article

Bax gene G(-248)A promoter polymorphism is associated with increased lifespan of the neutrophils of patients with osteomyelitis

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Background: Patients with osteomyelitis have a decreased rate of spontaneous apoptosis of their peripheral blood neutrophils. The G(-248)A polymorphism in the promoter region of the *bax* gene is associated with prolonged peripheral blood neutrophil survival in leukemic patients and may play some role in osteomyelitis. **Methods:** *Bax* G(-248)A promoter polymorphism was detected by DNA amplification using polymerase chain reaction, followed by restriction fragment length polymorphism analysis. Spontaneous apoptosis of peripheral blood neutrophils was measured by propidium iodide, annexin V, and flow cytometry, and Bax was quantified by Western blotting. **Results:** The *bax* promoter polymorphism A allele was significantly more frequent in 80 patients with osteomyelitis than in 220 healthy donors (18.1% vs. 10.6%, $\chi^2 = 4.84$, odds ratio = 1.81, 95% confidence interval = 1.06–3.07, $P = .028$). Carriers of the A allele had a lower apoptotic rate of their peripheral blood neutrophils compared with noncarriers (33.3 ± 16.7 vs. 43.1 ± 3.1, $P = .036$). Patients with the AA genotype showed a lower expression of the Bax protein compared with carriers of other genotypes ($P = .038$). **Conclusions:** Substitution of a nucleotide G→A at position -248 in the *bax* gene was more frequent in patients with osteomyelitis and was associated with a longer lifespan of their peripheral blood neutrophils and lower Bax protein expression. These findings may play a role in the pathogenesis of osteomyelitis. ***Genet Med* 2007:9(4):249–255.**

Key Words: *bax, polymorphism, osteomyelitis, neutrophils, apoptosis*

Osteomyelitis (OM) is a difficult-to-treat bone infection characterized by progressive inflammatory destruction of the bone, bone necrosis, and new bone formation. In adults, OM usually is a complication of open wounds involving the bone, from fractures, surgery, or both. The risk and severity of infection can be enhanced by the presence of a foreign body (metallic or prosthetic devices). It is reported that 0.4% to 7% of trauma and orthopedic operations are complicated by OM. This infection can also develop in a noninjured bone after bacteremia, mostly in prepubertal children and elderly patients, when the infection involves the axial skeleton. *Staphylococcus aureus* is the microorganism most frequently isolated in both posttraumatic and hematogenous OM. Despite appropriate combined medical and surgical therapies, up to 30% of OM becomes chronic, causing major economic losses and personal morbidity and mortality.[1] Much attention has been dedicated to improving the surgical and medical treatment of OM, but little progress has been made toward understanding its pathogenesis. It is clear that OM is multifactorial and mainly influenced by local factors related to the bone lesion and microorganisms inoculated into the bone, but inherited factors and cell immunity dysfunctions may play some role.[2–4]

Polymorphonuclear neutrophils are potent phagocytes that are the first line of the host immune defense against many infections. Clearance of neutrophils through apoptosis and their subsequent ingestion by macrophages play an important role in limiting the destructive potential of neutrophils leading to resolution of the inflammation.[5–7]

Neutrophil apoptosis is controlled by down- or up-regulation of the Bcl-2 family proteins, which may include the antiapoptotic proteins Mcl-1, A1, and Bcl-X_L, and the proapoptotic proteins Bax-α, Bid, Bak, and Bad.[8–13] The ratio of antiapoptotic to proapoptotic proteins is critical to regulate cell apoptosis. A high Bcl-2/Bax ratio and higher levels of Mcl-1 were strongly correlated with prolonged leukemic cell survival.[14,15] Mutations in the promoter and coding regions of the *bax* gene affected protein expression and function.[16,17] Recently, a novel single nucleotide polymorphism (SNP), G(-248)A, in the 5′-untranslated region of the *bax* gene was found in patients with chronic lymphocytic leukemia, a malignancy characterized by accumulation of lymphocytes resulting from failed apoptosis.[18,19] Delayed neutrophil apoptosis

