Does mammalian target of rapamycin or sestrin 1 protein signaling have a role in bone fracture healing?

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Background/aim: Fracture healing is a complex physiological process that involves a well-orchestrated series of biological events. The mammalian target of rapamycin (mTOR) and sestrin 1 (SESN 1) play a central role in cell metabolism, proliferation, and survival. The aim of our study is to present serum mTOR and SESN 1 levels by comparing patients with or without bone fractures. It is also a guide for further research on the roles of these proteins in fracture healing.

Materials and methods: A total of 34 patients (10 females, 24 males) with bone fractures and 32 controls (10 females, 22 males) participated in this study. After collecting serum venous blood samples, the quantitative sandwich ELISA technique was used for the determination of serum mTOR and SESN 1 levels.

Results: The mean serum mTOR level was significantly higher in the fracture group compared to the control group (P = 0.001). However, SESN 1 levels did not significantly differ between groups (P = 0.913).

Conclusion: We found that serum mTOR levels increased on the first day after fracture compared to the control group. However, we obtained no significant difference between groups in terms of SESN 1 levels. This study may guide further clinical studies investigating the potential role of mTOR signaling in the bone healing process.

Key words: mTOR, sestrin, bone, fracture

1. Introduction

Fractures, mostly arising from injury, are an important public health burden [1]. Approximately 5%–10% of the 6.2 million fractures occurring annually in the United States are associated with impaired healing including delayed healing or nonunion [2].

Fracture healing is a complex physiological process that involves a well-orchestrated series of biological events [3]. While our knowledge has vastly expanded, with the increasing understanding of the multiple factors and complex pathways involved, many new developments are anticipated in the years to come. Many local and systemic regulatory factors such as growth and differentiation factors, hormones, cytokines, and the extracellular matrix interact with several cell types including bone and cartilage, forming primary cells or even muscle mesenchymal cells, recruited at the fracture-injury site or from circulation [4].

The mammalian target of rapamycin (mTOR) protein, a 289-kDa serine-threonine kinase, has a central role in cell metabolism, proliferation, and survival [4,5]. Different factors including nutrients, growth factors, cellular energy, and stress regulate mTOR signaling [6]. Insulin and Ras (Rat sarcoma) signaling pathways are also important regulators of mTOR signaling activation [7]. The role of the mTOR pathway has been examined in age-related pathologies such as neurodegenerative disease, cancer, heart disease, and metabolic diseases [8–11]. Recent studies have implicated the potential role of mTOR in regulating multiple aspects of cartilage development and preosteoblast differentiation [12,13].

Sestrins (SESN) are highly conserved proteins that exhibit oxidoreductase activity and therefore the expression of SESNs is upregulated by DNA damage, oxidative stress, and hypoxia [14]. Independent of their antioxidant activity, SESNs are also negative regulators of mTOR signaling. Several studies revealed the importance of SESNs in insulin resistance, muscle degeneration, cardiac dysfunction, mitochondrial pathologies, and tumors, but little is known about the relationship between bone fractures and mTOR and SESN-1.

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Previous studies have emphasized the role of mTOR signaling in bone metabolism [2]. However, to date, we could find no clinical study investigating serum mTOR and SESN-1 concentrations in patients with the bone fracture. We hypothesized that defining serum mTOR and SESN-1 concentrations in patients with fractures may demonstrate their potential roles in the bone healing process. The evaluation of the relationship between these serum proteins and the pathway occurring in response to bone fracture may also guide further studies describing the bone healing process. Therefore, in this study, we aimed to compare serum mTOR and SESN-1 levels in patients with or without bone fracture.

2. Materials and methods

2.1. Study population
This cross-sectional study was completed after approval from our institutional ethical review board and was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from each participant.

Between May 2017 and October 2017, patients who were admitted to our university hospital and diagnosed with bone fracture were included in the study. Patients who were over 65 years old (52 patients) or had systemic comorbid diseases (25 patients) were excluded from the study. Patients with high-energy trauma (30 patients) and multiple fractures were also excluded from the study group. A control group consisting of healthy individuals between 18 and 65 years of age was created by a biochemistry laboratory physician who selected participants from consecutive healthy individuals. A total of 34 patients (10 females, 24 males) with bone fractures and 32 controls (10 females, 22 males) participated in the study.

