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A link between osteomyelitis and *IL1RN* and *IL1B* polymorphisms—a study in patients from Northeast Brazil

153 patients followed for 2 years

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Background and purpose — Treatment failure of osteomyelitis can result from genetic susceptibility, highlighting polymorphisms of the interleukin-1 (IL-1) family members, central mediators of innate immunity and inflammation. Polymorphisms are DNA sequence variations that are common in the population (1% or more) and represent multiple forms of a single gene. We investigated the association of *IL1RN*VNTR (rs2234663) and *IL1B*-511C>T (rs16944) polymorphisms with osteomyelitis development in patients operated on because of bone trauma.

Patients and methods — 153 patients who fulfilled the inclusion criteria were enrolled from a referral public hospital for trauma. All the patients were followed up daily until hospital discharge and, after this, on an outpatient basis. Patients were treated with prophylactic antimicrobials and surgery according to traumatology service protocol. The *IL1RN*VNTR and the *IL1B*-511C>T polymorphisms were determined by PCR and PCR-RFLP, respectively.

Results — The *IL1RN2/*2 genotype was associated (OR: 7; p < 0.001) with a higher risk of osteomyelitis and was also significantly associated with *Staphylococcus aureus* infection. The haplotypes (combination of different markers) *2-C and *2-T were also associated with osteomyelitis development.**

Interpretation — *IL1B*-511C>T and *IL1RN*VNTR polymorphisms were associated with osteomyelitis development, which may have implications for patients with bone traumas. These data may be relevant for new therapeutic strategies for this disease.

Osteomyelitis (OM) is an inflammatory process usually associated with an infectious agent (Walter et al. 2012). This difficult-to-treat bone infection has a significant morbidity and mortality (Valour et al. 2014), especially associated with accidents involving high-energy trauma and severe bone injuries, such as motorcycle accidents. *Staphylococcus aureus* is the most common microorganism associated with this disorder (Josse et al. 2015). The treatment for osteomyelitis is based on antibiotic therapy and surgical debridement of infected tissue (Wagner et al. 2016); nevertheless, the results can be unsuccessful. Besides bacterial resistance and the presence of avascular areas of bone necrosis, treatment failure can also result from genetic susceptibility (Lew and Waldvogel 2004, Walter et al. 2012).

There is evidence that inflammation can be modulated by polymorphisms in genes that encode cytokines (Akdis et al. 2011). Polymorphisms are DNA sequence variations that are common in the population. There are 2 or more alternatives forms of a gene (alleles) and to be classified as a polymorphism the least common allele must have a frequency of 1% or more in the population. The most common allele for a gene in a population is the wild type (Ford 1966). Cytokines are cell-signaling molecules that contribute to intercellular communication in immune responses and stimulate the movement of cells towards sites of inflammation, infection, and trauma (Balkwill 1997). Therefore, polymorphisms in genes that encode these cytokines are important candidates for susceptibility to osteomyelitis. Members of the interleukin-1 (IL-1) family are cytokines that are involved in inflammation and immunoregulation (Jaiswal et al. 2012). They are potent mediators of the inflammatory response (Achyut et al. 2007, Garlanda et al. 2013) playing an important role in innate and adaptive immunity. The IL-1 family includes 7 members with agonist activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ), 3 receptor antagonists (IL-1Ra, IL-36Ra, and IL-38), and an anti-inflammatory cytokine (IL-37) (Garlanda et al. 2013). IL-1α and IL-1β are major inducers of pro-

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inflammatory immune responses (Witkin et al. 2002). They both bind to the same receptor on the surface of many cells and initiate the inflammatory cascade. IL-1β is required to trigger inflammation and bone disorders (Lukens et al. 2014). On the other hand, IL-1Ra (interleukin-1 receptor antagonist) is a naturally occurring competitive inhibitor of IL-1α and IL-1β pro-inflammatory activities, since it binds to the same receptor of these cytokines but it does not initiate the inflammatory process. The levels of these 3 cytokines at an inflammatory site determine the beginning, the persistence, and the end of an inflammatory response (Witkin et al. 2002, Jaiswal et al. 2012).

