Fluorescence *in situ* hybridization analysis with subtelomere specific probes (12pter-15qter) showed no differences in deletion patterns between normotensive and essential hypertension

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**ABSTRACT.** Telomere biology is intimately linked to the genetic/environmental etiology of cardiovascular and metabolic diseases and telomere shortening is emerging as an important biomarker disease. The relationship between subtelomeric deletions and genetic hypertension was examined. Fluorescence *in situ* hybridization was used to directly assess whether there is a loss or gain of subtelomere copy number. Five subjects with essential hypertension and five normotensive controls were recruited from the outpatient population of the Cardiology Department of the Afyon Kocatepe University Medical School. Fluorescence *in situ* hybridization was performed using 12p(Tel12) and 15q(Tel15) Cytocell subtelomeric probes.
FISH analysis with subtelomere specific probes

on metaphase slides prepared from peripheral blood samples. No differences in subtelomeric region signals between the hypertensive and normotensive groups were found.

Key words: Telomere; Subtelomere; Fluorescence in situ hybridization; Essential hypertension; Deletion

INTRODUCTION

Essential hypertension is an escalating problem in industrialized and developing countries (Erbaşı et al., 1999; O’Shaughnessy, 2001) and reflects genetic-environmental interactions (Aviv and Aviv, 1999). It has been estimated that as much as 50% of mortality and 70% of morbidity in the elderly are attributable to hypertension (Erbaşı et al., 1999).

The genetic background of primary hypertension is complex and quite difficult to understand because many genes and environmental factors are involved, resulting in a ‘non-Mendelian’ inheritance pattern. However, lessons learned from the monogenic forms of hypertension have highlighted the clinical benefits if susceptibility genes were to be identified for primary hypertension (von Wowern et al., 2003). Candidate genes that represent major essential hypertension risk are on chromosomes 18 (Kristjansson et al., 2002), 2, 9, 12, 15, 19, and the sex chromosomes (Sharma et al., 2000). Telomeres cap and protect the ends of chromosomes. Associations with telomere length have been reported for diabetes (Jeanclos et al., 1998; Nakashima et al., 2004; Adaikalakoteswari et al., 2005; Sampson et al., 2006), insulin resistance (Gardner et al., 2005), obesity (Valdes et al., 2005), atherosclerosis (Okuda et al., 2000; von Zglinicki et al., 2000; Samani et al., 2001; Benetos et al., 2004), vascular dementia (Cawthon et al., 2003), essential hypertension (Aviv and Aviv, 1997, 1999), and mortality due to heart disease. A number of studies have examined the relationships between telomere length and indicators of vascular aging and cardiovascular risks in humans (Aviv, 2002; Edo and Andres, 2005). Their attrition, resulting from progressive rounds of cell division, is linked to replicative senescence of human somatic cells in culture (Blackburn, 2000; Wong and Collins, 2003). In vivo, the telomere length of replicating somatic cells is inversely correlated with age and is associated with age-related disorders, including cardiovascular disease (Aviv, 2004; Serrano and Andres, 2004). Gong et al. (2003) studied a large Chinese hypertensive kindred and discovered a locus on chromosome 12p. This region overlaps with the region containing the gene for autosomal dominant hypertension with type E brachydactyly, a condition observed a few years ago in a Turkish kindred. Gong et al. (2003) genotyped 94 members of a 387-member Chinese kindred with essential hypertension. Additionally, 32 Chinese nuclear families with essential hypertension were also recruited. Genomewide parametric linkage analysis identified a locus for primary hypertension on chromosome 12p12.2-p12.1 (parametric LOD score = 3.44). This locus overlaps the assigned locus that causes severe autosomal-dominant hypertension and brachydactyly (112410-OMIM), the only form of monogenic hypertension known at that time that resembles primary hypertension. The authors identified 2 candidate genes in the region: PDE3A (123805-OMIM), a cyclic nucleotide phosphodiesterase, and the sulfonylurea receptor SUR2 (601439-OMIM), a subunit of an ATP-sensitive potassium channel.

