

Polymorphisms in BMP4 and FGFR1 Genes Are Associated With Fracture Non-Union

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ABSTRACT: Fracture healing is a complex process influenced by a multitude of factors and expression of several thousand genes. Polymorphisms in these genes can lead to an extended healing process and explain why certain patients are more susceptible to develop non-union. A total of 16 SNPs within five genes involved in bone repair pathogenesis (*FAM5C*, *BMP4*, *FGF3*, *FGF10*, and *FGFR1*) were investigated in 167 patients with long bone fractures, 101 with uneventful healing, and 66 presenting aseptic non-unions. Exclusion criteria were patients presenting pathological fractures, osteoporosis, hypertrophic and infected non-unions, pregnancy, and children. All genetic markers were genotyped using TaqMan real-time PCR. Chi-square test was used to compare genotypes, allele frequencies, and haplotype differences between groups. Binary logistic regression analyzed the significance of many covariates and the incidence of non-union. Statistical analysis revealed open fracture to be a risk factor for non-union development ($p < 0.001$, OR 3.6 [1.70–7.67]). A significant association of haplotype GTAA in *BMP4* ($p = 0.01$) and *FGFR1* rs13317 ($p = 0.005$) with NU could be observed. Also, uneventful healing showed association with *FAM5C* rs1342913 ($p = 0.04$). Our work supported the role of *BMP4* and *FGFR1* in NU fracture independently of the presence of previously described risk factors. © 2013 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 31:1971–1979, 2013

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The biology of fracture healing is a complex process that follows specific regenerative patterns and involves changes in the expression of several thousand genes.¹ This process is influenced by a multitude of factors, some understood and others still to be delineated. Hematoma formation is the first step for bone repair, followed by an inflammatory response with the release of growth factors, such as platelet-derived growth factors (PDGF), tumor growth factor type- β (TGF- β), interleukins (IL) 1 and 6 and prostaglandin E2. In this early phase, osteoprogenitor cells and fibroblasts proliferate and are associated with bone morphogenetic proteins (BMP) and fibroblast growth factors (FGF) expressions. As the hematoma matures, a collagenous matrix develops with neovascularization, providing a scaffold for these cells.²

Although the high regenerative capacity of bone tissue, this biological process sometimes fails and fractures may heal in unfavorable anatomical positions, show a delay in healing, or even develop pseudoarthrosis or non-unions.³ Clinically, non-union (NU) is usually established between 6 and 8 months post-fracture,⁴ with a 14% frequency in high-grade open femoral fractures.⁵ However, despite the previ-

ously related risk factors associated with NU, such as poor mechanical stability, the presence of a large gap, soft tissue damage, infection, pharmacological agents and smoking, there is still the question of whether systemic factors or impairment of the cellular function may play a role in fracture healing.^{6,7} Taking into consideration the possible influence of genetic variation on the fracture healing response among individuals and a potential genetic predisposition to fractures NU, a few recent studies^{7,8} have assessed the correlation between related genes for bone healing and the development of NU. Among the results, polymorphisms in *PDGF*,⁸ *NOGGIN*, and *SMAD6* from the BMP pathway⁷ were appointed as contributors to fracture NU. However, it's important to consider that a possible explanation for the pathogenesis of non-unions is the existence of polymorphisms in specific regions of the gene mediators related to different phases of bone healing process, including inflammatory response, fibroblasts proliferation, and differentiation of osteoprogenitor cells.

Recent work⁹ has identified the involvement of the gene *FAM5C*, also known as bone morphogenetic protein and retinoic acid-inducible neural-specific protein-3, in the inflammatory response. *FAM5C* is a protein localized in mitochondria and identified as a gene that is induced by BMP and retinoic acid signaling on chromosome 1q31.1.¹⁰ Despite *FAM5C* enhances osteoblastic differentiation in vitro,¹¹ the function of *FAM5C* in bone healing remains unknown.