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is also associated with various proinflammatory diseases, including systemic inflammatory response syndrome, ischemia-reperfusion injury, and adult respiratory distress syndrome.[20–23] Increased levels of inflammatory cytokines have been reported in serum and surgically obtained bone fragments of humans[24,25] and mice with OM.[26,27] Some of the cytokines and growth factors, such as interleukin (IL)-1β, IL-2, IL-15, interferon-γ, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor, are reported to inhibit in vitro neutrophil apoptosis.[28–30] Others (e.g., tumor necrosis factor-α) induce or delay neutrophil apoptosis,[24,31,32] and the effect of IL-6 is variable as well.[33–38]

Our group recently showed that the apoptosis of peripheral neutrophils from patients with OM was decreased in relation to healthy controls and that this effect seemed to be the result, at least in part, of the high levels of circulating IL-6.[39] In the present work we tried to determine whether the *bax* G(-248)A promoter polymorphism (rs 4645878) could play a similar role in reducing apoptosis of neutrophils from patients with OM, perhaps leading to impaired resolution of the bone infection.

PATIENTS AND METHODS

Patients

Eighty patients (53 men and 27 women, with a mean age of 55.5 $\pm$ 17.7 years, range 16–89 years) who were admitted between January 1998 and June 2006 to the Hospital Central de Asturias, Oviedo, and to three other affiliated hospitals of the same northern Spanish region were studied. Patients with acute (55 cases) and chronic (25 cases) OM were included in the study and followed for 1 year. OM was diagnosed by clinical, roentgenographic, tomographic, and isotopic bone imaging criteria. Surgical and sinus tract pus samples were cultured in all the patients with OM. OM was considered chronic if it was present for more than 3 months and cured if did not relapse during 1 year of follow-up. In addition, 220 Blood Bank donors, matched for age and sex with the patients, were used as controls. Each participant gave informed consent for the study, which was approved by the ethics committee of the Hospital Central de Asturias.

Human neutrophil isolation

Ten milliliters of peripheral blood were collected for each assay from patients with OM or healthy individuals and deposited in Vacutainer plastic tubes containing potassium-EDTA (Vacuette, Greiner Bio-One, Kremsmuenster, Austria). Neutrophils were purified by Ficoll-Hypaque (Lymphoprep, Axis-Shield Poc AS, Oslo, Norway) centrifugation as previously described.[39] Cells collected from the gradient interface contained more than 95% neutrophils by Coulter identification and were more than 95% viable by trypan blue exclusion. Neutrophils were isolated before each experiment and used immediately.

Culture conditions

Culture conditions involved incubating 5×10^6 neutrophils with 200 μL of Ham's F-12 medium (Gibco, Paisley, Scotland, UK) at 37°C for 12 hours, and apoptosis was then measured.

Flow cytometry

Cell death of neutrophils was measured by propidium iodide (PI) staining as described by others.[40] The PI fluorescence of individual cells was measured with a flow cytometer (Cytomics FC500, Beckman-Coulter Inc., Miami, FL). Mean fluorescence values were determined from a minimum of 1×10^4 cells within an analysis region corresponding to nonfragmented neutrophils using Cytomics RXP Software (Beckman-Coulter Inc.) for data acquisition analysis. In some additional experiments, neutrophil apoptosis was measured by the annexin V-FITC Apoptosis Detection Kit (Sigma, St. Louis, MO) according to the protocol provided by the company.

Amplification of the *bax* promoter region

We collected 10 mL of blood from each patient and control individuals in tubes containing potassium-EDTA. Genomic DNA was extracted from peripheral leukocytes using a salting-out method.[41] To determine the *bax* genotype of the samples, the genomic DNA was amplified with primers (forward primer: 5′-CGGGGTTATCTCTTGGGC-3′; reverse primer, 5′-GTGAGAGCCCCGCTGAAC-3′).[42] The polymerase chain reaction (PCR) was performed in a final volume of 16 μL, containing 100 ng of genomic DNA, and consisting of an initial denaturation at 95°C for 5 minutes; followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 56°C, and 45 seconds 72°C; and a final extension of 5 minutes at 72°C.