Linkage analysis carried out by Bilginturan et al. (1973), for the two traits (hypertension-brachydactyly vs neurovascular contact) with the chromosome 12p marker showed an LOD
score of 9.2, making the odds that these two traits are linked to this locus at 1,000,000,000 to 1. Disse-Nicodeme et al. (2000) described a new locus on chromosome 12p13. They analyzed a large French family, in which 12 affected members over three generations confirmed the autosomal dominant inheritance. Affected subjects had hypertension together with long-term hyperkalemia, hyperchloremia, normal plasma creatinine, and low renin levels. PHA type II (Gordon’s syndrome) is characterized by familial hypertension with hyperkalemia, slight hyperchloremic metabolic acidosis, and otherwise normal renal function. PHA type II shows promise in leading to the cloning of two additional and as yet not appreciated genes which cause hypertension (Luft, 2003). Nagai et al. (1995) reported a Japanese child with a deletion syndrome on chromosome 12p. These findings, provide strong evidence for the more generalized importance of the chromosome 12p locus in blood pressure regulation and essential hypertension.

Frossard and Lestringant (1995) carried out association studies at a candidate locus, the pancreatic phospholipase A2 gene (PLA2A; 172410) located on chromosome 12. Positive associations were found between the presence of a TaqI dimorphic site located in the first intron of this gene and hypertension in 3 populations sampled: 2 from the USA and 1 from Germany. The results indicated that a quantitative trait locus implicated in determining an individual’s genetic susceptibility to hypertension may be present within up to 30 cM of the PLA2A gene. Phospholipase A2 is a rate-limiting enzyme in eicosanoid production. It is coupled to angiotensin II receptors and acts, upon activation by increased intracellular calcium, by releasing esterified arachidonic acid from membrane phospholipids.

In addition to brachydactyly with hypertension (112410-OMIM), which was shown by Schuster et al. (1996) to map to chromosome 12p in a large Turkish kindred, the disorder mapped within a region defined by markers D12S364 and D12S87.

Using rural Chinese sibling pairs with extreme blood pressure problems, Xu et al. (1999) identified suggestive linkage for regions on chromosomes 15 and 16. By refining the trait definition and genotyping additional markers, they detected significant linkage (maximum LOD score = 3.77) near D15S203 at the telomeric end of 15q in lower extremes of diastolic blood pressure in the sib pairs. Using a second independent data set from the same geographic area, they marginally replicated (P = 0.05) this result, suggesting that this locus is very likely to be involved in the regulation of diastolic blood pressure.

Fluorescence in situ hybridization (FISH) with telomeric probes has been used to assess directly whether there is a loss or gain of telomere copy number (Toka et al., 1998). The link between cytogenetics and Mendelism has received major impetus with the advent of methods such as interphase FISH (Knight et al., 1997). Our objective was to identify differences in subtelomeric deletions between telomeres 12p and 15q in hypertensive versus normotensive subjects by FISH staining.

**MATERIAL AND METHODS**

**Subjects**

The age of the subjects varied from 48 to 80 years. Blood samples were collected from 5 hypertensive and 5 normotensive subjects of the Afyon National Hospital, Cardiology Department. Blood pressure was carefully measured by a standard mercury sphygmomanometer, and the mean of 3 measurements was recorded. Measurements were performed with the
patient in a sitting position after 5 min of complete rest. Systolic blood pressure >140 mmHg and diastolic blood pressure >90 mmHg were considered to be hypertension. Metaphase slides from a peripheral blood sample of 5 hypertensive and 5 normotensive subjects were prepared and FISH was performed using 12p and 15q Cytocell telomere probes.

**Measurement of telomere shortening using subtelomeric FISH methods**

**Sample preparation**

Currently, all subtelomeres can be tested on a single-chromosome metaphase slide with a device developed by Knight et al. (1999). Subtelomere FISH using the Cytocell Subtelomeric Specific Probes (Cytocell, Ltd., Oxfordshire, UK) was performed in 10 patients referred for chromosome testing for a subtelomeric deletion for hypertension (Table 1). Metaphase cells were prepared from short-term blood cultures using standard cytogenetic procedures. The Cytocell Aquarius kit designed for use on cultured peripheral blood cells fixed in Carnoy’s fixative was used and metaphase slides were prepared according to our laboratory guidelines. Metaphases were arrested with 0.01 μg/mL colcemid for 18 h. Chromosome preparations were made by exposing the cells to 0.075 M KCl for 30 min and fixing in fresh fixative (methanol-glacial acetic acid at 3:1). The karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN, 1995).