One of the most important genetic polymorphisms in the gene that encodes the IL-1Ra cytokine (*IL1RN*) is the Variable Number Tandem Repeats (VNTR, 86-bp repeats) in intron 2. The short allele (polymorphic allele) with two repeats (*IL1RN**2) has been associated with more severe clinical outcome in various inflammatory diseases (Blakemore et al. 1994, van der Paardt et al. 2002, Witkin et al. 2002, Arend 2003, Carreira et al. 2005). Additionally, long alleles (IL1RN L) with 3 or more repeats seem to promote a regular immune response.

IL1B is a highly polymorphic gene that encodes the IL-1β cytokine. The polymorphic allele *IL1B*-511T has been associated with an increased secretion of IL-1β (Hwang et al. 2002), with atrophic gastritis, a chronic inflammation of the stomach lining (Furuta et al. 2002) and with the risk of type 2 diabetes mellitus (T2DM), a subclinical systemic inflammation characterized by persistent hyperglycemia, which contributes to a prolonged inflammatory state in T2DM (Achyut et al. 2007, Singh et al. 2016). Despite the fact that osteomyelitis is a frequent complication in patients with open fractures, there are few studies correlating genetic susceptibility with this disorder. An association has been reported between the polymorphism *IL1B* (rs16944) and pediatric hematogenous osteomyelitis in the Saudi population (Osman et al. 2016).

Considering the inflammatory nature of osteomyelitis, we investigated the association of *IL1RN*VNTR (rs2234663) and *IL1B*-511C>T (rs16944) polymorphisms with osteomyelitis development in patients operated on because of bone trauma in a Northeast Brazilian population.

Patients and methods

Study population

Patients from Dr. José Frota Institute, a public referral hospital for trauma cases, located in Fortaleza, Ceara (Northeast Brazil) were consecutively enrolled in the emergency room from January 2011 to January 2013. Dr. José Frota Institute is the most important public hospital for trauma cases in this geographical area. Most trauma patients are referred to this hospital. Thus this population can be considered representative for osteomyelitis following trauma.

Patients with any disease, neonates, those younger than 1 year, immunodeficient patients, or patients on immunosuppressive treatment and from other geographical regions were excluded from this study.

153 patients without any disease before the accident fulfilled the inclusion criteria for the study. All of them were bone trauma victims. There were no patients with comorbidities. Genotype analysis was performed in these patients. 39 individuals developed osteomyelitis. 114 patients did not develop osteomyelitis and represented the control group.

Clinical epidemiological data were collected from the hospital admission file of each patient. The patients were followed up daily until hospital discharge and after this on an outpatient basis for orthopedic and trauma surgery.

On admission, 146 patients presented with bone fractures as the major injury, and 7 other patients had soft tissue infection after bone trauma. 101 patients presented with open fractures, 16 were type I, 35 type II, and 50 type III fractures, as per Gustilo and Anderson Classification (1976). Among the 153 patients, 61 patients were operated with metalwork, 7 patients were operated with debridement, and 85 patients were operated with a combined procedure (debridement and metal work).

Patients were treated with prophylactic antimicrobials in addition to surgery when necessary, following the hospital traumatology service protocol. In the case of persistence of the infectious process, a second or third antibiotic protocol was initiated.

The osteomyelitis diagnosis was based on a detailed history, clinical examination, laboratory tests, and radiographs. A fistula to bone with draining pus, a pathognomonic sign of the disease (Walter et al. 2012) and a positive culture from bone biopsy were the microbiological criteria for the osteomyelitis diagnosis. Isolated microorganisms were identified by conventional methods (Koneman et al. 2001).

