Journal of Surgical Research **123,** 222–226 (2005)
doi:10.1016/j.jss.2004.08.003

Identification of Differentially Expressed Genes in Primary Varicose Veins

Dong-Ik Kim,*,[1] Hyun-Seon Eo,† and Jin-Hyun Joh*

**Division of Vascular Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, and †Samsung Biomedical Research Institute, Seoul, Korea*

Submitted for publication April 28, 2004

Background. **A number of changes in protein expression have been described in primary varicose veins, but the altered gene expressions in this disease are unknown. The aim of this study was to identify differentially expressed genes in primary varicose veins.**

Materials and methods. **Total RNAs were isolated from two groups of greater saphenous veins (four primary varicose veins and three normal) and then were reverse transcribed into cDNAs. We used the differential display reverse transcription–polymerase chain reaction technique to screen the differences in the mRNA expression profiles of the groups.**

Results. **We found that three cDNAs showed differences in expression patterns between normal and diseased saphenous veins. The cDNAs are prominently expressed only in patients with varicose veins. We identified that the cDNAs had significant similarities to the L1M4 repeat sequence of clone RP11-57L9, clone RP11-299H13, and Alu repetitive sequence of human tropomyosin 4 mRNA.**

Conclusions. **Our results suggest that the screened cDNA clones are useful disease markers in the genetic diagnosis of primary varicose vein and that the L1 and Alu elements possibly participated in the development of primary varicose veins through their expression patterns in genes encoded with structural proteins, such as collagen, elastin, and tropomyosin. Further studies are required to elucidate the potential relationship between repeat sequences and primary varicose veins.**

Key Words: **Alu elements; gene expression profiling; tropomyosin; veins.**

[1] To whom correspondence and reprint requests should be addressed at Division of Vascular Surgery, Samsung Medical Center, 50 Irwon-Dong, Kangnam-Ku, Seoul, Korea, 135-710. Fax: 82-2-3410-0040. E-mail: dikim@smc.samsung.co.kr.

INTRODUCTION

Primary varicose veins are the most common manifestation of chronic venous insufficiency, accompanying dilated, tortuous, and poorly contractile saphenous veins. A number of hypotheses have been proposed for the mechanism of development of primary varicose veins. But the etiology of primary varicose veins is still unknown.

The dilation and tortuosity of the veins are evidence of progressive venous wall remodeling associated with abnormalities of the extracellular matrix. Recently, it was reported that differences exist in the mRNA expression and synthesis levels of extracellular matrix components, such as collagen Types 1 and 3, in primary varicose veins [1]. In all blood vessels, the components of extracellular matrix determine the mechanical properties of the tissue to balance between rigidity and elasticity [2]. Therefore, alteration in the expression and synthesis levels of extracellular matrix components could be responsible for modifications of rigidity and elasticity in vein walls and may contribute to the development of venous insufficiency of primary varicose veins.

It has previously been reported that the elasticity of the venous wall in the lower limbs is reduced in patients with venous insufficiency, as well as in patients with a high risk of developing primary varicose veins [3] and in children with a family history of primary varicose veins [4]. This indicates the correlation between elasticity and genetic factors. Also, several studies have demonstrated the involvement of hereditary factors in the transmission of the primary varicose vein pathology and congenital weakness of the venous wall [5–7]. These findings suggest that a genetic defect in the regulation of the composition of the extracellular

0022-4804/05 $30.00

matrix might participate in the pathogenesis of primary varicose veins and could be inherited by the intergeneration.

Thus far, histological and immunohistologic methods have been used to investigate changes in the wall structure in primary varicose veins and some of the abnormalities in the metabolic processes were known to be involved in the development of varicosities. However, these methods cannot explain the part played by genetic defects in the metabolic processes of varicosities. In addition, little research has been done with newly available investigation methods to discover why varicose disease develops.

The present study was designed to investigate whether a genetic defect exists in the greater saphenous veins of patients with varicose veins compared to normal greater saphenous veins. To investigate genetic defects, we identified differentially expressed genes in primary varicose veins by the differential display reverse transcription–polymerase chain reaction (DDRT-PCR) method, as previously described, with some modifications [8, 9]. This method is a robust and reproducible method for identifying differentially expressed genes. It allows the comparison and verification of expressed mRNAs from different populations of tissues.

METHODS

Patients

The diagnosis of valvular insufficiency was made by confirming the presence of reflux in the greater saphenous vein using duplex sonography [10]. Patients were asked to stand, putting most of their weight on the leg not being examined. The incompetent veins were determined by measuring the amount of reflux following compression of the calf with the free hand of the sonographer. Three patients who had planned coronary surgery showed no reflux in the greater saphenous vein. Four patients who were scheduled for varicosectomy showed reflux in the greater saphenous vein. These four patients were classified as $C_2E_PA_{2,3}P_R$ according to the CEAP classification.

