

ORIGINAL ARTICLE

# CYR61—An angiogenic biomarker to early predict the impaired healing in diaphyseal tibial fractures



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## KEYWORDS

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**Summary** *Background:* Angiogenesis is a prerequisite for fracture repair, whereas insufficient blood supply is likely to result in impaired healing. In the present study, we aimed to determine the correlation of simple tibial fracture healing outcome with serial estimation of CYR61 expressions in the early phase of healing.

*Methods:* In total, 107 adult fractured patients and 97 healthy controls were analysed. Peripheral blood samples were taken from controls (at once) and fractured patients at 4<sup>th</sup>, 7<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup>, 20<sup>th</sup> and 28<sup>th</sup> days of post-fracture follow-ups to quantify the CYR61 mRNA and protein expression by qRT-PCR and Western blotting assay, respectively. Clinic-radiological follow-up was done at 6<sup>th</sup>, 10<sup>th</sup>, 16<sup>th</sup>, 20<sup>th</sup>, and 24<sup>th</sup> weeks of post-fracture follow-ups using RUST scores to analyse the fracture healing progression and their final outcomes.

*Results:* By considering controls as Group I ( $n = 97$ ), as per the clinico-radiological status at 24<sup>th</sup> week, fracture patients were divided into two groups: Group II (normal healing,  $n = 91$ ) and Group III (impaired healing,  $n = 16$ ). Both CYR61 mRNA and protein expressions were lower (baseline) in Group I than in Groups II and III; however, a significant difference was observed only with the Group II. In both groups, expressions of CYR61 mRNA as well as protein gradually upregulated from the baseline to a peak and then declined. Both, the CYR61 mRNA as well as protein expressions were significantly higher at all follow-ups in Group II than in Group III. Mean RUST scores between Group II and Group III showed a significant statistical difference at each follow-up. Significant correlation was found between the CYR61 expressions and the RUST score (fracture healing progression).

*Conclusion:* We conclude that CYR61 expression provides an early prediction of the healing outcomes of simple diaphyseal tibial fractures.

*The translational potential of this article:* Such an approach would benefit not only the patients' wellbeing but also the entire health care system in terms of the cost implications

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associated with long lasting treatment interventions and hospitalisation. However, the authors recommend further multicentric study with a large sample size to increase the validity, reliability, and generalisability of our observation and inferences.

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## Introduction

Angiogenesis is a process of the formation of new blood vessels from pre-existing ones. After fracture, it is stimulated to maintain oxygen homeostasis, supply of nutrients, removal of waste products, and provide cells and biological mediators. Angiogenesis plays a crucial role during intramembranous bone formation and endochondral ossification [1]. An adequate blood supply to the fracture is a prerequisite for the reconstitution of the bone tissue, whereas insufficient blood supply is likely to result in impaired bone healing [2]. However, there has been little evidence regarding the regulation of blood vessel formation in impaired bone healing. Amongst long bones, a shaft of the tibia is one of the commonest bones that are prone to fracture involving the relatively high incidence of impaired healing (2–10%) [3–6]. The Cysteine Rich Angiogenic Inducer 61 (CYR61) gene is a key indicator molecule involved in angiogenesis. In previous studies, it was found that CYR61 is an extracellular signaling molecule in human bone [7–9]. According to Wong et al [10] and O'Brien and Lau [11], CYR61 acts as a novel player in chondrogenesis. They also suggested that CYR61 may be important for the normal growth, differentiation, or morphogenesis of the cartilaginous skeleton of the embryo [10,11]. Hadjiargyrou et al [12] and Jasmin et al [13] primarily identified CYR61 to be upregulated during fracture healing. They suggested that CYR61 plays a vital role in cartilage and bone formation and may act as an important regulator of fracture healing. In a previous study, Ali et al [14] observed the significant effect of CYR61 genotype on its mRNA expression and concluded as a risk factor that could synergistically increase the susceptibility of a patient to develop fracture nonunion. Also, the CYR61 expressions were significantly higher in fractured patients than in the controls [15]. In the present study, we have analysed the correlation of tibial fracture healing outcomes with early serial estimation of CYR61 expression.

## Materials and methods

This is a prospective cohort study conducted between 2011 and 2016 at our institutional trauma center. After obtaining ethical clearance (Ref. Code: 55 E.C.M. IIB/P6) from the institutional ethical review committee and informed consent, demographic data of all enrolled patients were collected.