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Out of the 64 genes associated with bone metabolism, only *BMP4* fulfilled the criteria of suggestive evidence of linkage in a 126 affected sib-pairs study.¹² *BMP4* belongs to TGF- β superfamily and is located on chromosome 14q22–23. Haploinsufficiency can result in abnormalities in skeletal structures.¹³ Moreover, the *BMP4* expression increases significantly during fracture healing^{14,15} and in cases of fibrodysplasia ossificans progressiva in humans.¹⁶ Previous studies have shown that C > T polymorphism in *BMP4* rs17563 is associated with ossification of the posterior longitudinal ligament,^{17,18} but show no association with fracture NU.⁸

The antagonism between FGF and BMP signaling systems has been related in several studies.^{19–21} FGFs mediate their cellular responses by activating a family of four receptor tyrosine kinases, FGFR1 through FGFR4, which display different biologic functions.²²

Non-union is a well-described problem, though it is still unknown why certain patients are more susceptible to develop non-unions than others. Based on *FAM5C*, *BMP4*, and *FGF* functions and on their possible participation in multiple pathways involved in the bone repair pathogenesis, the purpose of this study was to investigate whether genetic variants within these genes can be correlated with impaired fracture healing. Greater knowledge about gene factors related to bone healing could serve as a foundation for novel therapies, addressing clinically challenging situations that often compromise the proper restoration of bone function.

MATERIALS AND METHODS

Patients

This study was conducted according to recommendations from the National Institute of Traumatology and Orthopaedic Research Ethics Committee (Registration number 0029.0.305.000-10). One hundred sixty-seven patients were randomly selected from an outpatient pool during 1 year. They underwent routine post-injury check-up at least 9 months after sustaining humeral, femoral, or tibial fractures. All patients were included in the study after the signature of an informed consent document. Subjects answered a personal and medical history anamnesis as well as had their smoking habits and alcohol consumption registered. The bone reparative process was evaluated clinically and with the use of digital X-rays. Exclusion criteria were patients presenting pathological fractures, osteoporosis, and other bone diseases that could interfere with calcium/phosphorus metabolism, hypertrophic and infected non-unions, pregnancy, and being younger than 18 years of age. The control group (U-union) consisted of 101 subjects with uneventful healed fractures, while the non-union (NU) group had 66 individuals with delayed bone healing. The baseline clinical parameters for the subject population are shown in Table 1.

Fracture Healing Diagnosis

Non-union was defined as failure of the fracture to heal within 9 months in a patient without radiographic signs of progression of the fracture callus between the third and sixth months after the fracture. Also, non-unions were scored

according to Calori et al.²³ In this scoring system, values are attributed to clinical features such as bone quality, type of initial lesion (open or closed), previous surgeries, mechanical stability, NU type, fracture alignment, gap size, soft tissue status at the time of the fracture, and patient's comorbidities. Union was diagnosed if, on clinical examination, there was no tenderness or pain at the fracture site with full weight bearing. Radiographic union was diagnosed in fractures with mature bone bridges crossing the fracture site in anteroposterior and lateral radiographs and the absence of a visible principal fracture line.

Single-Nucleotide Polymorphism Selection and Genotyping

Genomic DNA was obtained from saliva samples as previously described.²⁴ The amount and purity of the DNA was determined by the use of a Nanodrop[®] spectrophotometer (Thermo Scientific, Wilmington, DE). Only DNA samples presenting A260 nm/A280 nm ratio greater than 1.9 were used.

Sixteen single nucleotide polymorphisms (SNPs) in *BMP4*, *FAM5C*, *FGF3*, *FGF10*, and *FGFR1* were selected. These SNPs had previously been identified and reported in the database of the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/SNP/>), with minor allele frequencies greater than 0.2. Details of the studied genetic variants are shown in Table 2.

Real Time Polymerase Chain Reactions with TaqMan chemistry (Applied Biosystems, Foster City, CA) held in total 1.5 μ l/reaction were used for genotyping the selected markers in a PTC-225 tetrad thermocycler (Peltier Thermal Cycler, Bio-Rad Life Science, Corston, UK).

Since *BMP4*, *FAM5C*, *FGF3*, and *FGF10* are found in the same chromosome, it is believed that they are inherent as a unit. Polymorphisms in these genes were analyzed not only individually, but also in combination (haplotypes).