Genotype analysis

Genomic DNA was extracted from a 5 mL blood sample collected from all patients during routine blood tests, using the salting-out method (Miller et al. 1988). The *IL1RN*VNTR polymorphism (rs2234663) was determined by PCR (Rad et al. 2003) The primers for the *IL1RN*VNTR were (F) 5’ CTC AGC AAC ACT CCT AT 3’ and (R) 5’ TCC TGG TCT GCA GGT AA 3’. The *IL1RN* alleles were categorized as previously described (Witkin et al. 2002, Jaiswal et al. 2012). For the analysis, *IL1RN**2 and *IL1RN**6 alleles were considered short alleles. The other alleles *IL1RN**1(wild-genotype), *IL1RN**3, *IL1RN**4 and *IL1RN**5) were considered long alleles (*IL1RN* L).

IL1B-511C>T polymorphism (rs16944) was detected by PCR-RFLP according to published protocols (Rad et al. 2003). The region containing the polymorphic site was amplified using the primers 5′-TGG CAT TGA TCT GGT TCA TC-3′ (sense) and 5′- GTT TAG GAA TCT TCC CAC TT-3′ (antisense). The products were digested with 10 U Aval

Table 1. Demographics and trauma etiology

Characteristic	Total number (n = 153)	Patients with osteomyelitis (n = 39)	Healthy control group (n = 114)
Sex			
Male	116	30	86
Female	37	9	28
Age groups, year			
1–15	80	19	61
16–30	36	10	26
31–45	25	7	18
46–60	8	2	6
61–75	4	1	3
Trauma etiology			
Motorcycle accident	66	22	44
Run-over accident	41	14	27
Car accident	1	1	0
Fall from height	26	0	26
Crushing	11	2	9
Bicycle accident	2	0	2
Others	6	0	6

at 37°C overnight. The C/C wild-genotype corresponded to 114- and 190-bp fragments and the T/T polymorphic genotype to a product of 304 bp. For both polymorphisms, randomly selected samples were re-genotyped (15% of samples).

Statistics

Data were analyzed using Statistical Package for the Social Science for Windows software (SPSS) version 13.0 (SPSS Inc., Chicago, IL, USA), Epi Info 7.0 (CDC, Atlanta, GA, USA) and UNPHASED 3.1.7 (Frank Dudbridge, MRC Biostatistics Unit, Cambridge, UK; Dudbridge 2008). The frequencies were compared using the chi-square test ($\chi 2$) or Fisher's exact test. Odds ratios (ORs) and relative risks (RR) were calculated and reported within the 95% confidence intervals. Statistical significance was determined at a value of $p < 0.05$.

Ethics, funding, and potential conflicts of interest

This study was approved by the Ethics in Research Committee of Dr. José Frota Institute (nº 77830/2010 – 08/30/2010). Informed written consent was obtained from every participant or legal guardian. We had no external funding.

No competing interests declared.

Results

All the patients were successfully evaluated for *IL1RN*VNTR and *IL1B*-511C>T polymorphisms (Table 1).

Distribution and statistical analysis of *IL1RN*VNTR and *IL1B*-511C>T genotype frequencies are shown in Table 2. Allele frequencies of the total number of patients and patients without osteomyelitis for both polymorphisms were not in Hardy–Weinberg equilibrium (a mathematical model that provides a baseline to interpret genotype and allele frequencies in actual populations) (Douhovnikoff and Leventhal 2016). Allele frequency for *IL1RN*VNTR in patients with osteomyelitis was in Hardy–Weinberg equilibrium.

Among the 146 patients with bone fractures, 101 presented with open fractures. 81 patients had open fractures in long bones (Table 3). Among the 81 patients with open fractures in long bones, 33 developed osteomyelitis. As expected, only 2 patients with closed fractures and 4 patients with non-long bone fractures progressed to osteomyelitis.

When all the patients were taken into account, people carrying the *IL1RN**2/*2 genotype had a higher risk of osteomyelitis development. A risk of more than 2-fold was observed in the dominant model for the *IL1RN**2 allele (Table 2).

