Identification of Genomic Aberrations by Array Comparative Genomic Hybridization in Patients with Aortic Dissections

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Background: The aim of the present study was to identify chromosomal loci that contribute to the pathogenesis of aortic dissection (AD) in a Korean population using array comparative genomic hybridization (CGH) and to confirm the results using real-time polymerase chain reaction (PCR). Materials and Methods: Eighteen patients with ADs were enrolled in this study. Genomic DNA was extracted from individual blood samples, and array CGH analyses were performed. Four corresponding genes with obvious genomic changes were analyzed using real-time PCR in order to assess the level of genomic imbalance identified by array CGH. Results: Genomic gains were most frequently detected at 8q24.3 (56%), followed by regions 7q35, 11q12.2, and 15q25.2 (50%). Genomic losses were most frequently observed at 4q35.2 (56%). Real-time PCR confirmed the results of the array CGH studies of the COL6A2, DGCR14, PCSK6, and SDHA genes. Conclusion: This is the first study to identify candidate regions by array CGH in patients with ADs. The identification of genes that may predispose an individual to AD may lead to a better understanding of the mechanism of AD formation. Further multicenter studies comparing cohorts of patients of different ethnicities are warranted.

Key words: 1. Aorta 2. Aortic dissection 3. Genes 4. Polymerase chain reaction

INTRODUCTION

Aortic dissection (AD) is an uncommon but highly lethal disease that leads to the gradual deterioration of the mechanical properties of the aortic wall. It is caused by weak aortic structure, incompetent repair mechanisms and powerful destructive forces. Although genetic factors may play an important role in the pathogenesis of AD [1-3], studies investigating the genetic determinants of AD have been superficial, and reports of genetic risk factors for AD in Koreans are extremely rare.

Several chromosomal loci with evidence of linkage were found in studies of thoracic aortic diseases [4-6], but the mode of genetic transmission for AD remains uncertain. The identification of genetic markers associated with an increased risk of AD formation and growth will improve the treatment outcomes of patients with AD.

Array Comparative Genomic Hybridization (CGH) is a
powerful technique, allowing the simultaneous quantitative analysis of entire genomes, including a higher resolution, more dynamic range, direct mapping of aberrations to genomic sequences, and higher throughput [7,8]. Therefore, array CGH has been used to detect chromosomal aberrations in mental retardation, epilepsy, and schizophrenia [9-12]. Real-time polymerase chain reaction (PCR) has been used to empirically confirm the results of array CGH [13,14]. Moreover, quantitative real-time PCR has great advantages in measuring DNA copy-number changes owing to the economy, simplicity, and flexibility of the technique [15].

The aim of the present study was to identify possible chromosomal loci associated with the pathogenesis of AD in Koreans using array CGH and to confirm the results with real-time PCR.

### MATERIALS AND METHODS

**1) Patient population**

A total of 18 patients with ADs were recruited from the single hospital. The mean age of the patients was 54.17 years, ranging from 41 to 77 years. The demographic data and range of aortic pathologic disease are summarized in Table 1. Genomic DNA was extracted from individual blood samples using the Puregene DNA isolation kit (Qiagen, Hilden, Germany). Reference DNA was pooled from ten gender-matched (male), normal, healthy control subjects. Approval was obtained from the institutional review board of the College of Medicine of our university, and informed consent was provided according to the Declaration of Helsinki.

**2) Array-CGH analysis**

Array-CGH analyses were conducted on 18 individual samples. Array CGH was performed as described previously [16] using a commercial MACArray™-Karyo 4K BAC-chip (Macrogen, Seoul, Korea) with 4,030 bacterial artificial chromosome (BAC) clones duplicating the whole human genome with a resolution of 1 Mbp. DNA was labeled using the BioPrime labeling kit (Invitrogen, Carlsbad, CA). Each DNA (target and reference DNA) sample (500~700 ng) with random primers was boiled at 98~100°C for 5 min for denaturation and then cooled on ice for 5 min. The denatured DNA was differentially labeled with 3 μL of 1 mM Cy3-dCTP (references DNA), Cy5-dCTP (Target DNA), respectively (PerkinElmer, Boston, MA), and 1 μL Klenow fragments were added to the mixture (Invitrogen, Carlsbad, CA). DNA was incubated at 37°C overnight for labeling. Subsequently, unincorporated nucleotides were removed using MicroSpin G-50 columns (Amersham Biosciences, Buckinghamshire, England). Cy3 and Cy5 labeled DNA (Target DNA and reference DNA, respectively) was mixed with 50 μg of human Cot-1 DNA for blocking of repeat sequences. After purification, the mixture was resolved in hybridization buffer containing yeast tRNA for blocking binding of non-specific nucleotides. After the chips were in hybridization buffer with salmon sperm DNA for 1 h, the chips were hybridized with the purification mixture. Then they were incubated for 72 h in the 37°C hybridization chamber (BioMicro Systems, Salt Lake City, UT). After hybridization was complete, the array chips were washed and dried and then the arrays were scanned with a GenePix® (Axon Instruments, Foster City, CA).