Restriction enzyme analysis

The restriction enzyme Aci I (New England BioLabs Inc., Beverly, MA) was used to screen the samples for the SNP detection following the protocol of Moshynska et al.[19] Essentially, the PCR products were incubated for 3 hours at 37°C with the restriction enzyme Aci I. Aliquots of 10 μL of the digested PCR products were then electrophoresed on a 7.5% polyacrylamide gel at 165 V for 80 minutes in an electrophoresis apparatus (Mini-Protean II, Bio-Rad, Hercules, CA). PCR products were visualized after staining with ethidium bromide (Sigma, St. Louis, MO). Homozygous GG (wild-type) were visualized as three major bands of 352, 256, and 96 base pair (bp) (GG). It should be noted that the 256-bp band was the most intense. Heterozygous AG resulted in the loss of a restriction site for Aci I in one of the *bax* promoters and showed three bands, with the 352-bp band being the most intense, whereas the 96-bp band became almost invisible. Homozygous AA (homozygous carrier of the SNP) showed only the 352-bp band (Fig. 1).

Sequence analysis

The results of the restriction analysis were confirmed by sequence analysis of representative samples for each genotype (Fig. 1). PCR products were purified using spin columns (High Pure PCR Product Purification Kit, Roche, Mannheim, Germany) and sequenced on an ABI Prism 3100 Genetic Analyser (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. The sequences were compared with *bax* data in the GenBank (accession number U17193 for promoter).

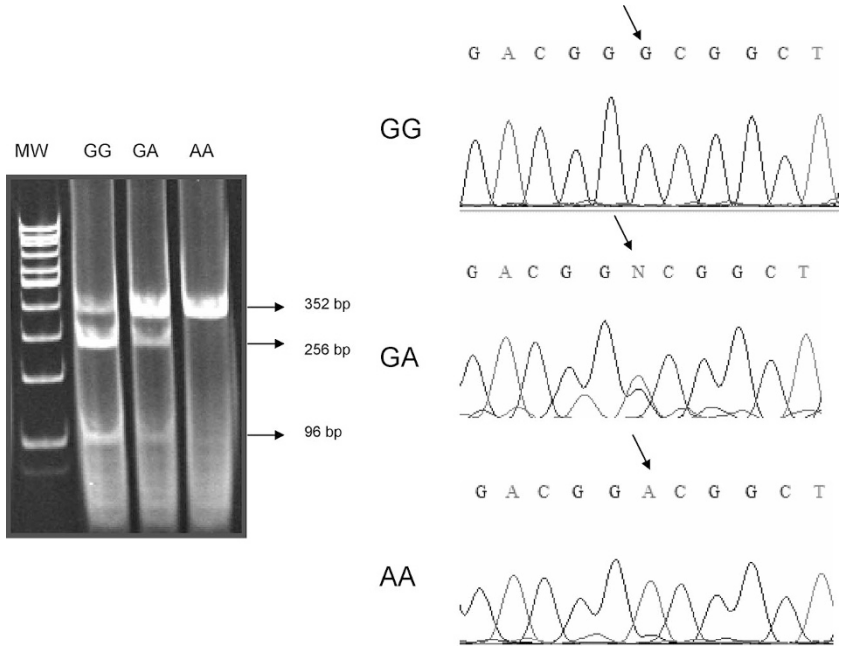

Fig. 1. Detection of the G(-248)A polymorphism in the *bax* gene. (Left) Genomic DNA of patients with OM was amplified, and PCR products were digested with the enzyme Aci I endonuclease followed by 7.5% polyacrylamide gel analysis. GG denotes a wild-type sample showing three distinct bands: 352, 296, and 96 bp; GA denotes a heterozygous sample showing the 352- and 296-bp bands and an almost invisible 96-bp band; AA denotes a homozygous SNP sample showing only the 35-bp band. MW, molecular weight marker, 100-bp DNA ladder (GeneRuler). (Right) Sequencing of the restriction fragment length polymorphism. Samples with no alteration: wild type (*upper*) with a heterozygous G-to-A replacement (*middle*) and a homozygous AA genotype (*lower*).