Statistical Analyses

The sample size needed for a study with 80% power was calculated using an online calculator at <http://statpages.org/proppowr.html>. Nominal variables were expressed as frequencies and percents. To access the significance of nominal variables between groups, the chi-square (χ^2) test was performed. Continuous variables were expressed as mean and standard deviation. Then, after the Shapiro–Wilk test showed normal distribution among variables, analysis of variance and Student's *t*-test were performed. Differences in the frequency of genotypes and alleles between NU and control groups were analyzed using the Fisher's exact and chi-square (χ^2) tests after fitting for Hardy–Weinberg equilibrium. Values of $p < 0.05$ were considered statistically significant and the risk associated to individual alleles and genotypes were calculated as the odds ratio (OR) with 95% confidence intervals (CI). Binary logistic regression analysis was performed to explore many covariates simultaneously. Statistical analysis was performed using STATA 11.1 (StataCorp, College Station, TX). In order to calculate linkage disequilibrium and haplotypes, the computer program package ARLEQUIN was used (v.20–<http://anthro.unige.ch/arlequin>) and Bonferroni correction was used to correct multiple comparisons (<http://www.quantitativeskills.com/sisa/calculations/bonfer.htm>).

RESULTS

Clinical Findings

From the total 167 subjects included in this study, 66 showed atrophic long bone NU (NU group), 25 females

Table 1. Baseline Demographic and Clinical Characteristics of the Study Population

Variables	Total (n = 167)	Union (n = 101)	NU (n = 66)	p-value	Odds Ratio	95% CI
	n (%)	n (%)	n (%)			
Ethnic group						
Caucasians	88 (52.7)	51 (50.5)	37 (56.1)	0.48	1.25	0.64–2.45
Non-Caucasians	79 (47.3)	50 (49.5)	29 (43.9)			
Age (years) ^a	46.72 ± 13.76	45.37 ± 13.26	48.76 ± 14.34	0.07		
Gender						
Female	70 (41.9)	45 (44.5)	25 (37.9)	0.39	0.39	0.38–1.50
Male	97 (58.1)	56 (55.5)	41 (62.1)			
Smoking						
Non-smoking	140 (83.8)	86 (85.1)	54 (81.9)	0.56	0.78	0.32–1.95
Smoking	27 (16.2)	15 (14.9)	12 (18.1)			
Alcohol consumption						
No	76 (45.5)	50 (49.5)	26 (39.4)	0.77	1.10	0.53–2.30
Yes	75 (44.9)	51 (50.5)	24 (36.4)*			
AUDIT ^a	3.41 ± 5.32	4.30 ± 6.1	2.69 ± 5.0	0.93		
General medical condition						
Systemic disease	39 (23.3)	27 (26.7)	12 (18.2)	0.20	0.61	0.26–1.39
Diabetes	5 (2.9)	4 (4.0)	1 (1.5)	0.36	0.37	0.02–3.66
High blood pressure	32 (19.2)	21 (20.8)	11 (16.6)	0.50	0.76	0.31–1.82
Hypotireoidism	2 (1.2)	2 (2.0)	0	0.25	0.00	0.00–6.29
Current medication						
Any medication	10 (5.9)	5 (5.0)	5 (7.5)	0.48	1.57	0.38–6.60
Antihypertension	29 (17.3)	20 (19.8)	9 (13.6)	0.30	0.64	0.25–1.62
Antimicrobials	0	0	0	—	—	—
NSAIDs ^b	5 (2.9)	2 (2.0)	3 (4.5)	0.34	2.36	0.31–20.8
SAIDs ^c	0	0	0	—	—	—
Hormony reposition	1 (0.5)	0	1 (1.5)	0.21		
Antidepressant	2 (1.1)	1 (1.0)	1 (1.5)	0.76	1.54	0.00–57.42
Fracture type						
Open	49 (29.3)	19 (18.8)	30 (45.5)	0.0002*	3.60	1.70–7.67
Closed	118 (70.7)	82 (81.2)	36 (54.5)			
Calori score ²³	—	—	20.7 ± 6.7			
Fracture site						
Humeral shaft	67 (40.1)	39 (38.6)	28 (42.4)	0.92	1.09	0.75–1.60
Femur diaphysis	42 (25.1)	25 (24.8)	17 (25.8)			
Femur neck	5 (3.0)	3 (2.9)	2 (3.0)			
Tibial diaphysis	53 (31.8)	34 (33.7)	19 (28.8)			

Control, patients with uneventful healing; NU, non-union group; chi-square test was used for nominal variables. ^aStudent *t*-test. ^bNon-steroidal anti-inflammatory drugs. ^cSteroidal anti-inflammatory drugs. ***p*-value < 0.05 are considered significant.

and 41 males, with mean age 48.76 ± 14.34 years. All these cases required further intervention to try to achieve union. The Union group consisted of 101 individuals, 45 females and 56 males, with mean age of 45.37 ± 13.26 . Clinical data from both groups are summarized in Table 1.