A total of 119 patients of both sexes aged between 18 and 40 years with simple, fresh (< 3 days) traumatic diaphyseal fractures of both bone leg managed

conservatively were included in the study. The exclusion criteria included age of < 18 years and > 40 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 3 post-fracture days; malnourished; and prolonged use of anabolic steroids, thiazides, diuretics, hormonal therapy, non-steroidal anti-inflammatories, calcium, fluorides, and immunosuppressive drugs. To exclude malnourished patients, the nutritional examination, such as haemoglobin percentage (manually), serum albumin (ELITech clinical system), and serum ferritin (Roche analyser) were done at the Department of Biochemistry. All patients included in this study were managed conservatively (reduction-setting and above knee plaster cast under general/regional anaesthesia). Prior to the management, the clinical and radiological examinations were done. All the patients were admitted for next 24–48 hours and then discharged with a standard advice. Simultaneously, total 97 healthy controls (without any fracture) were enrolled (Group I).

In biochemical examination, the CYR61 mRNA and protein expression in peripheral blood was conducted in enrolled fractured patients at following intervals, i.e., at 4<sup>th</sup>, 7<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup>, 20<sup>th</sup>, and 28<sup>th</sup> post-fracture days and once a time for the controls. The total CYR61 mRNA and serum protein from the whole blood was isolated as per the standard protocol using Trizol and the centrifugation method, respectively. The CYR61 mRNA expression was done by qRT-PCR analysis as per the standard protocol using primers and probe as follows: CYR61; forward primer, TGGAGTTATATTCACAGGGTCTG; reverse primer, GCAGCTCAACGAGGACTG; probe, CGCCG-AAGTTGCATTCCAGCC (IDT, Prime Time Standard qPCR Assay, FAM-TAMRA). Each gene of interest was normalised to the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward primer, GAAGGTGAAGTCCGGAGTC; reverse primer, GAAGATGTGATGGGATTC; probe, CAAGCTCCCGTTCTCAGCC) (IDT, Prime Time Standard qPCR Assay, FAM-TAMRA). The normalised 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 normalised with GAPDH (SC-25778), as per the standard protocol.

The clinico-radiological examination was performed at 6<sup>th</sup>, 10<sup>th</sup>, 16<sup>th</sup>, 20<sup>th</sup>, and 24<sup>th</sup> post-fracture weeks. The

radiological progression of healing was evaluated using RUST scores (Figure 1) [16,17]. The X-rays for the RUST score were examined separately by two orthopaedic surgeons blindly, and the findings were noted separately. The average of scores was taken for final decision/analysis. The clinic-radiological evaluation at 24<sup>th</sup> week was used to label the healing as normal (Group II) or impaired (Group III). Patients with normal bony healing were defined by RUST score  $\geq 7$  by the end of the 24<sup>th</sup> week along with painless (no tenderness), motionless (no abnormal mobility), with the presence of transmitted movements at the fracture site. Otherwise, they were labeled as impaired healing [16,17]. Figure 1 deals with the distribution of patients with normal and impaired healing. The clinical and radiological status (RUST Score) of union based on 24<sup>th</sup> week was then analysed against the expression of CYR61 (taken at 4<sup>th</sup>, 7<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup>, 20<sup>th</sup>, and 28<sup>th</sup> post-fracture days).

### Statistical analysis

Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA) for Windows program (15.0 version). 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 *t* test with 95% confidence interval, Mann–Whitney *U* test, and Spearman correlation was used. A *p* value of  $< 0.05$  or  $0.001$  was regarded as significant.

