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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 4th, 7th, 10th, 15th, 20th and 28th 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 6th, 10th, 16th, 20th, and 24th 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 24th 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.

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^{th}, 7^{th}, 10^{th}, 15^{th}, 20^{th}, and 28^{th} 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 pimer, GCAGCTCAACGAGGACTG; probe, CGCCGAAGTTGCATTCCAGCC (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, GAAGGTGAAGGTCGGAGTC; reverse primer, GAAGATGGTGATGGGATTTC; probe, CAAGCTTCCCGTTCTCAGCC (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^{th}, 10^{th}, 16^{th}, 20^{th}, and 24^{th} post-fracture weeks. The

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