2.2. Blood sample collection and analysis
In the fracture group, overnight fasting venous blood samples were taken from the patients on the first day of stay in the hospital. The control group consisted of healthy individuals between 18 and 65 years old without any comorbid disease and bone fracture. These individuals were called 1 day before and asked to attend after overnight fasting. Venous blood samples were collected from all participants into red-top tubes (Becton Dickinson, UK). The serum sample tubes were allowed to clot before centrifugation. After centrifugation at 4 °C for 15 min at 3500 rpm, the serum was aliquoted and immediately frozen at -80 °C (WiseCryo, South Korea). The quantitative sandwich ELISA technique was used for the determination of serum mTOR and SESN-1 (YL Biotech Co. Ltd., China). Tests were performed according to the manufacturer's recommendations. Patients with insufficient samples or incorrect results were excluded from the study.

2.3. Statistical analysis
The sample size estimation was calculated using GPower software. The minimum sample size was calculated by taking into account the large effect size of results for a false-positive rate of 5% (α = 0.05) and a power of at least 80% (β = 0.2) [15–17]. Using these parameters and adjusting for multiple comparisons, we required a minimum sample size of 26 patients for each group. A total of 32 patients per group was requested, taking into consideration the estimated 20% dropout rate (failure of test results); thus, a total of 64 patients were planned to be enrolled in the current study.

Statistical analysis was performed using SPSS 22.0 (IBM Corp., USA). Numerical variables are given as means and standard deviations, and categorical variables are given as frequencies and percentages. The comparison of means was completed with the t-test or Mann-Whitney U-test in accordance with the Shapiro–Wilk normality test. Comparison of frequencies was performed with the chi-square test and the Pearson correlation test was used to evaluate the correlation coefficient between mTOR and SESN-1 levels in both study groups. P < 0.05 was considered to be statistically significant.

3. Results
A total of 66 patients (46 females, 20 males) were enrolled in the study. The fracture group consisted of 34 patients (24 females, 10 males) with mean age of 45.8 ± 12.6 years (range: 18 to 64 years). The control group consisted of 32 individuals (22 females, 10 males) with mean age of 47.6 ± 10 years (range: 28 to 63 years). The fracture group and control group had no significant differences in terms of age or sex (P = 0.728 and P = 0.334, respectively). The mean serum mTOR level was significantly higher in the fracture group compared to the control group (P = 0.001). However, SESN-1 levels did not significantly differ between groups (P = 0.913) (Table 1). The distributions of fractures in the fracture group are shown in Table 2.

We observed a significant correlation between mTOR and SESN-1 levels in the fracture group (P = 0.015, r = 0.412) and in the control group (P = 0.043, r = 0.359).

4. Discussion
Bone fracture is a common injury that may initiate a series of physiological and pathological reactions. A number of promising therapeutic approaches have been developed, such as improvement of internal fixation devices and the application of novel biological materials; however, delayed healing or nonunion may occur in 5%–10% of fractures, adding further to patient morbidity and the expense of treatment [18]. The improvement of patient morbidity and reduction of costs is an incentive for the development of novel therapies to enhance fracture healing [19]. Recent

Table 1. mTOR and SESN-1 levels of the study groups.

ng/mL	Fracture group (n = 34)	Control group (n = 32)	P-values
mTOR	15.4 ± 6.2 (0.1–22.9)	9.6 ± 5.8 (0.1–22.9)	0.001*
	[13.2–17.5]	[7.2–12.1]	
SESN-1	19.4 ± 8.7 (0.2–33.8)	19.6 ± 7.3 (0.2–28)	0.913*
	[16.4–22.4]	[16.5–22.7]	

* P-values according to Student t-test.

Table 2. The distribution of fractures.