### Results

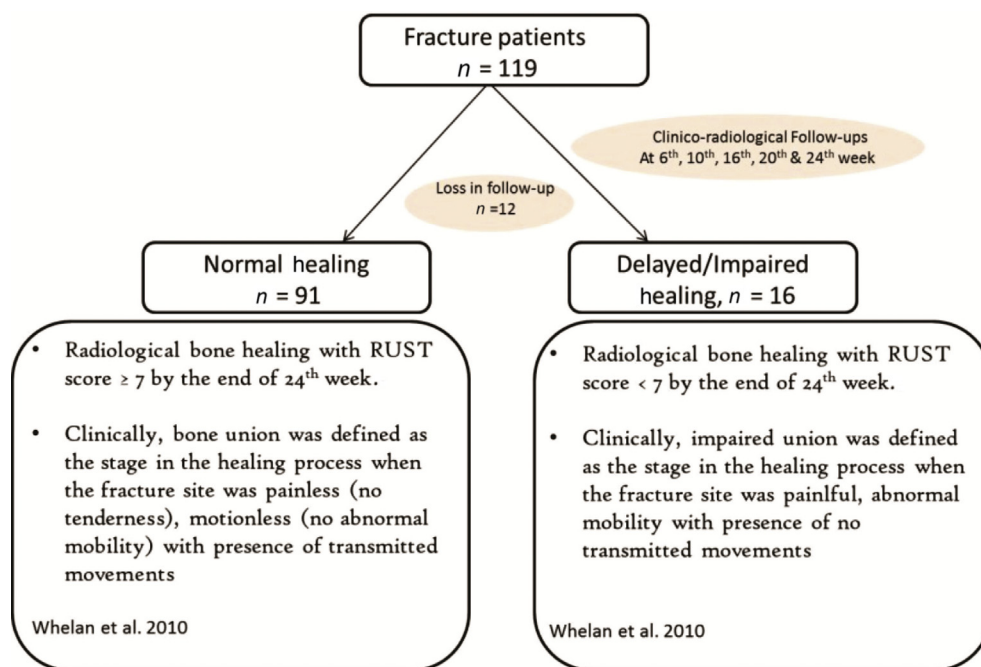
Of 128 patients who were eligible, 9 patients were excluded as per the inclusion–exclusion criteria. Of these 119

patients who were enrolled in our study, 12 patients were lost to follow-up. Thus, only 107 patients were analysed. Table 1 describes the baseline characteristics of the patients of different groups, which do not show any statistically significant difference.

All the enrolled non-fractured healthy controls as per the exclusion criteria were regarded as Group I ( $n = 97$ ). On the basis of the clinico-radiological status of fracture healing at 24<sup>th</sup> week, these 107 patients were distributed into two groups: Group II ( $n = 91$ ) with normal fracture healing and Group III ( $n = 16$ ) with impaired fracture healing. Mean RUST scores at 6<sup>th</sup>, 10<sup>th</sup>, 16<sup>th</sup>, 20<sup>th</sup>, and 24<sup>th</sup> weeks of post-fracture follow-up were  $6.32 \pm 0.49$ ,  $7.89 \pm 0.46$ ,  $8.41 \pm 0.60$ ,  $10.22 \pm 0.90$ , and  $11.08 \pm 0.86$ , respectively, in Group II and  $4.34 \pm 0.39$ ,  $4.65 \pm 0.43$ ,  $5.06 \pm 0.47$ ,  $5.62 \pm 0.46$ ,  $5.87 \pm 0.59$ , respectively, in Group III. The mean time of healing in Group II patients was  $17.2 \pm 3.7$  weeks. The mean RUST scores were significantly higher at each of the radiological follow-ups in Group II than in Group III ( $p < 0.0001$ ) (Figure 2).

In controls (Group I), the CYR61 mRNA and protein baseline expressions were  $1.97 \pm 0.34$  and  $0.25 \pm 0.17$ , respectively. Both CYR61 mRNA and protein baseline expression were lower in controls (Group I) than in baseline expression (at 4<sup>th</sup> post-fracture day) fracture groups (Groups II and III); however, significant difference was observed only with the Group II (Figure 3).

In Groups II and III, expressions of CYR61 mRNA gradually upregulated from the baseline to 20<sup>th</sup> week and then declined. Mean fold CYR61 mRNA expressions at 4<sup>th</sup>, 7<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup>, 20<sup>th</sup>, and 28<sup>th</sup> days of post-fracture biochemical follow-up were  $2.42 \pm 0.43$ ,  $3.48 \pm 0.47$ ,  $5.21 \pm 0.50$ ,  $7.29 \pm 0.69$ ,  $9.36 \pm 1.03$ , and  $9.05 \pm 0.67$ , respectively in normal healing patients (Group II) and  $2.13 \pm 0.37$ ,



**Figure 1** Labelling of cases as either normal healing or impaired healing group on the basis of 24<sup>th</sup> week's clinico-radiological evaluation.