Protein purification and Western blotting

Then, 5×10^6 neutrophils were collected and washed in phosphate-buffered saline. They were resuspended in a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton-X, 2 mM EDTA, 8 mM EGTA, 0.5 mM AEBSF), incubated for 30 minutes on ice, and centrifuged at 16,000*g* at 4°C. Protein concentration was measured from the supernatant using the BCA Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL). Western blot analysis of the whole cell lysates was performed, and a housekeeping gene product β-actin served as a control for equal loading. Samples were resuspended in a loading buffer (200 mM Tris, pH 6.8, 8% sodium dodecylsulfate, 0.4% bromophenol blue, 40% glycerol, 400 mM dithiothreitol) and heated 5 minutes at 90°C. Denatured proteins (80 μg/sample) were separated on 12% denaturing polyacrylamide gels (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to nitrocellulose membranes (Hybond C, Amersham Biosciences, Buckinghamshire, England). Membranes were blocked for 1 hour with a 5% (w/v) nonfat dry milk solution containing 10 mM Tris-Cl, pH 7.5, 140 mM NaCl, and 0.1% Tween 20 (TBS-T) before their incubation for 1 hour with a primary antibody against Bax or β-actin (Sigma), diluted 1:2500 with 2.5% nonfat dry milk in TBS-T. After washing, the membranes were incubated with a species-appropriate horseradish peroxidase-linked immunoglobulin-G antibody (Amersham Biosciences), diluted 1:4000 in TBS-T, and the labeled proteins were detected using enhanced chemiluminescence reagents as described by the manufacturer (Amersham Biosciences). The band intensity was quantitated using the software Image J (National Institutes of Health, Bethesda, MD).

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences Software version 12.0 (SPSS Inc., Chicago, IL). The chi-square and Fisher exact tests were used to compare the frequencies of genotypes and the clinical characteristics between the groups. Odds ratios and their 95% confidence intervals were also calculated. The observed genotype frequencies were compared with the expected Hardy-Weinberg distributions by chi-square analysis. Neutrophil apoptotic values between genotypes and allelic groups were compared with an analysis of variance test and presented as the mean $\pm$ standard deviation. All reported *P* values were two-sided. A *P* value less than .05 was considered statistically significant.

RESULTS

Frequency of the G(-248)A *bax* promoter polymorphisms in osteomyelitis

To determine the G(-248)A polymorphism in the *bax* promoter gene, genomic DNA from patients with OM was amplified and PCR products were subsequently digested with the enzyme Aci I. Restriction fragment length polymorphism analysis of the excised sections of the gene was then performed. The G(-248)A polymorphism in the *bax* promoter was in Hardy-Weinberg equilibrium among patients with OM and controls. Individuals with the G(-248)A *bax* promoter polymorphism A allele were significantly more frequent among the 80 patients with OM than in the 220 healthy controls (18.1% vs. 10.6%, $\chi^2 = 4.84$, odds ratio 1.81, 95% confidence interval 1.0–3.07,

Table 1
Genotypic and allelic frequencies of *bax* promoter G(-248)A polymorphism in patients with osteomyelitis and controls

	Genotypic frequencies			Allelic frequencies	
	GG	GA	AA	G	A
Patients with OM ($n = 80$)	53 (66.2%)	25 (31.3%)	2[a] (2.5%)	131 (81.9%)	29[b] (18.1%)
Controls ($n = 220$)	173 (78.6%)	46 (20.9%)	1 (0.5%)	392 (89.4%)	48 (10.6%)

OM, osteomyelitis.
[a]$P = .17$, $\chi^2 = 2.48$, odds ratio = 5.62, 95% confidence interval = 0.39–158.68 when comparing the genotypic frequencies between patients with OM and controls.
[b]$P = .028$, $\chi^2 = 4.84$, odds ratio = 1.81, 95% confidence interval = 1.06–3.07 when comparing the allelic frequencies between patients with OM and controls.

