TNF-alpha, TNF-beta, IL-6, IL-10, PECAM-1 and the *MPO* Inflammatory Gene Polymorphisms in Osteosarcoma

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Summary: The inflammatory microenvironment of tumors is characterized by the presence of cytokines and growth factor's network both in the supporting stroma and in tumor areas. These molecules may contribute to tumoral growth and progression, facilitating metastatic process. Therefore, cancer susceptibility and severity may be associated with the functional polymorphisms of inflammatory genes. We hypothesized that inflammatory gene polymorphisms may have important role for osteosarcoma patients. We studied −308G > A *TNF-α*, +252A > G *TNF-β*, −174G > C *IL-6*, −1082A > G *IL-10*, +125C > G *PECAM-1*, and the −463A > G *MPO* inflammatory gene polymorphisms in 80 osteosarcoma patients and 160 control individuals using polymerase chain reaction-restriction-fragment length polymorphism method. We found that the patients with variant genotype (GG) of the +252A > G *TNF-β* gene showed an event-free survival rate of 20% at 100 months. We suggest that the presence of the variant genotype (GG) of the +252A > G *TNF-β* polymorphism, which leads to higher level of cytokine production, could be a facilitator mechanism in tumor progression leading to a poor event-free survival.

Key Words: PCR-RFLP polymorphism, cytokines genes, osteosarcoma, *TNF-β*

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Osteosarcoma (OS) is the most common primary bone tumor in children and young adults.[1] Approximately, 10% to 20% of patients with OS have metastatic disease at diagnosis. The most frequent site of metastasis is the lung, however, a smaller percentage of patients have bone and soft tissues metastasis.[2] The presence of metastasis at diagnosis is a prognostic factor, with a strong impact in the global survival of these patients.[2] Patients without metastasis at diagnosis present 5 years overall survival of 70%, whereas in patients with metastasis at diagnosis the overall survival falls to 32%. In Brazil, 20% of patients present with metastatic disease at diagnosis, a rate that is twice as higher compared with the reported in developed countries.[3–5]

Despite the dramatic advances in OS treatment, patient survival reached a plateau. Recent clinical trials, which attempted to improve outcome through intensification of therapy or incorporation of new agents have not been widely successful. Therefore, increasing focus has been placed in achieving a greater understanding of the basic biology of OS, with the goal of using that information to improve treatment.[6]

The presence of polymorphisms in the human genome provides extensive genetic variation affecting both normal physiologic mechanisms and cancer pathogenesis. These polymorphic variants act through their products in various regulatory systems and metabolic chains at different levels of biologic organization.[7] In this manner, the proliferation, differentiation, and death of transformed and even malignant cells can be affected by preexisting polymorphisms in genes exerting the regulation of these basic processes. Consequently, understanding of the role of genetic variation affecting these diverse systems is crucial for the unraveling of differences in tumor behavior, prognosis, and response to therapeutic interventions.[7]

In OS, there is limited data on polymorphic alleles of inflammatory genes. Polymorphisms of cytokine and cell adhesion molecules genes, such as tumor necrosis factor-alpha (−308*TNF-α*), tumor necrosis factor-beta (+252*TNF-β*), interleucine-6 (−174*IL-6*), interleucine-10 (−1082*IL-10*), and platelet/endothelial cell adhesion molecule-1 (+125*PECAM-1*), have been reported to influence the level of secreted mediators. These alterations unbalance the inflammatory cascade, are involved in immune and inflammatory responses, and affect angiogenesis and tumor growth.[7–10] Myeloperoxidase (−483*MPO*) is an enzyme involved in inflammation and oxidative stress, and that plays an important role in human defense against microorganisms. *MPO* gene polymorphisms have been associated with the risk for lung cancer.[7–10]

The tumor inflammatory microenvironment is characterized by the presence of this cytokines and

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TABLE 1. Primers and Enzymes Used to Gene Polymorphism Analysis

Polymorphisms	Primers	Restriction Enzyme
− 308G > A *TNF-α*	5′- AGG CAA TAG GTT TTG AGG GCC AT –3 5′- TCC TCC CTG CTC CGA TTC CG -3′	*NcoI*
+ 252A > G *TNF-β*	5′- CTC CTG CAC CTG CTG CCT GGA TC -3′ 5′- GAA GAG ACG TTC AGG TGG TGT CAT -3′	*NcoI*
− 174G > C *IL-6*	5′- CTG ATT GGA AAC CTT ATT AAG -3′ 5′- GTG GTT CTG CTT CTT AGC -3′	*Nla III*
− 1082A > G *IL-10*	5′- AAT CCA AGA CAA CAC TAC TA -3′ 5′- CTC CAG CAC ATA GAA TGA AA -3′	*Nla III*
+ 125C > G *PECAM-1*	5′- ACG GTG CAA AAT GGG AAG AA -3′ 5′- AGA GGG TGA TGG GTG GAG AG –3′	*Alu I*
− 463A > G *MPO*	5′- CCG TAT AGG CAC ACA ATG GTG AG -3′ 5′- GAC ATG GTGT CAA GCG ATT CTT C -3′	*AciI*

growth factor network and may contribute to growth and tumor progression. In this manner, cancer susceptibility and severity may be associated with the functional polymorphisms of inflammatory genes.[9]