No differences were found for ethnic group, age, sex, alcohol consumption, or smoking habits. Considering the patient medical condition, the use of current medication and the fractures sites, no statistical differences could be found between groups. However, there was a significant high incidence of open fracture in the NU group compared to the Union group ($p = 0.0002$). The risk associated with developing fracture NU for patients with open fracture was 3.6 (95% CI = 1.7–7.67) times higher than in Union patients. Moreover,

the mean of Calori²³ score observed in patients showing NU was 20.7 ± 6.7 from the maximum 50.0 score.

Frequency Analysis of Genetic Polymorphism

Allele and genotype frequencies for all SNPs were within Hardy–Weinberg equilibrium in both groups (data not shown). The most significant genotyping results among the study participants are summarized in Table 3. Chi-square tests showed that *BMP4* rs17563 had a significant difference in the genotype frequency ($p = 0.02$). A borderline difference was found between *FAM5C* rs1342913 genotypes (AG + GG) and favorable healing ($p = 0.04$ /OR = 0.52 (95% CI = 0.26–1.02), as well as *FGFR1* rs13317, exhibited a higher frequency of the TT genotype in Union group ($p = 0.03$).

Table 2. Genetic Variants in Each Investigated Gene

Gene	Chromosome	SNP ID	SNP Type	Base Pair Position	Base Change ^a	MAF ^b
<i>BMP4</i>	Ch14	rs2761884	Intron	54421052	G > T	0.366
	Ch14	rs17563	Exon	54417522	C > T	0.371
	Ch14	rs2071047	Intron	54418411	A > G	0.401
	Ch14	rs762642	Intron	54423053	A > C	0.352
<i>FAM5C</i>	Ch1	rs1342913	Intron	190121025	A > G	0.379
	Ch1	rs1935881	Intergenic	190066386	C > T	0.209
<i>FGF3</i>	Ch11	rs7932320	Intron	69625511	C > T	0.486
	Ch11	rs1893047	Intron	69626472	A > G	0.428
	Ch11	rs4631909	Intergenic	69614847	C > T	0.478
	Ch11	rs4980700	Intergenic	69619417	A > G	0.439
<i>FGF10</i>	Ch5	rs1448037	Intron	44352344	C > T	0.361
	Ch5	rs900379	Intron	44369656	C > T	0.483
	Ch5	rs1011814	Intron	44335820	C > T	0.485
	Ch5	rs593307	Intron	44358915	A > G	0.364
	Ch5	rs11750845	Intron	44373060	C > T	0.379
<i>FGFR1</i>	Ch8	rs13317	3'UTR	38269514	C > T	0.275

Location, rs-IDs, base change and minor allele frequency of the 16 SNPs. ^aBase change according to Applied Biosystem. ^bMAF, minor allele frequency according to GenBank; Base change in *BMP4* rs 17563 had as reference the Ensembl.

Significant differences in the frequency of the *FAM5C* (rs1342913) G allele ($p = 0.01$) and *FGFR1* (rs13317) C allele ($p = 0.005$) between Union and NU individuals were observed (Table 4). The minor allele frequency in *FAM5C* rs1342913 showed association with Union ($p = 0.01$) as well as the *FGFR1* allele C, which showed significant higher incidence in the NU group ($p = 0.005$). Genotypes and alleles frequencies in *FGF3* and *FGF10* genes showed no significant association.

In order to assess factors concurrently, a binary logistic regression of individual parameters was performed. The initial univariate analysis demonstrated age ($p = 0.07$), fracture type ($p = 0.0002$), *BMP4* (rs17563) genotypes ($p = 0.02$), and *FGFR1* rs13317 genotypes ($p = 0.03$) as potential predictive factors for NU. Table 5 summarizes the binary logistic regression analysis and shows that age ($p = 0.03$), fracture type ($p < 0.001$), and *BMP4* (rs17563) genotypes ($p = 0.012$) as potential predictors associated with fracture NU.

Association Between Haplotypes and Occurrence of Fracture NU

The distribution of haplotypes arranged as alleles (Table 6) was constructed for four genes and assessed for association. There was a significant association between the GTAA haplotype of *BMP4* and the occurrence of NU ($p = 0.01$; Fig. 1). The odds ratio showed that there was an up to 3.34-fold increased risk for NU. The *FAM5C* GT diplotype was significantly associated with uneventful healing ($p = 0.001$), showing 2.98-fold increased protection against non-union fractures based on odds ratio calculations. With regard to the haplotypes of *FGF3* and *FGF10*, no significant associations were found. These results showed the same significance after strict Bonferroni correction.