### Table 1. Demographic characteristics of the 18 subjects

| Characteristic                      | n   |
|-------------------------------------|-----|
| No. of patients                     | 18  |
| Sex, female/male                    | 8/10|
| Age, yr                             | 54.17±11.76 |
| Operative therapy                   | 14  |
| Conservative therapy                | 4   |
| DeBakey classification              |     |
| Type I                              | 5   |
| Type II                             | 10  |
| Type III                            | 3   |
| Combined diseases                   |     |
| Coronary artery disease             | 5   |
| Atherosclerosis                     | 4   |
| Bicuspid aortic valve               | 2   |
| Hypertension                        | 15  |
| Hyperlipidemia                      | 2   |
| Diabetes mellitus                   | 2   |
| COPD                                | 0   |
| Current smoking                     | 5   |
| Peripheral vascular disease         | 0   |
| Stroke                              | 0   |
| Familial history of aortic disease  | 0   |

COPD=Chronic obstructive pulmonary disease.
Table 2. Recurrent Gains/Losses in aortic dissecting aneurysms (>30 frequencies) (UCSC genome browser: May 2004)

| Chromosomal region | Frequency (%) | Bac_start (bp) | Bac_end (bp) | Size (bp) | Contained genes |
|--------------------|--------------|----------------|--------------|-----------|----------------|
| **Gains**          |              |                |              |           |                |
| 1p1.12             | 6 (33%)      | 120390109      | 120485103    | 94994     | NOTCH2         |
| 1q23.1             | 6 (33%)      | 155045002      | 155148010    | 103008    | SH2D2A, NTRK1, INSRR |
| 2p13.1             | 8 (44%)      | 74539520       | 74620052     | 80532     | WBP1, MRPL53, LBX2, PCGF1, TLX2, DQX1, AUP1, HTRA2, LOXL3 |
| 2q31.1             | 6 (33%)      | 177310814      | 177395162    | 84348     | FUC1P          |
| 2q37.3             | 7 (39%)      | 240550837      | 240713841    | 163004    | NDUFA10, OR6B2, OR6B3, OR5S1P |
| 3q21.1             | 6 (33%)      | 131099654      | 131218467    | 118813    | TRH            |
| 4q13.3             | 8 (44%)      | 74563267       | 74654547     | 118280    | AFP, AFM       |
| 4q24               | 6 (33%)      | 105045356      | 105165560    | 120204    |                |
| 5p15.33            | 6 (33%)      | 203859         | 298137       | 94278     | SDHA*          |
| 5p15.33            | 6 (33%)      | 557250         | 688780       | 131530    | SLC9A3, CEP72  |
| 6p12.2             | 6 (33%)      | 51985699       | 52087584     | 101885    | PKHD1          |
| 7p14.3             | 7 (39%)      | 33392813       | 33486178     | 93365     |                |
| 7q11.23            | 7 (39%)      | 74705985       | 74764828     | 58843     |                |
| 7q11.23            | 8 (44%)      | 72089609       | 74747885     | 2658276   |                |
| 7q35               | 9 (50%)      | 143557352      | 143585922    | 28570     |                |
| 8p21.2             | 6 (33%)      | 23582247       | 23671417     | 89170     | NKX3-1         |
| 8p22               | 6 (33%)      | 18268730       | 18357165     | 88435     | AACP, NAT2     |
| 8p23.3             | 6 (33%)      | 649638         | 867290       | 217652    | ERICH1, C8orf68 |
| 8q13.2             | 6 (33%)      | 68343074       | 6844177      | 101103    | ARFGEF1        |
| 8q24.3             | 10 (56%)     | 145298570      | 145384455    | 85885     |                |
| 10p15.3            | 6 (33%)      | 795549         | 881206       | 85657     | LARP5          |
| 10q11.23           | 7 (39%)      | 52453706       | 52563182     | 109476    | PRKG1          |
| 10q22.3            | 6 (33%)      | 79941269       | 80019660     | 78391     |                |
| 10q23.1            | 6 (33%)      | 86354131       | 86459920     | 105789    |                |
| 10q26.3            | 6 (33%)      | 134654530      | 134754530    | 100000    | GPR123         |
| 11q12.2            | 9 (50%)      | 60669725       | 60731709     | 61984     | VPS37C         |