We hypothesized that polymorphisms of these genes may have an important role in the prognosis of OS patients. We thus studied *TNF-α, TNF-β*, *IL-6*, *IL-10*, *PECAM-1*, and *MPO* inflammatory gene polymorphisms in 80 OS patients and 160 control individuals. We analyzed the association of gene polymorphism with the incidence of metastasis at diagnosis, tumor histology, tumor necrosis grade, relapses of disease and survival analysis of the Brazilian OS patients.

MATERIALS AND METHODS

Patients and Controls

We selected 80 patients with the diagnosis of OS, admitted in the Oncology Pediatric Institute and 160 control individuals admitted in the Pediatric Department of the Federal University of São Paulo, Brazil. After the informed consent was obtained, 5.0 mL of peripheral blood was collected and each case was matched with a control for sex, age (± 3 y), and ethnic group. Each individual was examined clinically and the ethnic group was established by using a multivariate evaluation on the basis of the skin color, hair color and texture, and the shape of the nose and lips. In accordance to this multivariate analysis, those individuals with white skin, straight or wavy hair, prominent or upturned nose tip, thin or median lips, independently of hair or eye pigmentation, were called Euro-descendant, whereas those with black skin, black curly hair, black eyes, and thick lips were classified as Afro-descendant. All other patients, excepting Asio-descendant, were classified as intermediate.[11]

Gene Polymorphism Analysis

The peripheral blood leukocytes from patients and controls were stored in 4°C and were used as a source of DNA, which was extracted through the "Wizard Genomic DNA purification kit" Promega.

Previously published polymerase chain reaction and restriction digestion protocols were used to identify the − 308G > A *TNF-α*, + 252A > G *TNF-β*, − 174G > C *IL-6*, − 1082A > G *IL-10*, + 125C > G *PECAM-1*, and the − 463A > G *MPO* polymorphisms[8,10,12,13] (Table 1).

Statistical Analysis

Chi-square test was used for this study to check the possible association between the studied classes. The associations were considered significant when $P < 0.05$. We used Kaplan-Meier to analyze overall and event-free survival (EFS) of OS patients and Cox test for the multifactorial analyses.[14,15]

RESULTS

The mean age of OS patients was 15.8 years; 58.8% were male and 41.3% female; 63.8% were Euro-descendant, 18.3% there were no possibility to define the ethnic group, 16.3% were Afro-descendant, and 1.3% were Asian-descendant (Table 2).

Five years overall and EFS of OS patients was 70% and 30%, respectively (Figs. 1, 2). Curves of overall and EFS for *TNF-α*, *TNF-β*, *IL-6*, *IL-10*, *PECAM-1*, and *MPO* genotypes showed significant lower event-free survived only for variable *TNF-β* genotypes (Fig. 3).

Table 3 shows the allele and genotype frequencies, with the corresponding phenotypes of *TNF-α*, *TNF-β*, *IL-6*, *IL-10*, *PECAM-1*, and *MPO* inflammatory polymorphic genes in OS patients and controls. The distributions of these genotypes were in Hardy-Weinberg equilibrium, with exceptions of the − 308G > A *TNF-α* and + 125C > G *PECAM-1* polymorphisms. These polymorphisms revealed deviation from Hardy-Weinberg equilibrium due to the excess of heterozygosity, for the 2 polymorphisms (on either group, patients and controls) and of homozygosity for *TNF-α* polymorphism, on control group only. The expected frequencies were as follows: *TNF-α* (AG) = 0.43 patients and (AA) = 0.19 controls and *PECAM-1* (VL) = 0.37 patients and 0.55 controls (Table 3).