DISCUSSION

Evidence that fracture NU tend to increase in subsets of individuals may indicate that specific characteristics of the host, such as genetic factors, can jeopardize the bone healing process. Therefore, we decided to investigate the linkage of sixteen SNPs of genes involved in fracture healing in patients presenting fracture NU and uneventful healing, considering systemic and local characteristics as possible risk indicators. Our work supported the association of a *BMP4* haplotype and a *FGFR1* rs13317 C allele with fracture NU independent of the presence of previously described risk factors, which suggest that this disease can have its origin in different phases of the bone healing process.

In the herein study, all humeral, femoral and tibial fractures underwent routine check-up, during 1 year, were included in order to achieve 80% of power in the sample size. The inclusion of open fractures in both groups permitted to analyze these risk indicators in the studied population. Indeed, factors known to predispose to NU did not reveal any association except the covariates of open fracture and age. According to a previous study,⁷ this association can be attributed to the effect of aging at the cellular level, which decreases proliferative capacity of osteoprogenitor cells.²⁵ In addition, despite the open fracture to be a risk indicator for NU, the Union group showed almost 20% of cases with open fracture associated to uneventful healing, which can indicate that others factors, not previously described, can trigger the bone healing breakdown.

In order to elucidate the relationship between genetic background and fracture NU, we evaluated the distribution of genotypes using the data from the International HapMap Project.²⁶ The main results of the single marker and haplotype analyses can be

Table 3. Analysis of Genotype Distribution of 16 SNPs in Fractures NU Subjects and Union

Gene	SNP	Genotypes	Number (Frequency)		<i>p</i> -Value*	Odds Ratio	95% CI
			Union (<i>n</i> = 101)	NU (<i>n</i> = 66)			
<i>BMP4</i>	rs2761884	GG/GT/TT	47 (46.5)/42 (41.6)/12 (11.9)	32 (48.5)/23 (34.9)/11 (16.6)	0.51		
		GT + TT	54 (53.5)	34 (51.5)	0.82	1.08	0.55–2.11
	rs17563	CC/CT/TT	33 (32.7)/55 (54.5)/13 (12.8)	30 (45.4)/22 (33.4)/14 (21.2)	0.02		
		CT + TT	68 (67.3)	36 (54.5)	0.09	1.72	0.86–3.42
	rs2071047	AA/AG/GG	24 (23.8)/49 (48.5)/28 (27.7)	14 (21.2)/33 (50.0)/19 (28.8)	0.92		
		AG + GG	77 (76.2)	52 (78.8)	0.70	0.86	0.38–1.94
<i>FAM5C</i>	rs762642	AA/AC/CC	91 (90.1)/10 (9.9)/0	56 (84.8)/10 (15.2)/0	0.30		
		AC + CC	10 (9.9)	10 (15.2)	0.30	0.62	0.22–1.72
	rs1342913	AA/AG/GG	33 (32.6)/49 (48.5)/19 (18.9)	32 (48.4)/27 (41.0)/7 (10.6)	0.09		
		AG + GG	68 (67.3)	34 (51.5)	0.04	0.52	0.26–1.02
	rs1935881	CC/CT/TT	7 (7.0)/36 (35.6)/58 (57.4)	4 (6.1)/28 (42.3)/34 (51.6)	0.67		
		CT + TT	94 (93.0)	62 (94.0)	0.82	0.87	0.20–3.48
<i>FGF3</i>	rs7932320	CC/CT/TT	24 (23.8)/45 (44.5)/32 (31.7)	15 (22.7)/29 (43.9)/22 (33.4)	0.91		
		CT + TT	77 (76.2)	51 (77.2)	0.87	1.06	0.48–2.36
	rs1893047	AA/AG/GG	28 (27.7)/43 (42.6)/30 (29.7)	13 (19.7)/33 (50.0)/20 (30.3)	0.46		
		AG + GG	73 (72.3)	53 (80.3)	0.23	0.64	0.28–1.43
	rs4631909	CC/CT/TT	25 (24.7)/43 (42.6)/33 (32.7)	16 (24.2)/34 (51.6)/16 (24.2)	0.43		
		CT + TT	76 (75.2)	50 (75.7)	0.94	0.97	0.44–2.12
<i>FGF10</i>	rs4980700	AA/AG/GG	32 (31.7)/42 (41.6)/27 (26.7)	14 (21.2)/34 (51.5)/18 (27.3)	0.29		
		AG + GG	69 (68.3)	52 (78.8)	0.13	0.58	0.26–1.27
	rs1448037	CC/CT/TT	38 (37.6)/45 (44.5)/18 (17.9)	26 (39.4)/25 (37.9)/15 (22.7)	0.62		
		CT + TT	63 (62.4)	40 (60.6)	0.81	1.08	0.54–2.14
	rs900379	CC/CT/TT	30 (29.7)/46 (45.5)/25 (24.8)	19 (28.8)/29 (44.0)/18 (27.2)	0.93		
		CT + TT	71 (70.3)	47 (71.2)	0.89	0.96	0.46–2.00
<i>FGF10</i>	rs1011814	CC/CT/TT	25 (24.8)/46 (45.5)/30 (29.7)	19 (28.8)/29 (44.0)/18 (27.2)	0.83		
		CT + TT	76 (75.2)	47 (71.2)	0.56	0.81	0.38–1.73
	rs593307	AA/AG/GG	22 (21.8)/43 (42.6)/36 (35.6)	18 (27.3)/23 (34.8)/25 (37.9)	0.55		
		AG + GG	79 (78.2)	48 (72.3)	0.44	0.74	0.34–1.62
	rs11750845	CC/CT/TT	44 (43.6)/48 (47.5)/9 (8.9)	32 (48.5)/26 (39.4)/8 (12.1)	0.54		
		CT + TT	57 (56.4)	34 (51.5)	0.53	1.22	0.62–2.38
<i>FGFR1</i>	rs13317	CC/CT/TT	5 (5.0)/37 (36.6)/59 (58.4)	8 (12.1)/32 (48.5)/26 (39.4)	0.03		
		CT + TT	96 (95.0)	58 (87.9)	0.09	0.38	0.10–1.35