Bone	n	%
Humerus	3	8.8
Hand/forearm	2	5.9
Pelvis	4	11.8
Hip	2	5.9
Femur	5	14.7
Tibia	5	14.7
Foot/ankle	5	14.7
Others	8	23.5
Total	34	100

studies have indicated the complexity of the biological structure of bone [20]. The most important finding of this study was that serum mTOR levels increased with fractures while serum SESN-1 levels remained stable when patients with fractures were compared with a control group.

mTOR is identified as a key cellular signaling protein used to respond to diverse environmental stimuli, control numerous processes that generate or consume a mass of energy and nutrients, regulate most major cellular functions, and assist most organisms' inefficient transformation between anabolic and catabolic states. It plays an important role in regulating basic cell behaviors like growth and proliferation and also has an important role in bone growth and proliferation [21]. On the other hand, there is still debate about the effect of mTOR in osteogenesis. The negative and positive effects of mTOR inhibition on osteogenesis were also reported [21,22]. Interestingly, the activation of mTOR signaling has also been found to promote osteoblast differentiation [21]. In summary, studies showed that mTOR signaling serves as a double-edged sword in regulating cell differentiation [21]. These differences appear to depend on cell type and treatment conditions used [22]. Holstein et al. reported that rapamycin, an mTOR inhibitor, affected early fracture healing and inhibited callus formation in rats [16]. In another study by Yang et al., the authors mentioned that the inhibition of mTOR promoted bone fracture healing in rats [17]. In our study, mTOR levels were significantly higher in the fracture group compared to the control group.

Departing from in vitro and animal studies, we analyzed serum mTOR levels in our patients and found significantly higher mTOR levels. However, it should be kept in mind that stress and inflammatory response secondary to trauma may also lead to the increased mTOR levels. After fracture, the bone healing process begins immediately with increases in protein and growth factor synthesis, which play roles in fracture healing [23]. Previous studies showed the contribution of mTOR signaling to protein synthesis [24]. The increased levels of mTOR in our patient group may be due to increased protein synthesis secondary to fracture and trauma. In summary, the current literature agrees on the vital role of mTOR signaling for bone hemostasis [21].

In terms of demonstration of mTOR levels in fracture patients, this pathway has been investigated in terms of musculoskeletal disorders after abruptly leaving the mTOR pathway. The effect and significance of mTOR had been previously studied in various areas in the literature such as neurofibromatosis, rotator cuff tears, pathologies of nucleus pulposus, and osteosarcoma [25–29]. We think that this will be a guide for future studies about fractures in our work.

The SESNs are a class of proteins that are also induced by stress [14]. Currently, three isoforms of the SESN family are known: SESN-1, SESN-2, and SESN-3. Studies have shown that these proteins are important for the maintenance of metabolic homeostasis, for the protection of cells against age-related physiological damage, and, mainly, for the control of adenosine monophosphate kinase (AMPK)/mTOR signaling [30]. Previous studies showed that inhibition of SESN resulted in increases of age- and obesity-related pathologies [14]. SESNs suppress oxidative stress and regulate autophagy [31]. Some conditions have been shown to increase SESN levels such as hypoxia, oxidative stress, DNA damage,

and physical exercise [32]. In our study, we also evaluated levels of serum SESN-1 because of its relationship with mTOR signaling. According to our results, we found no significant difference in SESN-1 levels when patients with fractures were compared to healthy individuals. However, some studies reported that SESN overexpression potently suppresses mTOR signaling [30]. We found that there was a correlation between mTOR and SESN in both groups. This shows that more studies are needed to evaluate serum SESN levels on different days after fracture and to examine more patients.

The main limitation of this study is that serum mTOR and SESN-1 levels were only measured in the first 24 h after fracture. However, this study is the first in the literature evaluating serum mTOR and SESN-1 levels in patients with or without fracture. We also compared two homogeneous patient populations without any systemic comorbid disease to exclude other pathologies that may affect mTOR and SESN signaling pathways.

According to our results, the serum mTOR levels increased the first day after fracture compared to a control group. However, we obtained no significant difference between groups in terms of SESN-1 levels. This study may guide further clinical studies investigating the potential role of mTOR signaling in the bone healing process.

