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Investigation of polymorphisms in anti-inflammatory cytokine genes in hematogenous osteomyelitis

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ABSTRACT. Osteomyelitis is a progressive bone infection disease caused by destructive immunological inflammatory reactions following new bone formation. Anti-inflammatory cytokines are a series of immunoregulatory molecules that control the pro-inflammatory cytokine response. In this study, we investigated 9 single nucleotide polymorphisms in 5 different cytokine/cytokine receptor genes in hematogenous osteomyelitis (HO) patients, and compared their outcomes with normal healthy individuals. Sequence-specific forward and reverse primers and two TaqMan® MGB probes with dyes (VIC™ and FAM™) that specifically detect Allele 1 and Allele 2 of each SNP were utilized. The genotypes CC (P = 0.009) and CT (P = 0.041) of SNP rs2070874, and alleles A (P = 0.044) and G of SNP rs1800871 were significantly different between the patients and healthy controls. The expression of the CC genotype or C allele at rs2070874 was a risk factor for HO development, with higher frequencies of CT and T being found in the control samples. The expression of the A allele of rs1800871 was also significantly higher in patients than in controls, and was therefore considered a risk factor.

Key words: Hematogenous osteomyelitis; Anti-inflammatory cytokines; Single nucleotide polymorphisms

INTRODUCTION

Hematogenous osteomyelitis (HO) is a progressive bone infection disease caused by destructive immunological inflammatory reactions following new bone formation (Tsezoul et al., 2008). Blood-borne organisms gain access to the bone from known or unknown foci of infections, such as tonsillitis, otitis media, and upper respiratory tract infections. Microorganisms reach the bone through the bloodstream, leading to the destruction of cancellous (porous) bone and marrow, loss of blood supply, and bone death. The mechanism of bone loss or bone destruction in osteomyelitis patients remains unclear; however, one study has demonstrated that the *Staphylococcus aureus* protein A binds directly to osteoblasts, preventing cell proliferation, inducing apoptosis (Claro et al., 2011; Rabillard et al., 2011).

S. aureus, *Streptococci*, Gram-negative enteric organisms, and anaerobic bacteria are responsible for approximately 80% of the cases of osteomyelitis. However, *S. aureus* accounts for nearly 50% of the incidence of HO (Olson and Horswill, 2013). Anti-inflammatory cytokines are a series of immunoregulatory molecules that control the pro-inflammatory cytokine response (Opal and DePalo, 2000). The direct involvement of the cytokines interleukin 6 (IL-6) and 4 (IL-4) in bone desorption and the regulation of osteoclast activity indicates that they play a key role in the pathogenesis of osteomyelitis (Tsezoul et al., 2008). Bone-forming osteoblasts secrete several important immune mediators following exposure to *S. aureus*. Previous studies have reported that osteoblasts express IL-6 in organ cultures in response to progressive inflammatory damage; additionally, the elevation of IL-6 in a murine model of osteomyelitis was attributed to bone damage during the early phase of the infection. Furthermore, endogenous IL-6 expression has significant anti-inflammatory effects on the modulation of the *in vivo* stimulation of bone destruction. IL-4 is also associated with histopathological changes in osteomyelitis, including bone resorption and formation, during the later phases of osteomyelitis (Balto et al., 2001; Yoshii et al., 2002; Marriot et al., 2004; Tsezoul et al., 2008).

Single nucleotide polymorphisms (SNPs) are DNA sequence variations at single nucleotides (A, T, G or C); these differences between individuals result in important genetic variations that are associated with many diseases (Nachman, 2001).

Previous research has led to the development of many improved surgical and medical treatment methods for osteomyelitis; however, further research is required to better understand the immunological mechanisms of this disease. In this study, we investigated 9 SNPs (Kaur et al., 2007) in 5 different cytokine/cytokine receptor genes in HO patients and compared their outcomes with normal healthy individuals to determine if the genetic variability of SNPs was associated with the susceptibility to develop HO.

MATERIAL AND METHODS

Patients and control subjects

A total of 52 patients with HO were included in this study. The female-to-male ratio was 1.33, and the mean age was 6.5 years (range = 1-17 years). All patients were hospitalized at the Pediatric Orthopedic Department of King Fahad Medical City (KFMC), Riyadh, Saudi Arabia. The control group was composed of 103 healthy unrelated Saudi individuals with no history of HO or immune-compromised diseases at the time of blood collection. The study was approved by the Institutional Review Board (IRB) of KFMC, and signed informed consent forms were obtained from all participants or their guardians. The procedures for sample collection and analyses were in strict accordance with the rules and regulations of the government of Saudi Arabia and KFMC/IRB

policies and procedures.

All patients were clinically diagnosed by an expert orthopedic surgeon; this diagnosis was confirmed by numerous tests, including the measurement of C-reactive protein and erythrocyte sedimentation rate, culturing of blood and fluid/pus from suspected areas, plain X-rays, magnetic resonance imaging, and computerized tomography.