<table>
<thead>
<tr>
<th>Specific probe</th>
<th>Clone name</th>
<th>STS marker</th>
<th>Accession number (if available)</th>
<th>Max. physical distance from telomere (kb)</th>
<th>Clone (Ref.)</th>
<th>Aquarius cat. No.*</th>
<th>Choices cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>12p</td>
<td>496A11</td>
<td>12pte27</td>
<td>-</td>
<td>Unknown</td>
<td>9</td>
<td>LPT12PR/G</td>
<td>PCT112</td>
</tr>
<tr>
<td>15q</td>
<td>154P1</td>
<td>WI-5214</td>
<td>D15S936</td>
<td>300</td>
<td>14</td>
<td>LPT15QR/G</td>
<td>PCT115</td>
</tr>
</tbody>
</table>

*R specifies a red label, G specifies a green label.

**FISH protocol**

Following the harvest, metaphase cells were fixed in 3:1 methanol:acetic acid and dropped onto glass slides. The slides were allowed to air dry for several hours prior to use. The FISH procedure was carried out in accordance with a previously published procedure (Iqbal et al., 1999). In summary, we spotted the cell sample onto a cleaned microscope slide, which was then immersed in 2X SSC, pH 7.0, for 2 min, and thereafter dehydrated in ethanol series (70, 85, and 100%) for 2 min at each stage. FISH was performed with Spectrum Green-labeled subtelomeric (Tel12) DNA probes for chromosome 12p and Spectrum Green-labeled subtelomeric (Tel12) DNA probes for chromosome 15q. Each probe was then removed from a -20°C freezer and allowed to warm at room temperature. We ensured that the probe solution was homogeneous by repeated pipette mixing. Thereafter, 10 μL of the probe was removed and placed onto the slide of the cell sample. Slides were covered with a 24 x 24-mm glass coverslip, and were sealed with rubber cement. Slides were then placed on a 75°C (+ 1°C) hotplate and the samples were allowed to denature for 2 min. For hybridization, slides were
left overnight in a humid, lightproof container at 37°C (± 1°C). On the next day, we carefully removed the coverslip and all traces of glue and washed the slide in 0.4X SSC, pH 7.0, at 72°C (± 1°C) for 2 min. Slides were drained and washed again in 2X SSC, 0.05% Tween-20, pH 7.0, at room temperature for 30 s. After draining, 10 μL DAPI antifade was applied, the slides were covered with a coverslip, and allowed to color in the dark for 10 min.

**Statistical analysis**

The slides were examined using a Zeiss Axioplan 2 fluorescence microscope (Göttingen, Germany) with the following filters: SP-100 DAPI, FITC MF-101 for Spectrum Green (12p) and FITC MF-101 for Spectrum Green (15q). A minimum of 10-20 metaphases per patient were scored for the presence or absence of the critical region and control signals on both homologues for each of the probes (Tel12 and Tel15). For optimal visualization of the probes, a 100-W mercury lamp and plan apochromatic objectives of 63 or 100X were used. The images were acquired with a CCD camera and analyzed by the MetaSystem Isis software (Belmont, MA).

**RESULTS**

Details of age, gender, disease, use of medication, and blood pressure range values of hypertensive and normotensive patients analyzed in the present study are reported in Table 2. This study examined the relations between leukocyte subtelomeric length and hypertensive and normotensive blood pressures. The mean age of the hypertensive group was 59.8 years and that of the normotensive group was 58.6 years. Hypertensive and normotensive individuals had a history of other metabolic diseases, for example, ulcer, dyspnea and diabetes. No differences in age, gender, education, or other metabolic diseases were found between individuals selected for the hypertensive and normotensive groups.

**Table 2. Data for hypertensive and normotensive subjects.**

<table>
<thead>
<tr>
<th>Category</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Diseases</th>
<th>Use of medications</th>
<th>Blood pressure range (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertensive</td>
<td>Male</td>
<td>48</td>
<td>Cardiovascular disease</td>
<td>-</td>
<td>140/100</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>49</td>
<td>Cardiovascular disease</td>
<td>+</td>
<td>180/90</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>57</td>
<td>Ulcer</td>
<td>+</td>
<td>190/130</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>65</td>
<td>Diabetes</td>
<td>+</td>
<td>160/90</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>80</td>
<td>Diabetes</td>
<td>+</td>
<td>170/130</td>
</tr>
<tr>
<td>Normotensive</td>
<td>Female</td>
<td>48</td>
<td>Cardiovascular disease</td>
<td>+</td>
<td>120/70</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>48</td>
<td>Dyspnea</td>
<td>+</td>
<td>120/80</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>56</td>
<td>Diabetes</td>
<td>+</td>
<td>120/80</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>63</td>
<td>Dyspnea</td>
<td>+</td>
<td>120/70</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>78</td>
<td>Cardiovascular disease</td>
<td>+</td>
<td>120/80</td>
</tr>
</tbody>
</table>