**p*-Value calculated by chi-square test, *p*-values < 0.05 are considered significant.

summarized as follows: (1) the *BMP4* GTAA haplotype was highly associated with long bone fracture NU; (2) the *FGFR1* C allele was associated with NU; (3) uneventful healing was associated with *FAM5C* rs1342913; and (4) there seems to be no major

influence of the others genes encoding for mediators in fracture healing.

BMP4 rs17563 (C > T) is located in the translated region of the gene (exon 5). This polymorphism codes for a nonsynonymous amino acid change: the C allele

Table 4. Allele Distribution Analysis Showing Minor Allele Frequencies, *p*-Value, and Odds Ratio in Patients With Fracture NU and Union

Gene	SNP ID	A1	MAF_U	MAF_NU	<i>p</i> -Value	Odds Ratio	95% CI
<i>BMP4</i>	rs2761884	T	0.32	0.34	0.64	0.89	0.54–1.47
	rs17563	T	0.40	0.38	0.15	0.73	0.47–1.15
	rs2071047	A	0.48	0.46	0.77	0.94	0.58–1.51
	rs762642	C	0.04	0.08	0.52	0.52	0.18–1.49
<i>FAM5C</i>	rs1342913	G	0.43	0.31	0.01*	1.76	1.07–2.92
	rs1935881	C	0.23	0.27	0.50	1.18	0.68–2.05
<i>FGF3</i>	rs7932320	C	0.46	0.44	0.70	0.92	0.57–1.47
	rs1893047	A	0.45	0.48	0.58	0.88	0.55–1.41
	rs4631909	C	0.46	0.49	0.54	1.15	0.72–1.83
	rs4980700	G	0.47	0.53	0.27	0.78	0.48–1.25
<i>FGF10</i>	rs1448037	T	0.40	0.41	0.81	0.95	0.59–1.52
	rs900379	T	0.47	0.51	0.65	0.90	0.57–1.44
	rs1011814	C	0.47	0.51	0.65	1.11	0.69–1.77
	rs593307	A	0.43	0.44	0.74	1.08	0.67–1.72
	rs11750845	T	0.32	0.30	0.70	1.10	0.65–1.88
<i>FGFR1</i>	rs13317	C	0.22	0.37	0.005*	1.99	1.18–3.38