In patients with open fractures in long bones, an increased risk of osteomyelitis was associated with *IL1RN**2/*2 and *IL1RN* L/*2 genotypes, respectively. In the dominant model for the *IL1RN**2 allele, a more than 2-fold risk was observed (Table 3). It is interesting to note that all patients carrying the

Table 2. Distribution of patients according to *IL1RN* and *IL1B* genotypes

Genotype	Total number (n = 153)	Patients with osteomyelitis (n = 39)	Healthy control group (n = 114)	Odds ratio (95% CI)	Relative risk (95% CI)	p-value
IL1RN VNTR						
L/L (wild-genotype)	88	14	74	1	1	–
L/*2 (heterozygous genotype)	42	12	30	2.1 (0.9–5.1)	1.8 (0.9–3.5)	0.09
*2/*2 (polymorphic genotype)	23	13	10	6.9 (2.5–18.7)	3.6 (2.0–6.5)	< 0.001
L/L + L/*2	130	26	104	1.3 (0.7–2.7)	1.3 (0.7–2.3)	0.4
2*/2*+ L/*2	65	25	40	3.3 (1.6–7.1)	2.4 (1.4–4.3)	0.002
IL1B-511C/T						
C/C (wild-genotype)	64	14	50	1	1	–
C/T (heterozygous genotype)	49	9	40	0.8 (0.3–2.1)	0.8 (0.4–1.8)	0.6
T/T (polymorphic genotype)	40	16	24	2.4 (1.0–5.7)	1.8 (1.0–3.3)	0.05
C/C + C/T	113	23	90	0.9 (0.4–1.9)	0.9 (0.5–1.7)	0.8
T/T + C/T	89	25	64	1.4 (0.7–3.0)	1.3 (0.7–2.3)	0.4

Table 3. Distribution of patients with open fractures in long bones according to IL1RN and IL1B genotypes

Genotype	Total number (n = 81)	Patients with osteomyelitis (n = 33)	Healthy control group (n = 48)	Odds ratio (95% CI)	Relative risk (95% CI)	p-value
IL1RN						
L/L (wild-genotype)	46	12	34	1	1	–
L/*2 (heterozygous genotype)	23	12	11	3.1 (1.1–8.8)	2.0 (1.1–3.7)	0.03
*2/*2 (polymorphic genotype)	12	9	3	8.5 (2.0–37)	2.9 (1.6–5.2)	0.005 [a]
L/L + L/*2	69	24	45	1.5 (0.7–3.4)	1.3 (0.7–2.4)	0.3
2*/2*+ L/*2	35	21	14	4.3 (1.7–11)	2.3 (1.3–4.0)	0.002
IL1B						
C/C (wild-genotype)	33	12	21	1	1	–
C/T (heterozygous genotype)	22	8	14	1.0 (0.3–3.1)	1.0 (0.5–2.0)	1
T/T (polymorphic genotype)	26	13	13	1.8 (0.6–5.0)	1.4 (0.8–2.5)	0.3
C/C + C/T	55	20	35	1.0 (0.4–2.4)	1.0 (0.6–1.8)	1
T/T + C/T	48	21	27	1.4 (0.6–4)	1.2 (0.7–2.1)	0.5

[a] Fisher's exact test

Table 4. Distribution of patients with osteomyelitis according to associated *IL1RN* and *IL1B* genotypes

Association of *IL1RN* and *IL1B genotypes*	Total number (n = 153)	Patients with osteomyelitis (n = 39)	Healthy control group (n = 114)	Odds ratio (95% CI)	Relative risk (95% CI)	p-value
L/L + C/C (wild-genotypes)	31	1	30	1	1	–
L/L + C/T	30	5	25	6.0 (0.7–55)	5.2 (0.6–42)	0.1
L/L + T/T	27	8	19	13 (1.5–110)	9.2 (1.2–68)	0.009
L/*2 + C/C	20	5	15	10 (1.1–93)	7.8 (1–6.6)	0.03
L/*2 + C/T	13	3	10	9.0 (0.8–97)	7.2 (0.8–66)	0.07
L/*2 + T/T	9	4	5	24 (2.2–261)	14 (1.8–78)	0.006
*2/*2 + C/C	13	8	5	48 (4.9–471)	19 (2.6–138)	< 0.001
*2/*2 + C/T	6	1	5	6.0 (0.3–112)	5.2 (0.4–72)	0.3
*2/*2 + T/T (polymorphic genotypes)	4	4	0	Undefined	31 (4.5–213)	< 0.001