A limitation of this study was the small sample size; 5 normotensive and 5 hypertensive patients. FISH applying the specific subtelomeric probe for each case was carried out in all parents, giving normal results for both the normotensive and hyperten-
sive groups. Another limitation of this study was the lack of sufficient financial support, which restricted our ability to apply FISH methods to just 10 subjects. We wanted to use multiprobe FISH assay. First, polymorphic markers near the end of chromosomes were used to identify subtelomeric abnormalities, but since their development, multiprobe FISH has become the most widely used method for identifying these alterations. It allows the simultaneous analysis of all subtelomeric regions in a single hybridization experiment. The ages of the subjects varied between 48 and 80 years. It was not possible to evaluate a younger population because of the lack of funding. It is known that subtelomeric deletion is observed in both hypertension and aging. It is possible that the results of this study would be different if carried out on a group under 40 years.

Some of the submicroscopic subtelomeric deletions result in a specific phenotype, which may direct the clinician towards the diagnosis. In these patients, FISH analysis of a single and specific subtelomere would be sufficient to confirm the diagnosis. However, the majority of cases with subtelomeric defects lack a characteristic phenotype, so far. For these cases, a general subtelomeric screen is required to achieve a diagnosis. For this group, effective clinical preselection was essential because of the technical complexities and cost of screening for telomeric deletions.

Our results show that subtelomeric deletion does not differ between hypertensive and normotensive individuals in the 12p and 15q subtelomeric regions. We also found no differences in the results of FISH analysis according to the FISH signals observed between hypertensive and normotensive subjects (Figure 1A-D).
The increasing number of very small chromosomal aberrations that will be found in the near future will confront the clinicians with various problems. Some subtelomeric deletions may just not occur because of stability of the specific subtelomeric chromosome region. The future will tell us which of the above explanations/mechanisms are involved in these rare subtelomeric rearrangements. Such a collection will not only help patients and their families and clinicians but will also give more insight into molecular mechanisms involved in the etiology of hypertension or similar metabolic diseases.

Until now, no prior studies were found that have been conducted to examine the deletions of subtelomeres in individuals with essential hypertension using FISH methods. Thus, the current study represents the first analysis that compares the results of FISH applications of 12p and 15q subtelomeric probes in hypertensive and normotensive individuals.

This study investigated the association between deletions of subtelomeres and essential hypertension using FISH methods. Usually, telomere length is measured using conventional Southern blot analysis or Q-FISH (Baerlocher et al., 2002). However, in this study we wanted to use a different way, and therefore, the subtelomeric region of telomeres 12p and 15q was measured by using FISH. A subtelomeric probe is useful in detecting chromosomal rearrangements, which comprises a single-copy DNA sequence with a length of less than 25
kb, being capable of hybridizing to the terminal G-band or R-band of an arm of a single chromosome. The present investigation was concerned with chromosomal ends and subtelomeres and the detection of chromosomal rearrangements occurring in the subtelomeric regions of chromosomes. More particularly, this study examined probes that could be used to identify such chromosomal rearrangements in medical and cancer genetic diagnoses. Moreover, the present study focused on single copy probes effective for hybridization of a single location in the genome, where it would indicate whether the chromosome had undergone any rearrangement at the telomere or subtelomere region. Specifically, the present study was concerned with single copy probes that are useful for detecting a broader spectrum of abnormal chromosomal termini than currently detectable with existing cloned probes, providing insight into how the telomeric and subtelomeric regions of chromosomes are organized, correlating how the sequences of these chromosomal regions are related to each other and to other chromosomal regions, correlating rearrangements with specific clinical effects, and characterizing breakpoints in rare chromosomal rearrangements that are genetically balanced and unbalanced. Finally, the subtelomeric FISH methods may be informative for essential hypertension candidate genes.

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REFERENCES


FISH analysis with subtelomere specific probes