Cox regression model and χ^2 were used for the multivariate analyses and the analyses of the associations between all polymorphic genotypes identified (*TNF-α*,

TABLE 2. Clinical Characteristics of Studied Osteosarcoma Patients

Clinical Characteristics	Number Patients (%)
Sex	
Male	47 (58.8)
Female	33 (41.3)
Age	
First decade	8 (10)
Second decade	61 (76.3)
Third decade	10 (12.5)
Fourth decade	1 (1.3)
Ethnic group	
Euro-descendant	51 (63.8)
Intermediate	15 (18.8)
Afro-descendant	13 (16.3)
Asio-descendant	1 (1.3)
Metastasis at diagnosis	
Yes	26 (32.5)
No	54 (67.5)
Histology	
Osteoblastic	33 (50.8)
Chondroblastic	9 (13.8)
Fibroblastic	1 (1.5)
Telangectasic	5 (7.7)
Small cells	1 (1.5)
Giant cells	1 (1.5)
Others	15 (23)
Not identified	12
Tumor necrosis grade	
I Grade (0%-75%)	27 (51.9)
II Grade (75%-90%)	6 (11.5)
III Grade (90%-95%)	11 (21.2)
IV Grade (95%-100%)	8 (15.4)
Not identified	25
Relapses of disease	
Yes	36 (50.7)
No	35 (49.3)
Not identified	9

TNF-β, *IL-6*, *IL-10*, *PECAM-1*, and *MPO*) and the evaluated classes (sex, age, ethnic group, metastasis in the diagnosis, tumor histology, tumor necrosis grade, and relapses of disease). Neither of associations was significant.

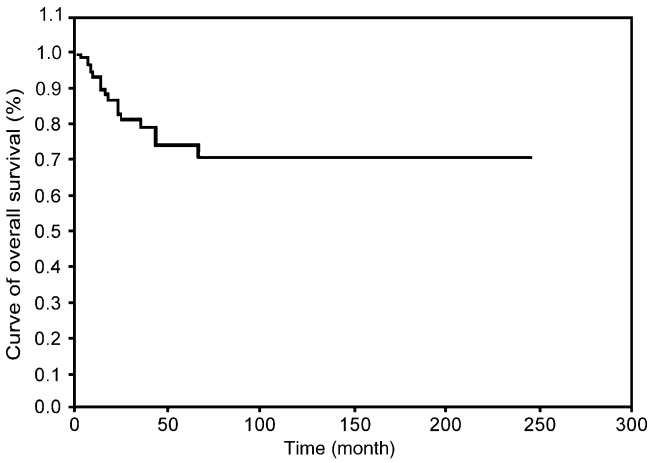

FIGURE 1. Kaplan-Meier curve of overall survival.

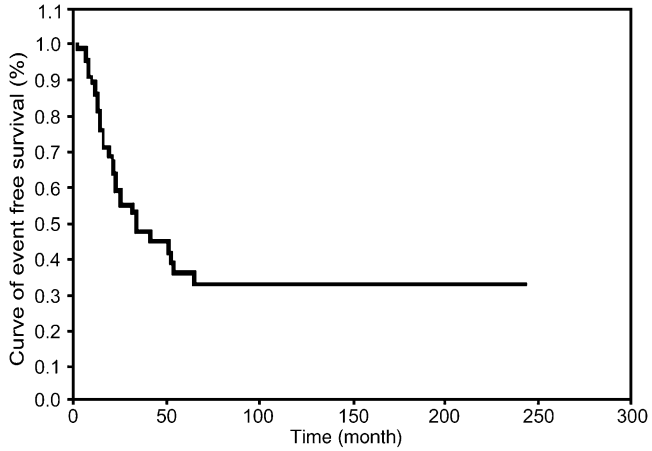

FIGURE 2. Kaplan-Meier curve of EFS.

DISCUSSION

Functional polymorphisms of inflammatory genes may play a role in cancer susceptibility and severity. The main genetic alterations involved in the development or progression of OS are still unknown.

Of a number of inflammatory gene polymorphisms and clinical parameters studied in patients with OS, only +252A > G *TNF-β* was noted to be associated with a reduced EFS. Patients with the variant genotype (GG) showed an EFS rate of 20% at 100 months, whereas all patients with wild genotype (AA) showed EFS rate of 60% after 100 months of observation (Fig. 3). Although this pilot study is small in numbers and includes multiple analyses, it suggests that further investigation is warranted.

The TNF cytokines have a paradoxal role in the evolution and treatment of malignant diseases. The high-dose local TNF management selectively destroys tumor blood vessels and has powerful anticancer action, but when chronically produced these cytokines may act as an endogenous tumor promoter, contributing to the tissue

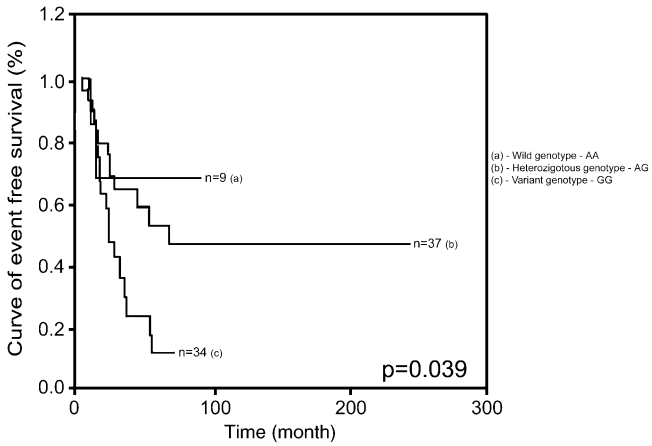

FIGURE 3. Kaplan-Meier curve of EFS for variable *TNF-β* genotypes for OS patients.

