ASSOCIATION OF ENDOSTATIN D104N WITH LEUKEMIA

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The bone marrow and/or peripheral blood from 126 patients with acute myeloid leukemia (AML), 57 with chronic myeloid leukemia (CML), 91 with acute lymphocytic leukemia (ALL), and 178 normal controls were analyzed using a polymerase chain reaction-restriction fragment length polymorphism (RFLP) assay to evaluate the association of the endostatin polymorphisms D104N (nucleotide 4349G→A) with leukemia. In the 178 normal Taiwanese, the allele frequency of 4349G was 98% (348/356) and that of 4349A was 2% (8/356). The frequencies of homozygous 4349G (104D/D) and heterozygous 4349G/A (104D/N) were 95.5% (170/178) and 4.5% (8/178), respectively. However, no individuals were homozygous 4349A (104N/N). Among the leukemia patients, 124/126 with AML (98.4%), 55/57 with CML (94.9%), and 89/91 with ALL (97.9%) were homozygous 4349G. In addition, 2/126 with AML (1.6%), 2/57 with CML (5.1%), and 2/91 with ALL (2.1%) were heterozygous 4349G/A. No patients were homozygous 4349A. Similar frequencies of endostatin polymorphisms were observed in leukemic patients and normal controls. This suggests that the endostatin polymorphism is not associated with the risk of leukemia.

Key Words: endostatin gene, polymorphism, leukemia
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The molecular pathogenesis of different kinds of cancer, including leukemia, involves a stepwise accumulation of mutations affecting both cellular oncogenes and tumor suppressor genes [1–3]. Other factors including genetic instability and genetic variations in drug metabolism have been explored [4, 5]. Other gene alterations may be associated with the risk or progression of leukemia.

Recently, angiogenesis has been suggested as a fundamental step in tumor progression and metastasis [6, 7]. Excessive angiogenesis is characteristic of a variety of pathologic conditions including cancer formation and metastasis [6, 8]. A series of angiogenic and antiangiogenic molecules that mediate the sequential steps involved in tumor angiogenesis have been described [6, 9]. Several angiogenic inhibitors have been identified: α-interferon, platelet factor 4, thrombospondin-1, angiostatin, the 16-kd fragment of prolactin, the 29-kd fragment of fibronectin, and endostatin [6, 9, 10]. Many of these inhibitors, including endostatin, are stored as cryptic parts of naturally occurring large precursor proteins that are not themselves angiogenic inhibitors [9, 10]. Endostatin, a potent inhibitor of angiogenesis, is a 20-kd fragment derived from the carboxyl-terminal of collagen XVIII (exons 41 to 43) that inhibits endothelial cell proliferation, migration, and angiogenesis [7, 11, 12].

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Endostatin binds to the heparan-sulfated proteoglycan situated on the endothelial cell surface, which induces apoptosis through tyrosine kinase signaling (via the Shb adaptor protein) and significant reduction of the anti-apoptotic proteins Bcl-2 and Bcl-X_L [10, 13]. Endostatin is present in the vascular basement membrane and frequently in elastic fibers and microfibrils [7, 10, 14]. The serum level of endostatin is sufficient to effectively inhibit endothelial cell proliferation *in vitro*. It has recently been reported that lower expression of the endostatin precursor (collagen XVIII) is associated with progression of human hepatocellular carcinoma [15]. Higher serum levels of endostatin induce regression of solid tumors in animals [16]. In addition, the endostatin polymorphism (D104N) predisposes to a higher risk of prostate adenocarcinoma [17]. Therefore, lower levels of endostatin could be associated with a higher risk of developing solid tumors. However, the association of endostatin polymorphisms with the risk of leukemia has not yet been studied. To elucidate endostatin polymorphisms in leukemia, cases of acute myeloid leukemia (AML), chronic myeloid leukemia (CML), and acute lymphocytic leukemia (ALL) were compared with normal controls. Polymorphism analysis was performed using a polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) assay; some representative cases were analyzed using direct sequencing.

Materials and Methods

Tumor samples and normal controls

Specimens of bone marrow and/or peripheral blood were obtained from 126 de novo cases of AML, 57 cases of CML, and 91 cases of ALL diagnosed at the Kaohsiung Medical University Hospital in 1996 through 2001. In addition, peripheral blood samples from 178 normal controls were also collected.

DNA amplification and restriction enzyme analysis

Total genomic DNA was prepared from bone marrow or peripheral blood leukocytes of subjects as described previously [18]. Oligonucleotide primer design and restriction enzyme analysis was conducted according to the methods of Iughetti et al [17]. We designed PCR primers to identify the endostatin D104N polymorphism (nucleotide 4349G→A of the collagen XVIII cDNA in NM_030582). The sequences of the forward and reverse primers used for PCR were 5'-CACGGTTTCTCTTCCAGGAC-3' and 5'-CTCTCAGAGCTGCTCACACG-3', respectively. DNA amplification was performed as described previously [19, 20], except that the annealing temperature was modified based on the melting temperature of the primers. The 169-bp amplified fragments were digested with the restriction enzyme *Mse* I and separated by electrophoresis in 2.5–3.5% agarose gel.