Genotyping

A MagNa pure compact instrument (Roche Diagnostics Ltd., Rotkreus, Switzerland) was used to extract genomic DNA from peripheral blood (collected in a tube with EDTA anti-coagulant); the DNA yields were measured using a Nanodrop® 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Sequence-specific forward and reverse primers and two TaqMan® MGB probes with dyes (VIC™ and FAM™) for the detection of Allele 1 and Allele 2 (Applied Biosystems, Foster City, CA, USA) were also employed in this study. The Light Cycler®480 SW 1.5.1 software was used to discriminate the alleles of each SNP. The following SNP genotypes were determined: *IL-4* (rs2070874), *IL-4* (rs2243248), *IL-10* (rs1800896), *IL-10* (rs1800871), *IL-10* (rs1800872), *TGF-B1* (rs1800470), *IL-4R* (1801275), *IL-2B* (rs3212227), and *IL-2* (rs2069762). Multiple-DNA samples with known SNP genotypes were run in parallel with our study samples for quality control, and the same results were obtained.

Statistical analysis

The Hardy-Weinberg equilibrium (HWE) test was performed on the control data, implementing a χ^2 distribution (1 degree of freedom) to indicate differences between the expected and observed values for the tested SNP genotypes as described by Rodriguez et al. (2009). The frequencies of alleles/genotypes for each SNP were derived using an algorithm based on a direct counting method in the SAS program (Shelton et al., 2004), and the results are reported as percentages at 95% confidence intervals. The Fisher exact test and logistic regression were used to compare the patients and controls. The log of the odds for the presence of each SNP was determined to detect significant differences, and the overall significance level was set at 0.05.

RESULTS

In this study, 155 individuals (103 controls and 52 patients) were genotyped for 9 SNPs distributed on 5 different chromosomes within the human genome (Table 1). The laboratory results for specimen cultures collected from the site of infection showed that *S. aureus* was the most common pathogen isolated (23.1%) from the patient samples; moreover, approximately 44% of the samples were sterile, which was attributed to the use of antibiotic medication at the time of sample collection. *MRSA* (13.5%) and *Streptococcus agalactiae* (3.8%) were also detected at lower frequencies.

The allele/genotype frequencies for these SNPs were calculated, and other statistical values were subsequently obtained for comparison of patients and controls. Among the SNPs genotyped in this study, only rs2070874 (C/T), rs1800871 (A/G), and rs2243248 (GT) showed significant differences (Table 2). Significant differences between patients and controls were observed for rs2070874 (C/T) at both the allelic and genotypic levels, whereas the rs1800871 (A/G) and rs2243248 SNPs differed only at the allelic level. Significantly, higher frequencies of the CC genotype and the C allele of rs2070874 were observed in patients than in controls, and individuals exhibiting these genetic components showed at least 2-fold greater susceptibility to HO. In contrast, the frequencies of the CT genotype

and the T allele of rs2070874 were significantly higher in controls than in patients, which may indicate a protective effect of these genetic markers against HO development among Saudi individuals.

Table 1. Cytokine, cytokine receptor, and Toll-like receptor characteristics.

Gene	Gene name	Chromosome	SNP ID	Polymorphism	Haplotypes	Dye tagged with probe [VIC/FAM]
IL-4	interleukin 4	5	rs2070874	C/T	CC/CT/TT	[C/T]
IL-4	interleukin 4	5	rs2243248	G/T	GG/GT/TT	[G/T]
IL-10	interleukin 10	1	rs1800896	T/C	TT/TC/CC	[T/C]
IL-10	interleukin 10	1	rs1800871	A/G	AA/AG/GG	[A/G]
IL-10	interleukin 10	1	rs1800872	T/G	TT/TG/GG	[T/G]
TGF-B1	transforming growth factor, B1	19	rs1800470	A/G	AA/AG/GG	[A/G]
IL-4R	interleukin 4, receptor	16	rs1801275	A/G	AA/AG/GG	[A/G]
IL-12B	interleukin 12B	5	rs3212227	G/T	GG/GT/TT	[G/T]
IL-2	interleukin 2	4	rs2069762	C/A	CC/CA/CC	[C/A]

SNP, single nucleotide polymorphism.

Table 2. Distribution of, and statistical results for, alleles and genotypes of studied single nucleotide polymorphisms (SNPs).