*IL1RN**2/*2 + *IL1B*-511T/T polymorphic genotypes developed osteomyelitis. Likewise, haplotype analysis showed significant associations of *2-C and *2-T with osteomyelitis development ($p = 0.004$ and $p < 0.001$, respectively). On the other hand, the L-C haplotype was more commonly observed in the control group. Of the 31 patients with *IL1RN* L/L + *IL1B* C/C wild-genotypes, only 1 progressed to osteomyelitis. The patient was a 21-year-old man who was admitted to hospital with an open fracture in a long bone, type III. Additionally, the 2 patients who had osteomyelitis associated with closed fractures in long bones carried the *IL1B*-511T/T genotype; 1 of them also showed the *IL1RN**2/*2 genotype. Moreover, 3 of the 4 patients with osteomyelitis in non-long bones carried the *IL1RN**2/*2 genotype and 1 of them showed the *IL1B*-511T/T genotype.

Association between IL1 markers and osteomyelitis due to Staphylococcus aureus

Regarding the infectious agent, *Staphylococcus aureus* was the most important isolated agent in osteomyelitis cases (35/39). A significant association was observed between *2/*2 patients and osteomyelitis due to *Staphylococcus aureus* (12/22) ($p < 0.001$).

Discussion

Polymorphisms in genes that encode proteins involved in the inflammatory response may explain some of the individual differences in susceptibility for osteomyelitis. It is important to highlight that in this study there were no patients with comorbidities beyond the traumatic injuries. Trauma etiology seems to be a risk factor for osteomyelitis. This disease is especially associated with high-energy traumas and severe injuries such as motorcycle accidents. One-third of our patients with this kind of accident developed osteomyelitis. On the other hand, none of the patients who were victims of fall from a height developed this disease. However, the most important finding of our study was the significant association of the *IL1RN*VNTR polymorphism (rs2234663) with osteomyelitis. To our knowledge