3) Data analysis

The scanned images were analyzed to determine Cy3 : Cy5 ratios for each array element using MAC Viewer™ v1.6.3 software (Macrogen, Seoul, Korea). Data are presented as log2 (Cy3 intensity/Cy5 intensity ratios) plotted against the position of clones within the particular chromosome in the current version of the genome. Ratios were normalized by using the median of fluorescence ratios computed as log2 values from the housekeeping DNA control fragments linearly distributed across the genome. Measurements flagged as unreliable by MAC Viewer™ v1.6.3 were excluded from subsequent analyses. The threshold corresponds to 2 standard deviation (SD) values from the mean. The information on each individual clone was obtained from the UCSC Genome Bioinformatics database (May 2004 freeze, http://genome.ucsc.edu). Then log2 ratios of chromosomal aberration regions were identified via t test analysis using the Sigma Plot program. All chromosome regions indicated as aberrant in Table 2 were calculated via t test analysis.

4) Quantitative real-time PCR

To validate genomic imbalances identified by array CGH in this study, four genes with obvious genomic changes were
Table 2. Continued

| Chromosomal region | Frequency (%) | Bac_start (bp) | Bac_end (bp) | Size (bp) | Contained genes |
|--------------------|---------------|----------------|--------------|-----------|-----------------|
| 12p13.33           | 7 (39%)       | 183679         | 257363       | 73684     | SLC6A12, SLC6A13|
| 12q13.13           | 6 (33%)       | 52345803       | 52436777     | 90974     | ATP5G2, CALCOCO1|
| 13q33.3            | 6 (33%)       | 107569780      | 107652035    | 82255     |
| 14q2.4             | 6 (33%)       | 71175267       | 71266409     | 91142     | SIPA1L1         |
| 14q32.33           | 7 (39%)       | 105541523      | 105648959    | 107436    |
| 15q2.5             | 9 (50%)       | 80539371       | 80776796     | 237425    | RPS17           |
| 15q26.3            | 6 (33%)       | 99653473       | 99758817     | 105344    | PCSK6*          |
| 17q12              | 6 (33%)       | 31081633       | 31171045     | 89412     | RASL10B, GASL2, C17orf50, MMP28, TAF15 |
| 17q21.31           | 7 (39%)       | 39133290       | 39273024     | 139734    | SOST, DSP3, MPP3|
| 17q25.3            | 6 (33%)       | 77755881       | 77849251     | 93370     | SLC16A3, CSNK1D |
| 19p13.11           | 8 (44%)       | 17691080       | 17784835     | 93755     | BPY2IP1, FCHO1, B3GNT3 |
| 19q13.43           | 6 (33%)       | 63514606       | 63629648     | 115402    | HKR2, A1BG, ZNF497, RPS5, ZNF584 |
| 21q22.11           | 6 (33%)       | 31352497       | 31450699     | 98202     | UBE3AP2, TIAM1  |
| 21q22.3            | 7 (39%)       | 46373142       | 46463667     | 90525     | COL6A2*, FTCD, C21orf56, LSS |

Losses

| Chromosomal region | Frequency (%) | Bac_start (bp) | Bac_end (bp) | Size (bp) | Contained genes |
|--------------------|---------------|----------------|--------------|-----------|-----------------|
| 4q35.2             | 10 (56%)      | 19096381       | 19100683     | 42865     | MUC12, MUC17    |
| 7q22.1             | 7 (39%)       | 100407386      | 100480418    | 73032     | GPR123, KDNC1, UTF1, VENTX |
| 10q26.3            | 7 (39%)       | 134755203      | 134904547    | 149344    | VSIG6          |
| 14q32.33           | 8 (44%)       | 105821330      | 105970946    | 86134     | NOMO3, PKD1P1, PKD1P2 |
| 15q12.3            | 8 (44%)       | 19958100       | 20053879     | 95779     | NOM01, PKD1P3, NPIP |
| 16p12.3            | 6 (33%)       | 16274784       | 16391850     | 117066    | GGT2, DGCR6, PRODH, DGCR5, DGCR2, TSSK7P, DGCR13, TSSK2, DGCR14*, GSC, SLC25A1, CLTCL1, HIRA, MRPL40, UF1L, CDC45L, CLDN5, SEPT5, GP1BB, TBX1, GNB1L, TNXR2, COMT, ARVCF, C22orf25, DGCR8, RANBP1, ZDHHC8, RTN4R, DGCR6L |
| 22q11.21           | 7 (39%)       | 17158480       | 18962782     | 1804302   | SDHA, forward, 5'-CTGAGTGATGCCCTGCAGTA-3'; reverse, 5'-TCCACACCGACAGAGGAAACT-3'. All real-time PCR procedures were performed three times and the normal control was different from each experiment (all three normal samples). The relative genomic copy number was calculated using the comparative Ct method [17]. Ct for each gene was determined using thermocycler software, and the average of three independent experiments was calculated. The fold change from normal samples was set at 1-fold and the ratio of the normalized fold change in TAD compared to that of control samples was calculated. |