Sequence analysis

Several wild and mutant endostatin alleles were further analyzed by direct sequencing to confirm the results. The amplicons were purified using a gel extraction kit (Geneclean III Kit, Bio101, Inc., CarlsbadCA, USA), then sequenced using the SequiTherm Excel™ II DNA Sequencing Kit (Epicentre®Technologies, Madison, WI, USA). The sequencing primers were those used for PCR.

Statistical methods

Descriptive analysis was performed to present the frequencies and distributions of demographic variables. Genotype frequencies in leukemia patients and normal controls were compared using contingency table analysis and the chi-square test. The odds ratio and its confidence interval were estimated using standard methods. The analysis was performed using SAS 6.11 statistical software.

Results

Changes in the D104N endostatin pattern after restriction enzyme digestion are shown in the Figure. The 169-bp PCR product was undigested in the 4349G

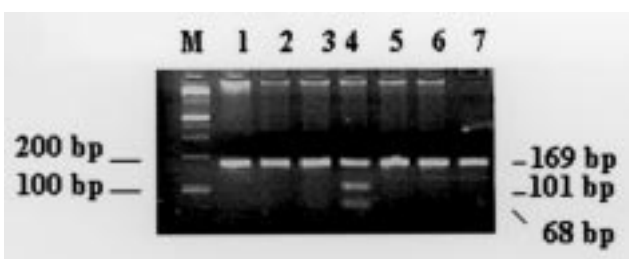

Figure. *Enzyme digestion analysis of the polymerase chain reaction product for endostatin D104N polymorphism. Lanes 1, 2, 3, 5, 6, and 7 are homozygous D/D and lane 4 is a D/N heterozygote. M is a 100-bp ladder marker.*

Table. Genotype distribution of endostatin D104N polymorphisms among controls and patient groups

Disease / Genotype	AML	CML	ALL	Control
DD	124 (98%)	55 (94.9%)	89 (97.9%)	170 (95.5%)
DN	2 (1.6%)	2 (5.1%)	2 (2.1%)	8 (4.5%)
NN	0	0	0	0
Total	126	57	91	178

AML = acute myelocytic leukemia; CML = chronic myelocytic leukemia; ALL = acute lymphocytic leukemia.

allele, but it was cleaved to 101- and 68-bp fragments in the 4349A allele.

The distribution of D104N polymorphisms is shown in the Table. Among the 178 normal Taiwanese, the 4349G (104D) allele was more common than the 4349A (104N) allele (98% *vs*. 2%), and homozygosity for 4349G was more common than heterozygosity (95.5% *vs*. 4.5%); there was no homozygosity for 4349A.

Of the leukemia patients, most were homozygous for 4349G (AML: 98.4%; CML: 94.9%; ALL: 97.9%). The remaining patients were heterozygous; there was no homozygosity for 4349A.

Discussion

In this study, we found that the frequency of endostatin D104N in the Taiwanese population ($q = 0.05$) is significantly different from that of Caucasians ($q = 0.16$) ($p < 0.001$), but is quite similar to that in blacks ($q = 0.08$) reported by Iughetti et al [17]. No controls or leukemia patients were homozygous for N104N endostatin, similar again to the data reported by Iughetti et al [17]. This result is consistent with our previous study on the CYP3A5 polymorphism, which suggested that inter-individual and interracial differences exist [21].

A number of polymorphic genes that may modify the effects of carcinogens, including the cytochrome p450, glutathione S-transferase, and N-acetyltransferase enzymes, are associated with a high risk of cancer susceptibility [22, 23]. Recently, a series of angiogenic and anti-angiogenic molecules involved in tumor angiogenesis have been described [6, 8, 9], and there is evidence that excessive angiogenesis is characteristic of cancer formation and metastasis. Leukemia and other hematological malignancies are also angiogenesis-dependent [24–26]. Padro et al noted increased angiogenesis in the bone marrow of AML patients and suggested the involvement of angiogenesis in the pathophysiology of AML [25]. Scappaticci et al reported angiogenesis-dependent leukemia in a mouse model [26], and the angiogenesis inhibitor endostatin was found to inhibit bone marrow angoiogenesis in leukemia in an animal study [11]. Polymorphisms in the angiogenesis-related endostatin gene and its precursor collagen XVIII are associated with a risk of cancer susceptibility in prostatic adenocarcinoma and hepatocellular carcinoma [15, 17]. These studies suggest that endostatin polymorphisms may be associated with a risk of leukemogenesis.

Endostatin is an important angiogenesis inhibitor, which acts by binding to heparin/heparan sulfate. Although D104 is not included in the binding epitope, Iughetti et al hypothesized that inhibition occurs through binding to other molecules [17]. We examined the frequencies of the endostatin D104N polymorphism in AML, CML, and ALL. There were no significant differences between any of these diseases and healthy controls ($p = 0.161$, 0.748, and 0.346, respectively).

The results showed that the endostatin D104N polymorphism in the Taiwanese population is not relevant to leukemogenesis. Consequently, we recommend that interracial differences in endostatin, differences in the pathogenesis of prostatic adenocarcinoma and leukemia, and the potential role of the endostatin in leukemia are studied further.

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