Gene	SNP ID	Haplotype	Case [% (N = 52)]	Control [% (N = 103)]	OR (95%CI)	P value
IL-4	rs2070874	CC	69.2	47.1	2.53 (1.25-5.13)	**0.009**
		CT	23.1	40.2	0.45 (0.21-0.97)	**0.041**
		TT	7.7	12.7	0.57 (0.18-1.85)	0.35
		C	81.1	67.5	2.1 (1.92-3.71)	**0.01**
		T	18.9	32.5	0.48 (0.27-0.84)	**0.01**
IL-4	rs2243248	GG	84.6	89.3	0.66 (0.25-1.75)	0.4
		GT	15.4	1.9	9.18 (1.87-45.0)	**0.0063**
		TT	0	8.8		
		G	92.3	90.3	1.29 (0.55-3.04)	0.59
		T	7.8	9.7	0.78 (0.33-1.82)	0.59
IL-10	rs1800896	TT	42.3	47.6	0.81 (0.41-1.58)	0.53
		TC	42.3	35.9	1.31 (0.66-2.59)	0.44
		CC	15.4	16.5	1.35 (0.53-3.44)	0.53
		T	63.3	65.2	0.93 (0.57-1.52)	0.76
		C	36.7	34.8	1.07 (0.66-1.76)	0.76
IL-10	rs1800871	AA	61.5	47.6	1.96 (0.98-3.93)	0.058
		AG	30.8	36.9	0.76 (0.037-1.55)	0.45
		GG	7.7	15.5	0.45 (0.14-1.43)	0.18
		A	76.9	65.7	1.7 (1.01-2.98)	**0.044**
		G	23.1	34.3	0.57 (0.34-0.99)	**0.044**
IL-10	rs1800872	TT	36	46.5	0.63 (0.32-1.28)	0.21
		TG	64	52.5	1.61 (0.79-3.23)	0.19
		GG	0	1	0	0
		T	68	73.5	0.77 (0.45-1.29)	0.32
		G	32	52	1.3 (0.77-2.21)	0.32
TGF-B1	rs1800470	AA	38.5	49	0.66 (0.34-1.31)	0.23
		AG	38.5	33.3	1.25 (0.62-2.5)	0.53
		GG	23	17.7	1.4 (0.62-3.18)	0.42
		A	57.7	65.7	0.7 (0.44-1.56)	0.17
		G	42.3	34.3	1.4 (0.86-2.28)	0.17
IL-4Rα	rs1081275	AA	76.9	63.5	1.91 (0.89-4.12)	0.098
		AG	23.1	32.3	0.63 (0.29-1.36)	0.24
		GG	0	4.2		
		A	88.5	79.7	1.9 (0.97-3.9)	0.0595
		G	11.5	20.3	0.51 (0.25-1.053)	0.0595
IL-12B	rs3212227	GG	68	50.9	2.04 (1.0-4.16)	**0.049**
		GT	24	38.2	0.51 (0.24-1.09)	0.083
		TT	8	10.9	0.72 (0.22-2.38)	0.59
		G	80	70.1	1.71 (0.96-3.03)	0.068
		T	20	29.9	0.59 (0.33-1.04)	0.068
IL-2	rs2069762	CC	30.7	35.9	0.79 (0.39-1.62)	0.52
		CA	50	47.6	1.1 (0.57-2.15)	0.78
		AA	19.3	16.5	1.2 (0.51-2.86)	0.67
		C	55.8	59.7	0.85 (0.52-1.37)	0.51
		A	54.2	40.3	1.18 (0.73-1.89)	0.51

OR, Odds ratio; CI, confidence interval. Values in bold are P < 0.05.

DISCUSSION

SNPs in many candidate genes have been linked to an increased risk of many diseases (Nachman, 2001). In this study, we investigated the significance of the relationship between polymorphisms in several cytokine genes and susceptibility to HO in a Saudi population. The rs2070874 SNP is an intragenic SNP located at the IL-4 gene on chromosome 5. IL-4 is a highly pleiotropic cytokine that acts as an important mediator and modulator of immune and inflammatory responses, including the ability to induce helper T (Th) cell differentiation. Early secretion of IL-4 induces the differentiation of Th cells into Th2-like cells, which secrete IL-4; moreover, autocrine production of IL-4 enhances cell proliferation (Tsezoul et al., 2008; Lu et al., 2014). Lu et al. (2014) demonstrated that rs2070874 (CC) may be a genetic risk factor for chronic hepatitis B in Chinese males, and Zhu et al. (2013) reported that individuals carrying the C allele of rs2070874 in the IL-4 gene are at a risk of asthma compared to TT homozygotes.

Only the A and G alleles of rs1800871 in the *IL-10* gene differed significantly between patients and controls. This SNP is also an intragenic SNP. The IL-10 cytokine has pleiotropic effects on immune regulation and inflammation, regulating the expression of Th1 cytokines and MHC class II antigens, co-stimulating macrophage surface molecules, and enhancing B cell survival, proliferation, and antibody production (Sowmya et al., 2014). The rs1800871 genotype is also significantly associated with an increased risk of tuberculosis in a Tunisian population and with increased susceptibility for rheumatoid arthritis in a Malaysian population (Ben-Selma et al., 2011).

Finally, this study revealed significant differences in the SNPs rs2070874 and rs1800871, which suggest possible modifications in response to immunological effector mechanisms in Saudi HO patients.

In conclusion, we detected significant variations in rs2070874 and rs1800871 among the genotyped SNPs in Saudi patients, despite the limited sample size. One possible limitation is that other unknown genetic factors are in linkage disequilibrium with our findings. Therefore, further genetic studies in larger samples are recommended to better understand the genetic markers associated with HO.

Conflicts of interest

The authors declare no conflict of interest.

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