References

1. Donaldson LJ, Reckless IP, Scholes S, Mindell JS, Shelton NJ. The epidemiology of fractures in England. Journal of Epidemiology & Community Health 2008; 62: 174-180. doi: 10.1136/jech.2006.056622

2. Dimitriou R, Tsiridis E, Giannoudis P V. Current concepts of molecular aspects of bone healing. Injury 2005; 36: 1392-1404. doi: 10.1016/j.injury.2005.07.019

3. Tsiridis E, Upadhyay N, Giannoudis P. Molecular aspects of fracture healing: which are the important molecules? Injury 2007; 38 (Suppl. 1): S11-25. doi: 10.1016/j.injury.2007.02.006

4. Hori K, Sen A, Artavanis-Tsakonas S. Notch signaling at a glance. Journal of Cell Science 2013; 126: 2135-2140. doi: 10.1242/jcs.127308

5. Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. Cancer Cell 2007; 12: 9-22. doi: 10.1016/j.ccr.2007.05.008

6. Sengupta S, Peterson TR, Sabatini DM. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Molecular Cell 2010; 40: 310-322. doi: 10.1016/j.molcel.2010.09.026

7. Takano A, Usui I, Haruta T, Kawahara J, Uno T et al. Mammalian target of rapamycin pathway regulates insulin signaling via subcellular redistribution of insulin receptor substrate 1 and integrates nutritional signals and metabolic signals of insulin. Molecular Cellular Biology 2001; 21 (15): 5050-5062. doi: 10.1128/MCB.21.15.5050-5062.2001

8. Sciarretta S, Volpe M, Sadoshima J. Mammalian target of rapamycin signaling in cardiac physiology and disease. Circulation Research 2014; 114: 549-564. doi: 10.1161/CIRCRESAHA.114.302022

9. Spilman P, Podlutskaya N, Hart MJ, Debnath J, Gorostiza O et al. Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-β levels in a mouse model of Alzheimer's disease. PLoS One 2010; 5 (4): e9979. doi: 10.1371/journal.pone.0009979

10. Yang SB, Tien AC, Boddupalli G, Xu AW, Jan YN et al. Rapamycin ameliorates age-dependent obesity associated with increased mTOR signaling in hypothalamic POMC neurons. Neuron 2012; 75: 425-436. doi: 10.1016/j.neuron.2012.03.043

11. Takayama K, Kawakami Y, Lavasani M, Mu X, Cummins JH et al. mTOR signaling plays a critical role in the defects observed in muscle-derived stem/progenitor cells isolated from a murine model of accelerated aging. Journal of Orthopaedic Research 2017; 35: 1375-1382. doi: 10.1002/jor.23409

12. Chen J, Long F. MTOR signaling in skeletal development and disease. Bone Research 2018; 6: 1. doi: 10.1038/s41413-017-0004-5

13. Fitter S, Matthews MP, Martin SK, Xie J, Ooi SS et al. mTORC1 plays an important role in skeletal development by controlling pre-osteoblast differentiation. Molecular and Cellular Biology 2017; 37: 1-20. doi: 10.1128/MCB.00668-16

14. Lee JH, Budanov AV, Karin M. Sestrins orchestrate cellular metabolism to attenuate aging. Cell Metabolism 2013; 18: 792-801. doi: 10.1016/j.cmet.2013.08.018

15. Alvarez-Garcia O, Carbajo-Perez E, Garcia E, Gil H, Molinos I et al. Rapamycin retards growth and causes marked alterations in the growth plate of young rats. Pediatric Nephrology 2007; 22: 954-961. doi: 10.1007/s00467-007-0456-8

16. Holstein JH, Klein M, Garcia P, Histing T, Culemann U et al. Rapamycin affects early fracture healing in mice. British Journal of Pharmacology 2008; 154: 1055-1062. doi: 10.1038/bjp.2008.167

17. Yang G, Duan X, Lin D, Li T, Luo D et al. Rapamycin-induced autophagy activity promotes bone fracture healing in rats. Experimental and Therapeutic Medicine 2015; 10: 1327-1333. doi: 10.3892/etm.2015.2660