Preparation of Veins

Two groups of human greater saphenous veins were harvested. Three normal veins were obtained from patients undergoing coronary bypass surgery. Four diseased greater saphenous veins were obtained during saphenectomy in patients with primary varicose veins. About 5 cm of the proximal portion of the diseased greater saphenous vein was used for this study.

RNA Isolation

Veins tissue samples were pulverized under liquid nitrogen, and total RNAs were isolated with Trizol reagent (Life Technologies, Inc., Grand Island, NY), as described [11]. The RNAs were further treated with DNase I to remove residual DNA.

DDRT–PCR

Differentially expressed bands were cloned and isolated from cDNAs or genomic libraries. The cDNAs were synthesized by using three 1-base-anchored oligo(dT) primers to subdivide the mRNA population. We performed three reverse transcriptions for each RNA sample, using 0.2 μg of total RNA in 1×reverse transcription buffer, 250 μM dNTP, and 2 μM H-T11A, H-T11C, and H-T11G oligonucleotides (Genhunter, Brookline, MA). After the mixture was heated for 5 min at 65°C and cooled to 37°C, 100 units of reverse transcriptase was added and incubated for 50 min. PCR was performed in a reaction mixture containing 2 μl of reverse transcription reaction mixture, PCR buffer, 25 μM dNTP, 2 μM relevant H-T11M oligonucleotide, eight kinds of arbitrary 13-mer nucleotide (AP-1: 5′-AAGCTTGATTGCC-3′; AP-2: 5′-AAGCTTCGACTGT-3′; AP-3: 5′-AAGCTTTGGTCAG-3′; AP-4: 5′-AAGCTTCTCAACG-3′; AP-5: 5′-AAGCTTAGTAGGC-3′; AP-6: 5′-AAGCTTGCACCAT-3′; AP-7: 5′-AAGCTTAAGCAGG-3′; AP-7: 5′-AAGCTTAACGAGG-3′; AP-8: 5′-AAGCTTTTACCGC-3′), 1 unit of *Taq* polymerase (Qiagen), and 1 μCi of 33P-dATP. Light mineral oil was overlaid and the first denaturation was done at 94°C for 5 min, and 40 rounds of PCR were done at 94°C for 30 s, 37°C for 2 min, and 72°C for 40 s. The amplified cDNA was then separated on a 6% DNA sequencing gel containing 7 M urea. The gel was dried without fixing and autoradiographed on X-ray film.

Recovery and Reamplification of cDNA Probes

Differences in cDNA patterns were identified and the bands corresponding to these cDNAs were excised from the dried gel. Each gel slice was incubated in 100 μl of water at room temperature for 10 min and boiled for 15 min to release the PCR products. The cDNA was recovered by ethanol precipitation in the presence of 0.3 M sodium acetate and 5 μl of 10 mg/ml of glycogen. Four microliters of an eluted cDNA probe was reamplified in a 40-μl reaction volume using the same primer set and PCR conditions as used in the mRNA display. However, the dNTP concentration was adjusted to 250 μM instead of 25 μM and no isotope was added.

Cloning and Sequencing of Differentially Expressed cDNAs

Amplicons exhibiting differential expression following the reverse Northern screen were subsequently subcloned into the TA cloning site of the pCR® 2.1-TOPO vector (Invitrogen) and insert-containing vectors from multiple positive transformants sequenced using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (version 2.0). All NCBI (National Center for Biotechnology Information, Bethesda, MD)-maintained nucleotide databases were searched for homologies using BLAST (basic local alignment search tool; located at http://www.ncbi.nlm.nih.gov/BLAST/index.html).

RESULTS

Differential Display of cDNAs

Eight arbitrary 13-mer primers were screened against three 1-base-anchored oligo(dT) primers. A differential display reaction yielded between 100 and 200 discrete cDNA bands ranging in size from 100 to over 500 nucleotides. We identified that two cDNA bands showed a difference in expression patterns between normal greater saphenous veins and diseased saphenous veins (Fig. 1). Both the cDNAs were highly expressed in all four diseased saphenous veins, but not in the three normal veins. The cDNAs were later designated C6 and G6. The cDNA bands were selected, excised, and reamplified using the corresponding primer sets. The reamplified cDNAs were inserted into a pCR® 2.1-TOPO vector, transformed, and subjected to colony hybridization to eliminate the possibility that