Polymorphisms of Prostate-Specific Antigen Gene Promoter: Determination From Cord Blood Collected on Filter Paper

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Abstract. Recent studies have shown that a single nucleotide polymorphism of A/G substitution in the androgen response element-1 (ARE-1) of the promoter for the prostate-specific antigen gene is a biomarker of prostate cancer. Portuguese men with prostate cancer have a high percentage (43%) of the AA polymorphism of the gene (41% AG, 16% GG), whereas healthy Japanese men have a much lower rate (5%) of the AA polymorphism, (31% AG, 64% GG). The goal of the present study was to see whether or not the Chinese also have a low rate of the AA polymorphism. This study used 94 specimens of cord blood that were the leftover waste of cord blood banking. The samples were collected from Chinese infants onto filter paper, dried, and shipped to Rochester, MN, USA, for PCR amplification and analysis. The observed rate of the AA polymorphism in the samples was very low (5%), with 26% AG, 69% GG. The low incidence of AA polymorphism appears to be a trait of Asians that may reduce their risk of prostate cancer. (received 26 March 2003; accepted 9 June 2003)

Keywords: PSA gene promoter, androgen response element-1, prostate cancer, cord blood, Chinese subjects

Introduction

Prostate cancer, a major public health concern in the USA and around the world, is the most common cancer and second highest cause of death from cancer, after lung cancer, in the male population [1]. In the USA, African Americans have the highest incidence of prostate cancer, followed by Caucasian Americans and Asian Americans. The incidence in African Americans is 2-fold greater than in Caucasian Americans and 4- to 5-fold greater than in Asian Americans [2-5]. The risk of prostate cancer in African Americans is 50 to 60 times greater than in Japanese or Chinese living in Asia. The risk is increased 10-fold in Asians who have migrated to the United States [6]. Prostate cancer may be related to ethnic, genetic, environmental, dietary, or lifestyle factors, or a combination of these factors [4-7].

Current treatments for prostate cancer, such as radiation, prostatectomy, or hormones, can have adverse effects on urinary, bowel, and sexual functions [8,9]. Thus an understanding of the low risk in Asians might help to improve the treatment or even reduce the risk of prostate cancer. Adjusting the diet by reducing fat consumption and increasing fruit and vegetable consumption may reduce the risk of cancer [10]. The incidence of prostate cancer increased from 1.6 per 100,000 per yr in the 1970s to 2.3 in the 1990s in Shanghai, China, possibly as a consequence of westernization. Actually, the rate of prostate cancer in developed countries increased steadily from 1975 to 1990 [6]. Diet-related factors are believed to account for 30% of all cancers in developed countries [11].

In a recent study [12], polymorphism of the androgen response element-1 (ARE-1) of the prostate-specific antigen (PSA) gene promoter showed promise as a biomarker for the risk of prostate cancer. High incidence of homozygosity for the AA allele was related to the risk of prostate
cancer in a European population, whereas in Japanese men, a low risk population, only 5% have polymorphism of the AA allele [13]. These results lead to the question of whether this is a genetic characteristic of Asians.

This study used cord blood, a waste product of cord-blood banking, from 94 Chinese babies. The waste cord blood was applied to a filter paper for subsequent analysis to determine the frequency of the AA polymorphism. This method of sample collection is similar to that used in our previous study of insulin-like growth factor I, in which blood specimens were collected on filter paper in distant locations (ie, Inner Mongolia or Taiwan) and the dried specimens were sent to the United States for assay [14]. We aimed to determine whether the Chinese population has a low frequency of the AA polymorphism, a finding that might indicate that most Asians have a low risk factor for prostate cancer.

Material and Methods

**Specimens.** With the consent of their parents, cord blood specimens were collected for cord-blood banking from Chinese infants born at the Women and Children Hospital of Guangdong Province in China. In total, 94 cord-blood units were collected; 44 units from male infants and 50 units from female infants. After the cord-blood banking process was completed, residual waste blood, including granulocytes and red blood cells, was applied to collection filter papers. The amount applied to each filter paper was about 0.1 ml. The paper was air-dried and kept at room temperature for >2 yr.

**DNA purification.** A disk 3 mm in diameter was punched out of the filter paper. The disk was washed by adding 200 µl of DNA Solution 2 (Gentra System, Inc., Minneapolis, MN), and the disk was
incubated 15 min at room temperature. The washing solution was removed as completely as possible. After the washing procedure was repeated twice, 750 µl of absolute ethanol was added, the disk was incubated for 1 min, and then the ethanol was removed. The ethanol washing was repeated once. The disk with remaining DNA was air-dried at room temperature overnight. It was then ready for PCR amplification.