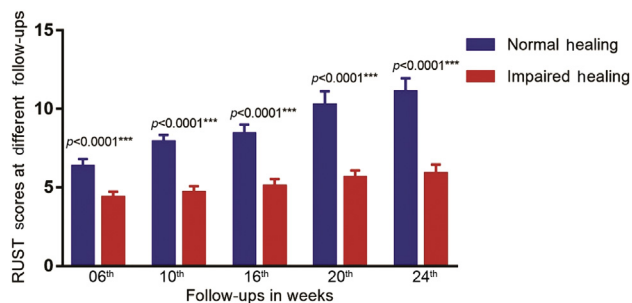
**Table 1** Comparison of baseline characteristics between controls, normal, and impaired healing patients.

Characteristics	Group I (Controls) (n = 97)	Group II (Normal healing) (n = 91)	Group III (Impaired healing) (n = 16)	Significance of difference
Mean age $\pm$ SD (range), y	29.36 $\pm$ 8.11(18–40)	31.08 $\pm$ 7.33 (18–40)	31.25 $\pm$ 6.93 (19–40)	$p = 0.2706^{\dagger}$
Sex				
Male	84 (86.59)	85 (79.44)	13 (12.15)	$\chi^2 = 3.407$
Female	13 (13.4)	6 (5.61)	3 (2.80)	$p = 0.1820^{\ddagger}$
Side of fracture				
Left	—	50 (46.73)	6 (5.61)	$p = 0.2785^{\ddagger}$
Right	—	41 (38.31)	10 (9.35)	
Mode of injury				
Fall from height	—	24 (22.42)	05 (4.67)	$\chi^2 = 6.047$ ;
Road traffic accident	—	46 (42.99)	07 (6.54)	$p = 0.1093^{\#}$
Simple fall	—	21 (19.62)	3 (2.80)	
Slip on ground	—	0 (0)	1 (0.93)	
AO Type				
A1	—	29 (27.10)	4 (3.73)	$\chi^2 = 1.246$ ;
A2	—	27 (25.23)	7 (6.54)	$p = 0.5362^{\#}$
A3	—	35(32.71)	5 (4.67)	
Haemoglobin $\pm$ SD; g/dL (range)	10.92 $\pm$ 1.35(8.9–14.2)	10.58 $\pm$ 1.23(8.4–13.5)	10.62 $\pm$ 0.95 (9.4–12.3)	$p = 0.1740^{\dagger}$
Albumin level $\pm$ SD g/dL (range)	3.81 $\pm$ 0.51 (3.6–4.6)	3.75 $\pm$ 0.22 (3.4–4.5)	3.66 $\pm$ 0.16 (3.4–4.0)	$p = 0.1212^{\dagger}$
Ferritin level $\pm$ SD; ng/mL (range)	102.67 $\pm$ 42.8 (32–197.8)	105.19 $\pm$ 36.6 (28–190.2)	91.81 $\pm$ 34.2 (25–136)	$p = 0.4579^{\dagger}$

$\dagger$ ANOVA (Two-tailed).

$\ddagger$ Fisher exact test;  $\#$ Chi square test.

Data are presented as  $n$  (%) or mean  $\pm$  SD.



