypothesis was that the fracture healing is a complex phenomenon that comprise of different overlapped but sequential biological events in which process like angiogenesis plays a vital role in early phase. Therefore, it might happen that biochemical markers like CYR61, that play essential role in angiogenesis may show enhanced expression pattern during the healing phase of fracture.

In the present study, the difference in demographic data like mean age, genders, side of fracture, Muller's AO classification and mode of injuries of different groups were found to be statistically insignificant. This observation suggested that the impaired healing outcome of fracture tibial bone was independent of age, gender, mode of injuries and side as well as pattern of fracture. In this study, we observed higher mean fold CYR61 mRNA and protein expression in cases as compare to controls and shows a significant statistical difference. This observation indicates a crucial role of CYR61 in fracture healing process. However, insignificant correlation was observed while comparing of expression between CYR61 mRNA with protein in cases and controls. These might be due to differential splicing, turnover as well as post-translational modifications. By

these finding we have suggested that the transcriptomic and proteomic data of CYR61 cannot be compared.

To the best of our limited knowledge, no clinical study has been done that could have showed simple diaphyseal tibial fracture healing outcome in relation to serial estimation of CYR61 gene (mRNA & protein). However, two animal studies done by Hadjiargyrou et al. and Jasmin et al. [9,10] had analyzed tibial fracture healing outcome in relation to serial expression of CYR61. And both of them observed elevation in the CYR61 mRNA and protein expression during the fracture healing progression. However, we are unable to compare our study observation as none of them could compare the CYR61 expression in fracture cases with the non-fracture controls.

However as we observed higher CYR61 mRNA and protein expression then the controls group showed significant difference. This observation might be an indication for CYR61 to play an essential role in early phase of fracture healing. Simultaneously, as the difference in expression obtained in early phase of fracture healing, we hypothesised that the CYR61 might be used as a potential biomarker to predict the healing outcomes early. However, future study with the serial estimation of CYR61 throughout the fracture healing phase and then correlation of CYR61 with fracture healing progression as well as their outcome will be needed to better prove the CYR61 as a biomarker to

predict the fracture outcomes.

This may open new horizons for innovations in this field with an addition to our armamentarium to deal with complications associated with impaired fracture healing especially in tibial bone fractures. Since this biomarker measurement in peripheral blood are relatively less invasive, inexpensive, and can be repeated more often, it can also be used as an important prognostic tool for early identification of patients who are prone to impaired fracture healing in future. Such an approach would benefit not only to the patients' wellbeing but also to the entire health care system in terms of the cost implications associated with long lasting treatment interventions and hospitalization. However, small sample size as well as single centric study was the limitation of the present study. Therefore author recommend further multicentric study with a large sample size to increase the validity, reliability, and generalizability of our observation and inferences.

## CONCLUSION

Fracture healing is a complex phenomenon involved angiogenesis process. The CYR61 is key indicative molecule involved in angiogenesis. Therefore, we plan to analyse the CYR61 expressions in controls and diaphyseal tibial fractures patients. In the present study, the post fracture CYR61 mRNA and protein expressions were higher in fractures cases than the controls. By this we might be able to conclude that CYR61 has a vital role in early fracture healing phase and as the difference in expression obtained in early phase of fracture healing this might be used as a potential biomarker to predict the healing outcomes early. However, further multi-centric study was needed to increase the generalizability of our observation.

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**Conflict of interest:** The authors have no conflict of interests in this article.

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