Genotyping of polymorphisms. The alleles of the single nucleotide polymorphism of the A/G substitution at position -158 in the ARE-1 region of the PSA gene promoter can be detected with the NheI restriction enzyme on the product of PCR amplification. The polymorphism site was amplified with forward primer (5′-TTG TAT GAA GAA TCG GGG ATC GT-3′) and reverse primer (5′-TCC CCC AGG AGC CCT ATA AAA-3′) [15]. In brief, PCR was performed in a 50-µl amplification solution containing 20 pmol of each primer, PCR buffer (1x), 1 mM MgSO4, 300 nM of each deoxynucleotide triphosphate, and 0.5 U of Taq polymerase Pfx (Invitrogen, Carlsbad, CA). It is important that the 3-mm disk be submerged completely in the amplification solution.

The PCR cycling-conditions were 95°C for 5 min, followed by 5 cycles at 94°C for 30 sec, 60°C for 30 sec, and 70°C for 30 sec, followed by 32 cycles at 95°C for 30 sec, 55°C for 20 sec, and 72°C for 40 sec, with a final cycle at 72°C for 9 min. After amplification, 5 µl of amplified product was digested with 5 units of NheI restriction enzyme (New England Biolabs, Beverly, MA) at 37°C overnight, and the digested product was separated on a 2.5% agarose gel containing ethidium bromide. The enzyme NheI recognizes and cuts a unique DNA sequence of G↓CTAGC.

Results

There are 3 possible genotypes for prostate-specific antigen polymorphisms as shown by 3 distinct banding patterns: homozygous alleles both without the restriction enzyme NheI cutting site, AA (300 bp); heterozygous alleles, one with the enzyme site, AG (150, 300 bp); and homozygous alleles both with the restriction enzyme site, GG (150 bp) (Fig. 1).

In Chinese infants, the frequencies of the homozygous and heterozygous genotypes were 6.8% AA, 27.3% AG, and 65.9% GG in males and 4% AA, 20% AG, and 76% GG in females (Table 1). The total frequencies in the Chinese infants were 5.3% AA, 23.4% AG, and 71.2% GG.

Discussion

Previously, we successfully used filter paper to collect plasma for insulin-like growth factor I (IGF-I) testing in healthy middle school students from Inner Mongolia and in patients with acromegaly from Taiwan [14]. We dubbed the use of laboratory resources on one continent to help another continent as “Laboratories Without Borders,” which is somewhat analogous to the international program “Doctors Without Borders.” Collection of samples on filter paper made this program possible. IGF-I testing is advantageous because of the relatively high plasma concentration of the hormone (ie, ng/ml). Many other hormones also have high concentrations and can be tested with the filter paper collection procedure. For hormones with low plasma concentrations (ie, pg/ml), such testing may be impractical and the sensitivity of assays will have to be increased.

The filter paper procedure is suitable for collection of whole blood samples for DNA analysis with PCR amplification. For the present study, only

Table 1. Frequency of homozygous and heterozygous A/G substitutions at the region of the prostate-specific antigen gene promoter in DNA from cord-blood samples of 94 Chinese newborn infants.

<table>
<thead>
<tr>
<th>Promoter genotype</th>
<th>Male (n = 44) number (%)</th>
<th>Female (n = 50) number (%)</th>
<th>Male &amp; female (n = 94) number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG (150 bp)</td>
<td>29 (65.9%)</td>
<td>38 (76%)</td>
<td>67 (71.2%)</td>
</tr>
<tr>
<td>AG (150, 300 bp)</td>
<td>12 (27.3%)</td>
<td>10 (20%)</td>
<td>22 (23.4%)</td>
</tr>
<tr>
<td>AA (300 bp)</td>
<td>3 (6.8%)</td>
<td>2 (4%)</td>
<td>5 (5.3%)</td>
</tr>
</tbody>
</table>
100 µl of cord blood (which otherwise would have been discarded) was needed. It was collected on filter paper, and a 3-mm disk was punched from the filter paper. After unwanted material was washed from the paper disk, the DNA remained and was used for DNA amplification by PCR without elution from the disk. Clear bands of enzyme-digested DNA were detected for polymorphism analysis (Fig. 1).

Many 3-mm disks can be punched out from a single 100-µl blood collection. Collection on filter paper not only facilitates the transport but also prolongs the storage of blood specimens. The samples used in this study had been stored for >2 yr at room temperature. This collection process also reduces the shipping weight of wet or frozen samples and saves costs for dry ice and postage.