this is the first study that associates posttraumatic osteomyelitis and polymorphisms in *IL1B*-511C>T and *IL1RN*VNTR genes. Another study detected an association between the rs16944 (G/A) and rs3804099 (C/T) polymorphisms and the susceptibility to pediatric hematogenous osteomyelitis (Osman et al. 2016). Both studies demonstrate that disturbance of the IL-1 system seems to be an important factor for osteomyelitis development. In our study, as described in the literature (Carek et al. 2001), osteomyelitis was strongly associated with open fractures and long bones. The presence of the *IL1RN**2/*2 genotype in 3 of 4 patients with fractures in non-long bones corroborates the relevance of this allele in osteomyelitis development, since this disease rarely affects these types of bones (Carek et al. 2001, Witkin et al. 2002). We observed a higher frequency of the T/T genotype in patients with osteomyelitis and that all people carrying the *2/*2-T/T genotypes developed osteomyelitis, corroborating an association between a more severe inflammatory state (*2/*2) and an increased secretion of 1 of the major inducers of inflammation (T/T). These data were also corroborated with haplotype analysis, where the *2-C and *2-T haplotypes were associated with osteomyelitis development contrasting with the L-C haplotype, which was more frequent in patients without osteomyelitis. The presence of both alleles (*2/T) would increase the inflammatory response, explaining the risk. Similar results were observed in other diseases such as T2DM (Achyut et al. 2007) and gastritis (Kulmambetova et al. 2014). These 2 polymorphisms (*IL1RN*-VNTR and *IL1B*-511C>T) are located within different regions of genes. The frequency of association of their different alleles is not higher or lower than what would be expected (Slatkin 2008) and *IL1B* and *IL1RN* are separated by 280 kb (Wang et al. 2015). Therefore polymorphisms in *IL1B* and in *IL1RN* are in general in low-linkage disequilibrium (LD). Based on this fact it is more plausible that they are causal and not due to linkage disequilibrium and that there is an independent segregation of these 2 markers. It is acknowledged that IL-1Ra is a receptor antagonist of IL-1. IL1Ra binds to the IL-1 receptor with equal or greater affinity than IL-α and IL-1β. Thus, IL1Ra works as a naturally occurring anti-inflammatory protein (Arend 2003). During the course of an inflammatory process IL-1Ra concentrations increase late and prevent maintenance of the acute inflammation (Witkin et al. 2002). In this context, it is the IL-1Ra/IL-1β ratio and the infectious agent that will determine whether a pro-inflammatory response will persist or will be finished (Arend and Guthridge 2000). Polymorphisms in the IL-1 interleukins genes have a substantial impact on the regulation of the IL-1 system, particularly the *ILRN**2 alelle, which has been associated with a longer and more aggressive inflammatory response (Zapata-Tarrés et al. 2013). It could be speculated that a lower IL-1Ra/IL-1β ratio in *IL1RN**2 carriers could determine increased activity of pro-inflammatory cytokines and hence a more severe inflammatory response. As documented in the literature, *Staphylococcus aureus* was the main isolated agent in this study. This microorganism can live inofensively on the skin, in the nose and in other mucous membranes of some healthy individuals (Lobati et al. 2001), which may favor the dissemination of this infectious agent, particularly in open wounds. Here, it is important to point out that, in our study, all individuals with soft tissue infectious processes and open fractures and who underwent surgery on admission to the hospital, following the hospital protocol, were treated with the same group of antibiotics during the same time. The above procedure prevented the development of osteomyelitis resulting from the non-use of an antibiotic or use of different antibiotics. The significant association of *IL1RN**2/*2 patients with osteomyelitis development could suggest a less effective response to these infectious agents in the presence of this genotype.

An important aspect resulting from potential involvement of *IL1RN**2 for osteomyelitis risk is the possibility of a new approach for treatment with drugs to block IL-1 activities (Dinarello and van der Meer 2013) with successful results in a broad spectrum of inflammatory syndromes. Additionally, good results were reached using this strategy for treatment of severe hidradenitis suppurativa in patients who had also episodes of pustular folliculitis due to *S. aureus*, the most important microorganism associated with posttraumatic osteomyelitis (Zarchi et al. 2013).

In summary, this study suggests that the *IL1RN**2/*2 genotype and *IL1RN**2 allele are significantly associated with an increased risk of osteomyelitis development in northeastern Brazilians. Potential involvement can also be attributed to the *IL1B*-511T allele, indicating that disturbance of the IL-1 system is an important factor for osteomyelitis development. This finding can potentially be used to aid in the follow-up of patients with bone traumas and in guidance of therapeutic strategies. Since IL-1 antagonism is effective against many inflammatory diseases, inhibition of the inflammation pathway involving this interleukin could be an interesting strategy for the treatment of osteomyelitis.

Additional studies with other populations across the world will be necessary to corroborate these results. The small sample size, other genetic factors and the control group may represent limitations of this study. Despite these limitations, this work could have detected important findings about genetic variations in the inflammatory process of posttraumatic osteomyelitis.

CAS, MSQAS and SHBR designed the study. CAS, AQAS and MSQAS participated in sample collection. CAS, JAL and AQAS reviewed the diagnosis of the patients. MSM and SHBR performed the DNA genotype. MSQAS, AQAS and SHBR performed the statistical analysis. CAS, AQAS, MSQAS and SHBR wrote the first draft and took care of manuscript revisions. SHBR offered expert comments on the genetic procedures.

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