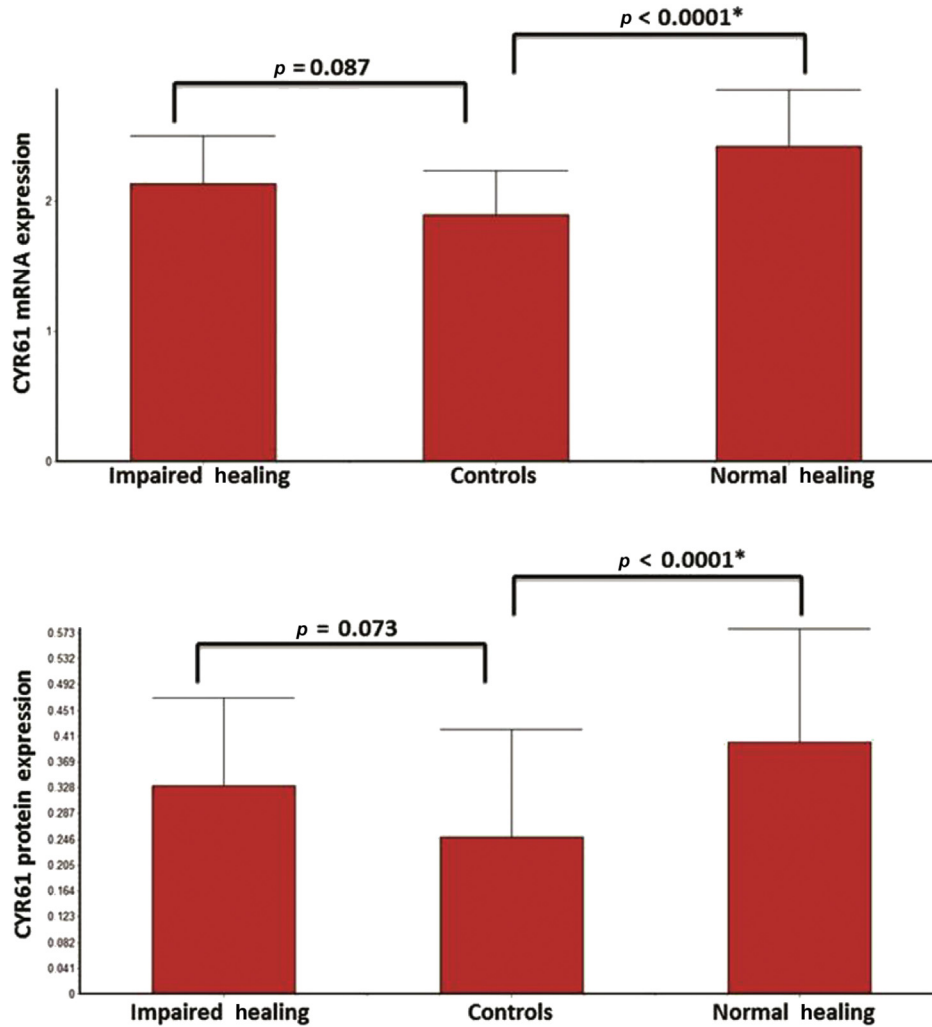
**Figure 2** Graph showing mean RUST score between normal and impaired healing patients.

3.20  $\pm$  0.57, 4.92  $\pm$  0.63, 6.69  $\pm$  0.90, 8.73  $\pm$  0.98, and 8.22  $\pm$  0.90, respectively in impaired healing patients (Group III). Similar to CYR61 mRNA expression, CYR61 protein expression gradually upregulated from the baseline to 20<sup>th</sup> day of follow-up in both the groups and then declined. Mean fold CYR61 protein expressions at 4<sup>th</sup>, 7<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup>, 20<sup>th</sup>, and 28<sup>th</sup> days of post-fracture biochemical follow-up were 0.40  $\pm$  0.18, 0.67  $\pm$  0.21, 1.31  $\pm$  0.33, 1.68  $\pm$  0.35, 2.01  $\pm$  0.43, and 1.81  $\pm$  0.25, respectively in Group II and 0.33  $\pm$  0.14, 0.54  $\pm$  0.27, 1.08  $\pm$  0.32, 1.42  $\pm$  0.47, 1.67  $\pm$  0.57, and 1.52  $\pm$  0.43, respectively, in Group III. The peak expressions of CYR61 (mRNA and protein) were obtained at 20<sup>th</sup> day of post-fracture. The CYR61 mRNA as well as protein expressions were significantly higher at all

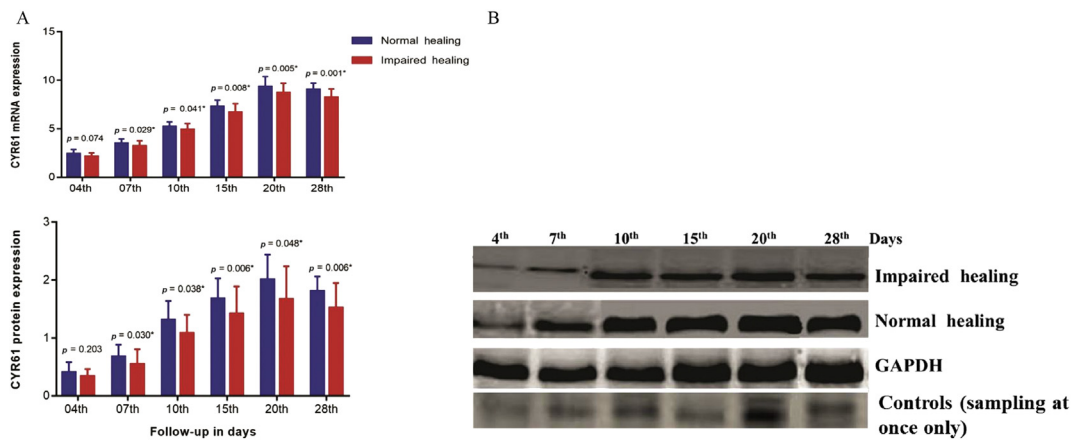
follow-ups in Group II than in Group III except for the 4<sup>th</sup> day of post-fracture (Figures 4A and 4B). However, the expressions of CYR61 mRNA as well as protein in all follow-ups within Group II and most of the follow-ups in Group III also showed statistically significant difference (ANOVA–Dunn’s multiple comparison test). Similarly, Kruskal–Wallis Test (ANOVA) also showed statistically significant difference in both groups among median values of expression ( $< 0.0001$ ). A significant positive correlation was found between the peak mean CYR61 mRNA and protein expression level (at 20<sup>th</sup> day) with the fracture healing progression at different follow-up measured using RUST scoring, except at 6<sup>th</sup> and 10<sup>th</sup> week of post-fracture follow-ups. However, while analyzing the expressions of CYR61 mRNA with CYR61 protein expressions at each biochemical follow-ups, insignificant correlation were found.