TABLE 3. Genotype and Allele Frequencies of Inflammatory Gene Polymorphisms in the Osteosarcoma Patient Samples

Genes	Genotype Frequency Patients	Genotype Frequency Controls	Allele Frequency Patients	Allele Frequency Controls	Genotypes-associated Phenotypes	No. Patients (%)	No. Controls (%)
−308 TNF-α	AA (0.07)	AA (0.01)	A (0.26)	A (0.26)	Higher production	6 (7.5)	5 (3.1)
	AG (0.43)	AG (0.19)				19 (23.7)	25 (15.6)
	GG (0.64)	GG (0.79)	G (0.80)	G (0.80)	Lower production	55 (68.7)	130 (81.25)
+252 TNF-β	GG (0.43)	GG (0.47)	G (0.65)	G (0.68)	Higher production	34 (42.5)	77 (48.1)
	AG (0.45)	AG (0.42)				37 (46.2)	66 (41.2)
	AA (0.11)	AA (0.09)	A (0.34)	A (0.31)	Lower production	9 (11.25)	17 (10.6)
−174 IL-6	GG (0.10)	GG (0.07)	G (0.32)	G (0.27)	Higher production	9 (2.8)	10 (3.8)
	GC (0.42)	GC (0.38)				23 (30.7)	68 (43.1)
	CC (0.46)	CC (0.51)	C (0.67)	C (0.72)	Lower production	32 (56.4)	82 (52.2)
−1082 IL-10	AA (0.12)	AA (0.15)	A (0.35)	A (0.38)	Lower production	5 (6.4)	23 (14.6)
	AG (0.45)	AG (0.46)				44 (56.4)	76 (48.4)
	GG (0.42)	GG (0.37)	G (0.64)	G (0.61)	Higher production	29 (37.1)	58 (36.9)
+125 PECAM-1	LL (0.31)	LL (0.25)	L (0.56)	L (0.25)	Not applicable	9 (11.2)	48 (30)
	VL (0.48)	VL (0.45)			Not applicable	52 (65)	65 (40.6)
	VV (0.19)	VV (0.21)	V (0.43)	V (0.45)	Not applicable	19 (23.5)	41 (25.6)
−463 MPO	GG (0.51)	GG (0.57)	G (0.71)	G (0.76)		44 (37.1)	93 (59.2)
	AG (0.39)	AG (0.36)			Decreased intracellular concentration	29 (56.4)	53 (33.7)
	AA (0.07)	AA (0.05)	A (0.28)	A (0.23)	Decreased intracellular concentration	5 (5.2)	11 (7.0)

remodeling and stromal development necessary for tumor growth and spread.[9,16]

Moreover, the TNF cytokine are present in various tumor cell types that use the same molecular tools (cytokines, adhesion molecules, chemokines), typical of migrating leukocytes, to seed at distant anatomic sites during inflammation.[9] We suggest that the presence of the variant genotype (GG) of the gene +252A > G *TNF-β*, which can increase TNF-β cytokine levels, could act as an endogenous tumor promoter and result in reduced EFS in patients with OS. The high level of *TNF-β* cytokine produced could be making the metastatic process easier in OS patients.

Research regarding involvement of polymorphic genes encoding key factors that mediate immune reactions, inflammation, angiogenesis, extracellular matrix degradation, and cell adhesion/communication still is scarce. Relatively convincing results associating certain polymorphic variants with cancer progression and prognosis have been reported for only a handful of genes such as *TNF-α* and *TNF-β*, however, it has to be stressed that studies in this direction have been initiated only a few years ago. Although lack of conclusive results does not allow making serious conclusions, it is important to notice that most of the pathogenetically important polymorphisms in this highly heterogenous group of genes affect regulatory (especially promoter) sequences. Therefore, it can be suggested that gene expression changes, rather than structural changes, in relevant protein products are likely to be involved in complex biologic machinery driving cancer invasion and metastasis.[7]

Research about interindividual genomic variations in general population can collaborate to improve the understanding of the complex diseases like cancer. In OS the knowledge about the tumoral development and progression, and the drug metabolism differences between the patients is scarce. Therefore, polymorphism studies with its variants can contribute to better clarify this complicated malignancy.

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