18. Einhorn TA. Enhancement of fracture-healing. Journal of Bone and Joint Surgery-American Volume 1995; 77: 940-956. doi: 10.2106/00004623-199506000-00016

19. O'Neill KR, Stutz CM, Mignemi NA, Burns MC, Murry MR et al. Micro-computed tomography assessment of the progression of fracture healing in mice. Bone 2012; 50: 1357-1367. doi: 10.1016/j.bone.2012.03.008

20. Guntur AR, Rosen CJ. Bone as an endocrine organ. Endocrine Practice 2012; 18 (5): 758-762. doi: 10.4158/EP12141.RA

21. Shen G, Ren H, Qiu T, Zhang Z, Zhao W et al. Mammalian target of rapamycin as a therapeutic target in osteoporosis. Journal of Cellular Physiology 2018; 233 (5): 3929-3944. doi: 10.1002/jcp.26161

22. Martin SK, Fitter S, Bong LF, Drew JJ, Gronthos S et al. NVP-BEZ235, a dual pan class I PI3 kinase and mTOR inhibitor, promotes osteogenic differentiation in human mesenchymal stromal cells. Journal of Bone and Mineral Research 2010; 25: 2126-2137. doi: 10.1002/jbmr.114

23. Ghiasi MS, Chen J, Vaziri A, Rodriguez EK, Nazarian A. Bone fracture healing in mechanobiological modeling: a review of principles and methods. Bone Reports 2017; 6: 87-100. doi: 10.1016/j.bonr.2017.03.002

24. You JS, Anderson GB, Dooley MS, Hornberger TA. The role of mTOR signaling in the regulation of protein synthesis and muscle mass during immobilization in mice. Disease Models & Mechanisms 2015; 8: 1059-1069. doi: 10.1242/dmm.019414

25. Ma J, Li M, Hock J, Yu X. Hyperactivation of mTOR critically regulates abnormal osteoclastogenesis in neurofibromatosis type 1. Journal of Orthopaedic Research 2012; 30: 144-152. doi: 10.1002/jor.21497

26. Liu X, Joshi SK, Samagh SP, Dang YX, Laron D et al. Evaluation of Akt/mTOR activity in muscle atrophy after rotator cuff tears in a rat model. Journal of Orthopaedic Research 2012; 30: 1440-1446. doi: 10.1002/jor.22096

27. Zhou R, Zhang Z, Zhao L, Jia C, Xu S et al. Inhibition of mTOR signaling by oleanolic acid contributes to its anti-tumor activity in osteosarcoma cells. Journal of Orthopaedic Research 2011; 29: 846-852. doi: 10.1002/jor.21311

28. Jiang LB, Jin YL, Wang HR, Jiang YQ, Dong J. Glucosamine protects nucleus pulposus cells and induces autophagy via the mTOR-dependent pathway. Journal of Orthopaedic Research 2014; 32: 1532-1542. doi: 10.1002/jor.22699

29. Joshi SK, Liu X, Samagh SP, Lovett DH, Bodine SC et al. MTOR regulates fatty infiltration through SREBP-1 and PPARγ after a combined massive rotator cuff tear and suprascapular nerve injury in rats. Journal of Orthopaedic Research 2013; 31: 724-730. doi: 10.1002/jor.22254

30. Budanov AV, Karin M. p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. Cell 2008; 134: 451-460. doi: 10.1016/j.cell.2008.06.028

31. Ho A, Cho CS, Namkoong S, Cho US, Lee JH. Biochemical basis underlying sestrins' physiological activities. Trends in Biochemical Sciences 2016; 41 (7): 621-632. doi: 10.1016/j.tibs.2016.04.005

32. Crisol BM, Lenhare L, Gaspar RS, Gaspar RC, Muñoz VR et al. The role of physical exercise on Sestrin1 and 2 accumulations in the skeletal muscle of mice. Life Sciences 2018; 194: 98-103. doi: 10.1016/j.lfs.2017.12.023