$P = .028$ by the Yates correction). However, although homozygotes for the G(-248)A *bax* promoter polymorphism (AA genotype) were more frequent among the 80 patients with OM than in the 220 healthy controls, the difference between groups was not statistically significant (2/80 [2.5%] vs. 1/220 [0.5%], $P = .17$) (Table 1).

Effect of the G(-248)A *bax* promoter polymorphism on the cause and pathogenesis of osteomyelitis

A possible correlation between the *bax* polymorphism and the clinical presentation of the patients with OM was studied. There were no differences in the age, gender, source of infection (posttraumatic or hematogenous), microorganisms found (*S. aureus* vs. Gram-negative bacteria), or evolution (chronic or acute) between carriers and noncarriers of the A allele of the *bax* polymorphism (Table 2). Finally, there were no significant differences in different pathogenic predisposing factors to OM among the carriers of the different G(-248)A *bax* promoter polymorphism genotypes (Table 3). Therefore, the predisposition to OM is due to the presence of this *bax* polymorphism.

Neutrophil apoptosis

To determine whether the G(-248)A *bax* promoter polymorphism was associated with abnormal lifespan of neutrophils, we examined the spontaneous cell death and apoptosis of peripheral neutrophils after 12 hours of incubation in Ham's medium. Cell death and apoptosis of neutrophils from patients with OM and carriers and noncarriers of the A allele were significantly decreased in relation to controls, as we previously reported.[39] Cell death rate for the AA group differs from that in the heterozygous GA (15.2% ± 5.4% vs. 26.8% ± 18.2%, $P = .066$) and the wild-type GG groups (15.2% ± 5.4% vs. 50.2% ± 26.5%, $P = .107$) by PI staining (Fig. 2A). Patients with OM who were carriers of the A mutated allele had a significantly lower cell death rate of their neutrophils compared with patients who were carriers of the wild-type G allele (24.2% ± 16.6% vs. 50.2% ± 26.5%, $P = .026$) (Fig. 2B). When binding of Annexin V was used as a marker for apoptotic cells,[42] patients with OM who were carriers of the mutated A allele of this G (-248) A *bax* promoter polymorphism showed a significantly lower apoptotic rate of their neutrophils compared with patients with the wild-type GG genotype (33.3% ± 16.7% vs. 43.1% ± 3.1%, $P = .036$) (Fig. 2C).

Table 2
Clinical characteristics of patients with osteomyelitis with different genotypes of *bax* promoter G(-248)A polymorphism

Bax G(-248)A genotype	GG	GA/AA	*P* value
No. of cases	53	27	Not applicable
Mean age, y	56.6 ± 18.6	54.8 ± 16.6	.9
Male/female	36/17	19/8	.8
Chronic/acute OM	14/39	11/16	.2
Hematogenous/post traumatic OM	14/39	7/20	.9
Gram-negative bacteria/*S. aureus* OM	16/37	6/21	.5

OM, osteomyelitis.

Table 3
Predisposing factors for osteomyelitis in carriers of the different genotypes of *bax* promoter G(-248)A polymorphism

Bax G(-248)A genotype	GG ($n = 53$)	GA ($n = 25$)	AA ($n = 2$)	*P* value
Paraplegia (%)	7/53 (13.2)	3/25 (12.0)	0/2 (0.0)	.88
Peripheral vascular disease (%)	3/53 (5.7)	1/25 (4.0)	0/2 (0.0)	.75
Cavus foot (%)	2/53 (3.8)	1/25 (4.0)	0/2 (0.0)	.96
Diabetes	7/53 (13.2)	1/25 (4.0)	0/2 (0.0)	.21
Other factors[a] (%)	3/53 (5.7)	4/25 (16.0)	0/2 (0.0)	.13
Total factors (%)	21/53 (39.6)	10/25 (40.0)	0/2 (0.0)	.97

NS, not significant.
[a]Sensitive polyneuropathy, Ewing sarcoma, prostate cancer, cerebrovascular stroke, liver cirrhosis, morbid obesity, hemophilia.