## Discussion

The primary aim of this study was to quantify the expression of CYR61 in blood at the initial phase of fracture healing and to correlate their expression levels at selected intervals with the healing outcome of the tibial fracture. Our research hypothesis was that the fracture healing is a complex phenomenon that consists of different overlapped but sequential biological events in which process like angiogenesis plays a vital role in the early phase. Therefore, it might happen that biochemical markers that play an essential role in angiogenesis may show an optimal



**Figure 3** Mean fold change (baseline) of CYR61 mRNA and protein expression level between controls, normal, and impaired healing patients.



**Figure 4** (A) Mean fold change of CYR61 mRNA and protein expression level and (B) Western blot bands of CYR61 protein expression between controls, normal, and impaired healing patients.

differential expression pattern throughout the healing phase of the fracture. Any quantifiable alteration in their expression may predict impaired fracture healing early.

In the present study, the difference in demographic data, such as mean age, sex, side of the fracture, Muller's AO classification, and mode of injuries of different groups were statistically insignificant. This observation suggested that the impaired healing outcome of the fractured tibial bone was independent of age, sex, mode of injuries and side, as well as the pattern of fracture. In the present study, we observed that the expression of CYR61 mRNA as well as protein gradually upregulated from the baseline to 20<sup>th</sup> week in both groups and then declined. In comparison to Group III, CYR61 mRNA and protein expressions remained higher in Group II at all biochemical follow-ups. While analyzing the CYR61 mRNA and protein expression using Mann–Whitney *U* test, the statistical significant difference was observed between both groups at all biochemical follow-ups except at baseline value. In controls, the CYR61 mRNA and protein expressions were lower in expression (baseline) than in fracture groups (normal and impaired healing) and showed significant difference only with the normal healing group. When ANOVA was applied at mean mRNA and protein expression level at different follow-ups within Group II as well as Group III, a significant increase in CYR61 mRNA as well as protein expression was found at the majority of follow-ups within each group. A positive correlation was found between the peak mean value of CYR61 mRNA as well as protein expression with the RUST score at different radiological follow-up at 16<sup>th</sup>, 20<sup>th</sup>, and 24<sup>th</sup> weeks. However, in comparison to CYR61 protein expression, the CYR61 mRNA showed a strong correlation with the RUST score at different radiological follow-ups. An insignificant correlation was found while analyzing the expressions of CYR61 mRNA with CYR61 protein expressions at each biochemical follow up.

To the best of our limited knowledge, no clinical study has been conducted that showing simple diaphyseal tibial fracture healing outcome in relation to the serial estimation of CYR61 gene (mRNA and protein). However, two animal studies performed by Hadjiargyrou et al [12] and Jasmin et al [13] had analysed tibial fracture healing outcome in relation to serial expression of CYR61. In 2000, Hadjiargyrou et al [12] observed that the mRNA expression of CYR61 during fracture repair was temporally expressed. Elevated level of CYR61 is seen as early as 3<sup>rd</sup> and 5<sup>th</sup> post-fracture days. It rises dramatically at 7<sup>th</sup> and 10<sup>th</sup> post-fracture days and finally declines at 14<sup>th</sup> and 21<sup>st</sup> post-fracture days. These results suggest that CYR61 plays a significant role in cartilage and bone formation and may serve as an important regulator of fracture healing. In 2005, Jasmin et al [13] quantified the expression of CYR61 protein during fracture healing in an ovine tibial model. According to them, CYR61 protein expressed during the early phase of fracture healing is indicative to play a significant role in cartilage and bone formation. Its expression generally upregulated at the early phase of fracture healing (2 weeks) and then decreased over the healing time. Decreased fixation stability was associated with a reduced upregulation of the CYR61 protein expression and a reduced vascularisation at 2 weeks leading to a slower healing. In the present study, we also found a similar gradual increase