Earlier studies have investigated polymorphisms related to androgen receptor function and androgen potency. Examples include the number of CAG trinucleotide repeats on the androgen receptor gene, which translate into polyglutamines in the N-terminal region of the androgen receptor, and the polymorphism of the 5α-reductase gene that encodes the enzyme that converts testosterone to DHT. In extensive investigations with large groups of healthy controls and patients with prostate cancer in Shanghai, China, polymorphisms of the androgen receptor and 5α-reductase were not statistically significant biomarkers for a low risk of prostate cancer in Asian men [16,17]. That the androgen receptor polymorphisms are not useful as biomarkers for prostate cancer was further confirmed in a different ethnic group, the Finns in Finland [18].

A new biomarker for increased risk of prostate cancer was found in southern Europeans [12]. Restriction fragment length polymorphisms of alleles lacking the endonuclease Nhe I site within the ARE-1 region of the PSA gene promoter were shown to be a risk factor for prostate cancer. It is only a single nucleotide of the A or G substitution at position -158 in the prostate-specific antigen gene promoter. An A allele does not have the enzyme-cutting site G↓CTAGC, but the G allele does. Polymorphisms can be detected by gel separation after digestion (Fig. 1). In homozygous subjects without the endonuclease site, the AA pair alleles (a single band of 300 bp) was clearly a risk factor [12].

The percentage of AA homozygous subjects is low in the low-risk Japanese population, but high in the high-risk African-American and Caucasian-American populations. The percentage of homozygous subjects with the endonuclease site of GG substitution is high in the low-risk Japanese population but low in the high-risk African-American and Caucasian-American populations [13]. In a multiethnic cohort study of healthy men, the respective percentages of homozygous subjects AA, heterozygous subjects AG, and homozygous subjects GG, were 5%, 31%, and 64% in Japanese men (n = 99); 28%, 48%, and 24% in African-American men (n = 100), and 25%, 46%, and 29% in Caucasian-American men (n = 113) [13].

In a study of Portuguese men younger than 67 yr, polymorphisms of AA, AG, and GG, respectively, were 25%, 47%, and 27% for healthy controls (n = 83) and 43%, 41%, and 16% for men with prostate cancer (n = 75). Polymorphism AA was higher (P<0.013) in patients with prostate cancer, and it was considered a risk factor for prostate cancer [12].

In this study, 94 cord blood samples (from 44 male infants and 50 female infants whose parents were Chinese) were tested to determine whether Chinese have a low percentage of AA polymorphism. We included female infants in our cohort even though they will not have prostate cancer; however, their genes represent a Chinese cohort and will affect future generations of the male Chinese population. Moreover, PSA expression in women is useful in the prognosis of breast cancer. It may be a risk-protective factor and a hereditary factor for women with breast cancer [19,20]. Our results in the Chinese cohort, male and female, indicated that 5% were AA polymorphisms, 23% AG, and 71% GG; the corresponding results reported in a Japanese population were 5%, 31%, and 64% [13]. Evidently, Asians have a low frequency of the high-risk AA alleles.

The low frequency of AA polymorphism may be protective for Asians, but environmental and dietary factors should not be ignored. In Asians who have migrated to the United States and adopted the Western diet and lifestyle, the incidence rates of prostate and breast cancer have increased to 50% that of Caucasian Americans and 20% that of African Americans [21]. The large increase of
prostate cancer within a short time suggests that a mutation or polymorphism is not the cause; it may reflect the changes of environment, diet, or lifestyle.

What is the characteristic diet or lifestyle of the Chinese population? Soybeans are the staple of the daily diet of Chinese. Many food products, such as bean curd, flavored dried bean curd, fermented bean curd, and soybean milk, are made from soybeans containing high concentrations of genistein, daidzein, and isoflavonoids [22], which inhibit the activity of 5α-reductase (an enzyme that converts testosterone to the potent androgen, DHT). DHT has major functions in the development of the masculine sex, and also stimulates prostate cancer [23]. Chinese and Japanese also drink green tea containing isoflavonoids of epigallocatechin gallate (EGCG) and other catechins. EGCG inhibits 5α-reductase and urokinase, a proteolytic enzyme that promotes cancer growth by invading cells and forming metastases [24]. An average Chinese tea drinker may drink 10 cups of green tea a day, which may contain 1,420 mg of EGCG, the amount needed to be effective for reducing cancer risk [25].

Most Chinese and Japanese drink green or oolong tea, while Europeans consume black tea, which contains very little EGCG after the complete fermentation process. Although there are many other dietary and lifestyle differences between the Chinese and Western cultures, the consumption of soybeans and drinking of green tea are two known diet factors that have been tested in animals and in epidemiological studies. It would be exceedingly difficult to evaluate these factors in a double-blind study of large human populations.

We favor the hypothesis that hereditary factors, in combination with environmental, dietary, and lifestyle factors, influence the rate of prostate cancer in Asian men who reside in the West and help to maintain the low rate of prostate cancer in the East.

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