A1: Minor allele (based on whole sample)/MAF_U: minor allele frequency in union group/MAF_NU: minor allele frequency in non-union group.***p*-value calculated by chi-square test, *p*-value < 0.05 are considered significant.

forms part of a valine codon whereas the T allele is part of an alanine codon. Capasso et al.²⁷ performed an association study of the C > T substitution in *BMP4* on the development of cutaneous melanoma, reporting that the polymorphic risk allele predicted a change in the structure of the mRNA and that *BMP4* levels were significantly higher in T allele carriers. Ren et al.¹⁸ showed that the *BMP4* TGGGCTT haplotype included the polymorphic allele of SNP rs17563 and it was associated with the overexpression of *BMP4* mRNA in patients with ossification of the posterior longitudinal ligament. Our results showed that the GTAA haplotype, including the putative functional polymorphic *BMP4* allele, was significantly associated with NU. In contrast with recent research⁸, our results suggest this polymorphism clearly related to NU. Individuals with GTAA haplotype seemed to be three times more

susceptible to fracture NU. In addition, genotype analysis of *BMP4* rs17563 showed significant association between wild type genotypes and Union group. Another important detail is that a previous study in fracture NU genetic background⁸ analyzed just one *BMP4* gene region. Our study was the first to analyze multiple *BMP4* variants. We hypothesize that this *BMP4* haplotype can trigger a mRNA *BMP4* overexpression which can interfere in bone homeostasis.

The role of *FAM5C* in osteoblastic differentiation and muscle-bone interactions has been recently studied.¹¹ The reduction of endogenous *FAM5C* reduced the levels of osterix, alkaline phosphatase, and osteocalcin mRNA as well as the levels of type 1 collagen and β -catenin in mouse osteoblastic MC3T3-E1 cells.¹¹ Therefore, *FAM5C* was named as a humoral bone anabolic factor. *FAM5C* is localized in mitochondria which has the ability to activate multiple pathways that modulate both cell proliferation and, inversely, promote programmed cell death²⁸—all phenomena relevant to the bone healing process. The *FAM5C* rs1935881 was associated with norepinephrine changes during exercise²⁹ and aggressive periodontitis.³⁰ In addition, *FAM5C* expression was correlated to high mRNA levels of IL-1 β , IL-17A, IL-4, and RANKL (receptor activator of nuclear factor kappa-B ligand) in gingival samples, suggesting the involvement of *FAM5C* with inflammation. However, there is no published evidence linking *FAM5C* to the pathogenesis of fracture NU. Our genetic analysis found significant association between *FAM5C* rs1342913 (A > G) and uneventful healing. The haplotype analysis confirmed these findings, showing the GT diplotype, including the G polymorphic allele, associated with Union group. Our work is the first to implicate *FAM5C* in NU, suggesting that *FAM5C* acts as a protective factor

Table 5. Binary Logistic Regression Analysis

Parameter	Z	<i>p</i> -value	Odds Ratio	95% CI
Age	−2.09	0.03	0.97	0.94–0.99
Fracture type	3.48	<0.001	3.96	1.82–8.59
<i>BMP4</i> rs17563				
CC	—	1.0	—	—
CT	2.53	0.012	2.73	1.25–5.95
TT	0.16	0.87	1.08	0.39–3.02
<i>FAM5C</i> rs1342913				
AA	—	1.0	—	—
AG	0.86	0.38	1.39	0.65–2.99
GG	1.19	0.23	1.92	0.65–5.63
<i>FGFR1</i> rs13317				
CC	—	1.0	—	—
CT	0.48	0.63	1.36	0.38–4.92
TT	1.68	0.09	2.95	0.83–10.47