in CYR61 expression in the post-fracture follow-ups. This may prove the role of CYR61 in early angiogenesis as well as chondrogenesis. However, instead of 15<sup>th</sup> and 20<sup>th</sup> post-fracture day, we could observe the decline of expression by end of 28<sup>th</sup> post-fracture day. This may be because the previous studies involved small animal models which have shorter healing time than human beings.

As our finding shows a significant statistical difference of CYR61 mRNA as well as protein expression between both groups at 7<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup>, 20<sup>th</sup>, and 28<sup>th</sup> post-fracture days, it might act as a prognostic biomarker to predict impaired healing early. In the present study, a positive correlation was observed between peak CYR61 expression (mRNA and protein) and mean RUST score at 16<sup>th</sup>, 20<sup>th</sup>, 24<sup>th</sup> post-fracture weeks. This observation suggests that expression of CYR61 can be correlated with new bone formation (callus at fracture site). However, in the present study, we recommend CYR61 mRNA as a better predictor of impaired fracture healing than CYR61 protein expression as they showed a strong correlation to RUST score. We suggest that an insignificant correlation of expression of CYR61 with RUST score at 6<sup>th</sup> and 10<sup>th</sup> week was because of the fact that radiological early callus appears late on conventional plain radiographs and may be due to the circadian rhythm of bone remodelling, seasonal effects, as well as high range of variation in CYR61 expression level. Furthermore, we also found an insignificant correlation while analyzing the expressions of CYR61 mRNA with CYR61 protein expressions at each biochemical follow-ups. These might be due to differential splicing, turnover, and post-translational modifications. By these findings, we have suggested that the transcriptomic and proteomic data of CYR61 cannot be compared.

Proving our study hypothesis, we found that CYR61 showed different temporal mRNA as well as protein expression patterns at the initial healing phase of a fractured tibia at different intervals. Also, they showed higher expression (both at mRNA and protein level) in Group I (normal healing) than in Group II (impaired healing) and were statistically significant in most of the post-fracture biochemical follow-ups. Beside the above observations, we also observed that CYR61, an angiogenic marker, showed gradual upregulation of expression till 20<sup>th</sup> day of the follow-up. The authors recommend estimation of CYR61 at different intervals during early fracture healing phase to predict early impaired healing cases as a prognostic marker.

However, due to the large intra and inter-individual variability of CYR61, we may not be able to strongly predict the healing outcome of the fracture by taking these biomarkers expressions alone. However, at the same time, the authors realised that instead of selecting a single biomarker, if we use two or combinations of different biomarkers having a vital role in fracture healing, we may be able to strongly predict the healing outcome early. If the role of any of the biochemical marker or combination of markers in relation to the healing of the fracture is further proved, it 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 the biomarker measurements 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 the future. Such an approach would not only benefit the patients' wellbeing but also the entire health care system in terms of the cost implications associated with long lasting treatment interventions and hospitalisation. However, small sample size and the single centric study are the limitations of the present study. Therefore, we recommend further multicentric study with a large sample size to increase the validity, reliability, and generalisability of our observation and inferences.

## Conclusion

Fracture healing is a very complex process involving expression of thousands of biomarkers. Since these biomarkers are derived from both cortical and trabecular bone, they may reflect the metabolic activity of the entire skeleton. Therefore, the detail explorations of the role as well as the correlation of the biomarkers with fracture healing or bone remodelling process are in demand. These markers may not only predict the impaired healing of the fractured bone but also predict the same for various other skeleton disorders. In the present study, most of the post-fracture follow-ups observed a significantly higher CYR61 expression in the normal healing group than in the impaired healing group and showed a positive correlation with fracture healing measured using the RUST score. Thus, it suggests that the CYR61 expression may provide an early prediction of the healing outcomes of simple diaphyseal tibial fractures. However, further multicentric study is needed to increase the generalisability of our observation.

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## Conflicts of interest

The authors have no conflict of interest in this article.

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