Bax protein expression in freshly isolated human neutrophils

To determine whether the G(-248)A *bax* promoter polymorphism was associated with abnormal Bax expression, neutrophil lysates of patients with OM with different *bax* genotypes were assessed by Western blotting with a commercially available antibody to Bax. Patients with the AA genotype showed a significantly lower expression of Bax compared with those with the GA and GG genotypes (46.7 ± 25.1 vs. 79.2 ± 24.3 optical density arbitrary units, $P = .038$) (Fig. 3).

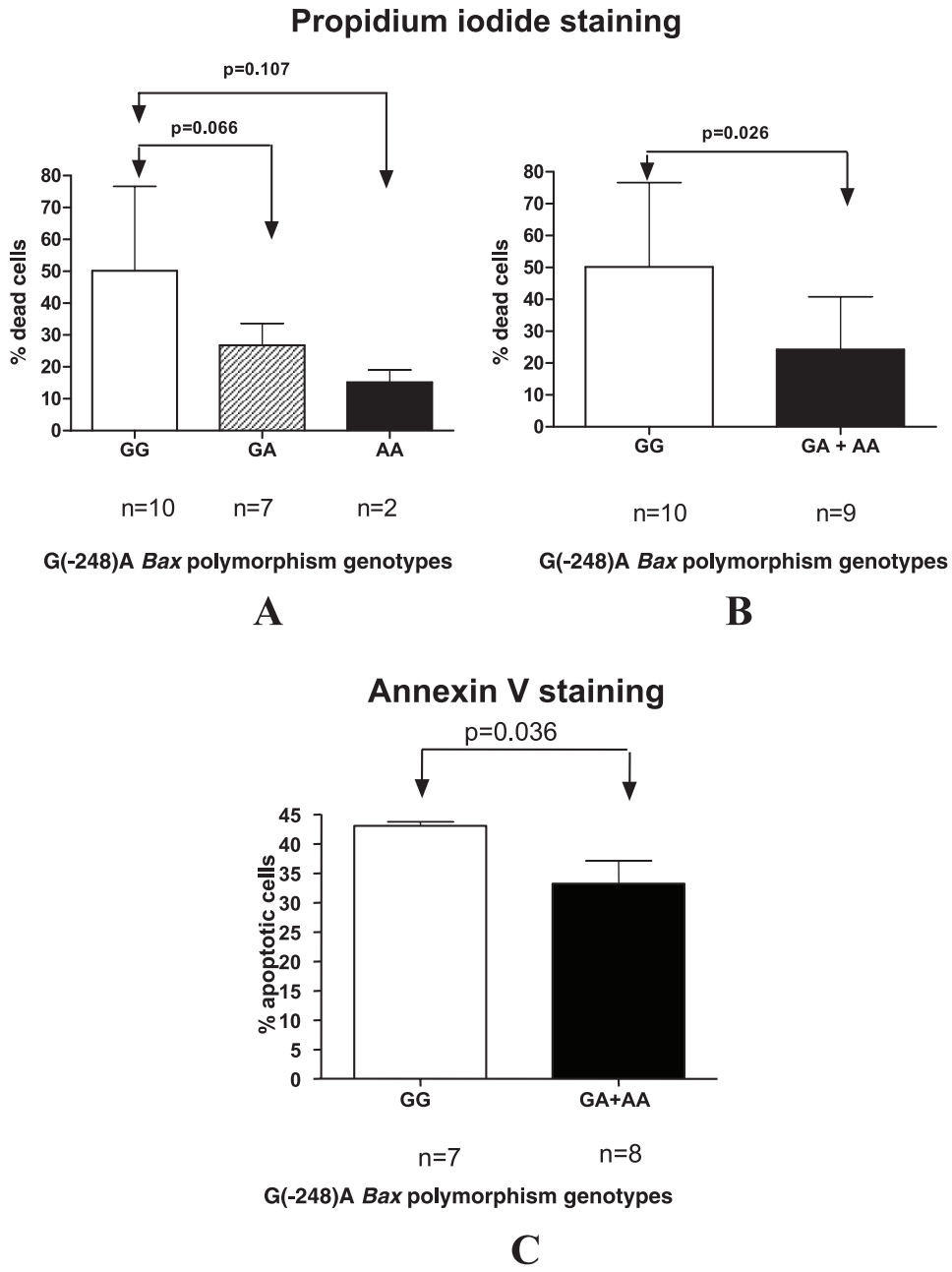

Fig. 2. Cell death and apoptosis of neutrophils of patients with OM with different *bax* G(-248)A polymorphism genotypes. Neutrophils ($0.5–1 \times 10^7$) of patients with OM were incubated at 37°C in Ham's medium, and cell death (A and B) and apoptosis (C) were measured after 12 hours with PI/annexin V-fluorescein isothiocyanate staining and flow cytometry. Results represent the mean ± standard deviation of the patients.

DISCUSSION

Bacterial OM causes substantial morbidity worldwide, despite improvements in medical and surgical management. The clinical course and outcome of this bone infection differs from patient to patient even among those of the same age, gender, microorganism, bone involved, and source of infection. For these reasons, perhaps the genetic background of patients with OM may be responsible for this variability that could signify disease susceptibility and/or poor clinical outcome. To date, several gene polymorphisms have been reported as predisposing one to develop OM.[2–4] The present work brings additional information to this subject by finding that a polymorphism in the *bax* promoter G(-248)A was more frequent among patients with OM (18.1%) compared with controls (10.6%). Our findings regarding the allelic frequency of this polymorphism are in agreement with previous reports. Thus, the A allele frequency of the G(-248)A *bax* promoter polymorphism found in our control white population of 220 Blood Bank donors was similar to that reported by Starcynski et al.[43] in 135 British white volunteers (8.0%), and it was a little higher than that reported by Saxena et al.[18] in 25 healthy Canadians (3%), although they did not report their racial background. Unlike