Table 6. Examined *BMP4*, *FAM5C*, *FGF3*, *FGF10*, and *FGFR1* Haplotypes

Gene	Haplotype	Frequency Estimation		Odds Ratio	95% CI	<i>p</i> -Value	Bonferroni Corrected <i>p</i> -Value
		Union (<i>n</i> = 101)	NU (<i>n</i> = 66)				
<i>BMP4</i> (rs2761884; rs17563; rs2071047; rs762642)	GCAA	0.28	0.17	1.00	—	—	—
	GCGA	0.19	0.24	0.54	0.23–1.24	0.15	0.47
	TTGA	0.13	0.14	0.50	0.20–1.28	0.15	0.47
	GTGA	0.11	0.04	2.04	0.47–8.85	0.34	0.81
	TTAA	0.09	0.06	1.14	0.32–4.04	0.84	0.99
	GTAA	0.04	0.11	3.34	0.86–13.91	0.01*	0.03
	TCAA	0.04	0.08	0.22	0.04–1.20	0.08	0.28
	TCGA	0.02	0.06	0.70	0.16–3.13	0.65	0.98
	GCGC	0.04	0.03	0.22	0.05–1.08	0.06	0.21
<i>FAM5C</i> ** (rs1342913; rs1935881)	AT	0.37	0.53	1.00	—	—	—
	GT	0.39	0.19	2.98	1.51–5.86	0.001*	0.002
	AC	0.19	0.16	1.74	0.82–3.72	0.15	0.27
	GC	0.03	0.10	0.36	0.10–1.35	0.13	0.24
<i>FGF3</i> (rs7932320; rs1893047; rs4631909; rs4980700)	TATA	0.46	0.42	1.00	—	—	—
	CGCG	0.36	0.36	0.95	0.58–1.56	0.84	0.99
	TGCG	0.06	0.10	0.55	0.24–1.25	0.16	0.50
	CGTG	0.05	0.05	0.79	0.29–2.15	0.64	0.98
	TGTA	0.01	0.01	0.90	0.14–5.92	0.91	0.99
<i>FGF10</i> (rs1448037; rs900379; rs1011814; rs593307; rs11750845)	TCTAC	0.39	0.39	1.00	—	—	—
	CTCGT	0.30	0.29	1.05	0.61–1.81	0.85	0.99
	CTCGC	0.15	0.19	0.80	0.43–1.50	0.49	0.96
	CCTGC	0.10	0.06	1.43	0.65–3.17	0.38	0.90
	CCTAC	0.01	0.01	2.27	0.19–27.42	0.52	0.97

**p*-value < 0.05 are considered significant. **For *FAM5C* diplotype analyses.

against NU. Based on previous studies,^{29,30} we suggest that *FAM5C* is associated with normal osteoblastic differentiation, which is crucial to uneventful consolidation process. In addition, *FAM5C* gene possibly participates in inflammation regulation during fracture healing. Our findings may provide potential target for the investigation of the NU pathogenesis. However, future studies are necessary in order to clarify the exact function of *FAM5C* in bone healing and the expression of this mediator in tissues samples collected during NU treatment.

The antagonism between BMP and FGF contributes to bone healing.²¹ FGF/FGFR signaling is present in endochondral and intramembranous bones and plays important roles in regulating their development and growth.^{31,32} In our study, it was evident that the *FGFR1* rs13317 C allele is associated with fracture NU. Carrying the C allele increases twice the risk of NU, in contrast to TT genotype, which acts as a protective factor. Also, *FGFR1* is a positive regulator for skeletal formation: it has a synergistic effect with *FGFR2* and stimulates osteoblastic differentiation,^{33,34} regulating the morphology and patent of craniofacial sutures.^{35,36} Gain-function mutations in *FGFRs* genes

cause craniosynostosis which can explain why, in our study, *FGFR1* rs13317 was associated with uneventful healing. It is possible that the variant allele of *FGFR1* favors an adequate bone repair, in contrast of that observed in individuals carrying the C allele.

FGF3 and FGF10 interact with *FGFR1* and display various biological functions, including angiogenesis and wound healing.^{22,31} However, no significant associations were observed in *FGF3* and *FGF10* polymorphisms and NU process. It is possible that specific alterations in the receptor, despite FGFs functions, are triggering the pathologic process during fracture healing.

In summary, we obtained supporting evidence for an association between the occurrence of NU and *BMP4* and *FGFR1*. In addition, we found evidence of *FAM5C* participation in bone healing, acting as a protective factor against NU. However, future studies are necessary, including ones with larger sample sizes and in different populations, in order to confirm our findings and identify other genes responsible for genetic susceptibility to NU fracture. It can therefore be speculated that the “inert or deficient local biology” at the fracture site seen in these cases may



Figure 1. Radiographic image showing NU of a femoral diaphyseal fracture treated with intramedullary nail that failed to heal 62 months post-fracture. The GTAA haplotype for *BMP4* gene were identified in this patient.

represent a genetically predisposed environment with reduced potentials for bone regeneration. Consequently, greater knowledge of the genes involved in fracture repair may provide new approaches at the molecular level in the treatment of these patients and the on-time intervention in the biological aspects of bone healing.

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