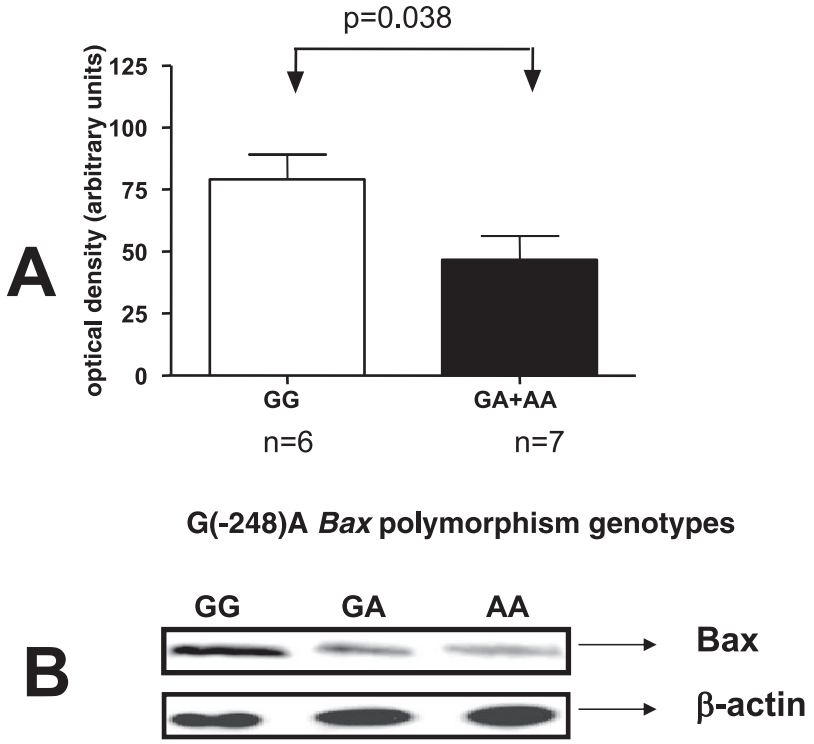

Fig. 3. Detection of Bax protein in neutrophils in patients with OM with different *bax* G(-248)A polymorphism genotypes. A: Bax expression in the samples of each genotype of *bax* was assessed by Western blotting and normalized to β-actin. Band intensities were registered in arbitrary units using the software Image J (National Institutes of Health, Bethesda, MD). Results represent the mean ± standard deviation of the patients. B: Results of the Western blot assay of a representative sample of each genotype. β-actin is used as a loading control.

other cytokine polymorphisms, such as the IL-1α (-889) associated with a younger age at diagnosis of OM,[2] or the TLR4 (Asp299Gly) associated with Gram-negative bacteria and hematogenous OM,[4] carriers of the A allele of the G(-248)A *bax* promoter polymorphism did not show differences in their age, gender, source of infection, microorganism involved, or condition predisposing to OM when compared with noncarriers of the allele.

Bax is a death-promoting protein shown to be a tumor suppressor that stimulates cellular apoptosis in vivo.[44,45] The *bax* gene is located on chromosome 19 and consists of six exons and a promotor region with four p53 binding sites.[46] Sequence variations in the promotor region and coding sequence can abolish its proapoptotic function.[17] The promoter region with G-to-A SNP consists of potential binding sites for c-Myb, and single nucleotide substitutions in this region may affect c-myb–induced transcriptional activation.[18,47] The G(-248)A *bax* promoter polymorphism is associated with disease progression, treatment resistance, and shorter survival in patients with chronic lymphocytic leukemia and with decreased cell Bax expression.[18,43,47] It is known that changes in the 5′-untranslated region sequence can inhibit initiation of translation and that the altered expression of Bax protein more likely involves a posttranscriptional mechanism.[18] Other genetic variants in the *bax* gene have been found in B-cell lymphomas and several cell lines of human hematopoietic malignancies.[16,48,49] However, SNPs of the *bax* gene are rare, and the G(-248)A promoter SNP, studied in the present work, is the most important among them. Ours is the first report of an association between a *bax* gene SNP and an infection, OM. We also found that the G(-248)A *bax* promoter polymorphism in patients with OM was associated with an increased survival of their neutrophils. This *bax* polymorphism was also associated with reduced Bax protein expression by neutrophils. Therefore, it seems likely that this polymorphism is responsible at least in part for the dysregulation of the apoptotic cascade that we observed in the neutrophils of patients with OM. It could be speculated that neutrophils of carriers of the A allele, which have a blunted cellular response to p53 activation and a low constitutive expression of Bax,[43,49] may have an increased response to IL-6 and other cytokines found at increased levels in serum of patients with OM that delays neutrophils apoptosis. IL-6 enhances the expression of the antiapoptotic proteins Mcl-1, A1, and Bcl-X_l and inhibits constitutive Bax expression, therefore enhancing the antiapoptotic effect of A allele of the G(-248)A *bax* promoter polymorphism on peripheral neutrophils of patients with OM.[8,33,38,39] Neutrophils with an extended lifespan in the infected bone may help to perpetuate the bone infection by release of their proteolytic enzymes.

Finally, we cannot rule out that this G(-248)A *bax* promoter polymorphism could be in linkage disequilibrium with an unidentified variant responsible for the observed effect on neutrophil lifespan and Bax expression.

More research is needed to clarify in depth how this antiapoptotic effect of the G(-248)A *bax* promoter polymorphism may affect the susceptibility to develop OM.

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