* = Verified by real-time PCR.
RESULTS

1) Pattern of aberration in individual chromosomal region

Chromosomal copy-number changes were detected by array CGH in blood samples obtained from AD patients. The identified regions of genomic aberrations, ranging from 6 in 18 samples (33% frequency of chromosomal gain and loss) to 10 in 18 samples (56% frequency of chromosomal gain at 8q and loss at 4q) (Table 2). Overall, the individual chromosomal aberration pattern was not random and tended to be consistent, showing DNA gains at 1p, 1q, 2p, 2q, 3q, 4q, 5p, 6p, 7p, 7q, 8q, 10p, 10q, 11q, 12p, 12q, 13q, 14q, 15q, 17q, 19p, 19q and 21q, and DNA losses at 4q, 7q, 10q, 14q, 15q, 16p and 22q. Chromosomal copy number gains were observed more frequently than copy number losses. The most frequently detected gains were at 8q24.3 (56%). DNA gains were also frequently observed at 7q35, 11q12.2 and 15q25.2 in 9 samples (50%), and in 2p13.1, 4q13.3, 7q11.23 and 19p13.11 in 8 samples (44%). Regions with a 39% frequency of gains were 2q37.3, 7p14.3, 7q11.23, 10q11.23, 12p13.33, 14q32.33, 17q21.31 and 21q22.3. Other regions with 33% frequency of DNA gains included 1p11.2, 1q23.1, 2q31.1, 3q21.3, 4q24, 5p15.33, 6p12.2, 8p21.2, 8p22, 8q23.3, 8q13.2, 10p15.3, 10q22.3, 10q23.1, 10q26.3, 12q13.13, 13q33.3, 14q24.2, 15q26.3, 17q12, 17q25.3, 19q13.43 and 21q22.11. Genomic losses were most frequently observed in 4q35.2 (56%). Regions with 44% frequency of losses were 1q41.13, 21q22.3, 22q11.21 (Table 2).

To attempt to correlate a diagnosis with a specific region, we applied statistics for each potential loci. All aberrant chromosomal locations (>30%) detected in array CGH (Table 2) were selected in t test analysis. Although all 48 regions were calculated, only t test analysis of five regions is shown in Table 3. Because a high association was detected in all regions, samples which indicated higher p-value (>50% frequency) are shown in the Tables.

2) Real-time PCR

Four genes with obvious genomic changes were analyzed using real-time PCR in order to assess the level of genomic imbalance identified by array CGH in this study. Of the more than 30% frequency clones, those considered to have vascular related aberrations in the chromosome were selected for real-time PCR. We evaluated the change in fold difference in samples from normal and AD patients. Fig. 1 shows the regions of chromosomal aberration in the four genes. Gains were seen in 5p15.33, 15q26.3, and 21q22.3, which contain the SDHA, PCSK6, and COL6A2 genes (A, B, and C), while a loss was observed at 22q11.21, which contains the DGCR14 genes (D). The fold difference obtained from the real-time PCR was in accordance with the array CGH results and the p-values of all samples was <0.05. The gain regions (A ∼ C; COL6A2, PCSK6, and SDHA) in the results from the real-time PCR were equal to all of the gain regions in the array CGH. The relative fold decreases in DGCR14 (D) were reflected similarly.

DISCUSSION

Aortic dissection is a life-threatening disease that develops without warning. Despite significant advances in diagnostic and therapeutic techniques, the overall in-hospital mortality was 27.4% according to the international registry of aortic dissection [19]. It occurs in 2 ∼ 9% of the population over 65 years old, and the prevalence may be increasing [20]. Modern diagnostic methods, such as computed or magnetic resonance tomography, are able to show an aortic wall hematoma at the acute onset of the disease. This hematoma will develop into an aortic wall dissection over time [6]. Early recognition and management are crucial, and it is therefore important to be able to rapidly identify aortic dissection, initiate supportive therapy, and refer patients to appropriate specialty care [2].

Table 3. Examples of t test analysis in specific alternative regions

| Chromosome region | Log2 ratio means±SD | T value | p-value |
|--------------------|---------------------|---------|---------|
| 7q35               | 0.43±0.12           | −11.06  | 6.6E-09 |
| 8q24.3             | 0.35±0.13           | −8.40   | 1.21E-07|
| 11q12.2            | 0.38±0.13           | −8.78   | 1.64E-07|
| 15q25.2            | 0.28±0.06           | −13.54  | 4E-10   |
| 4q35.2             | −0.63±0.34          | 5.88    | 1.45E-05|
Fig. 1. Relative fold differences selected from 4 genes in which the most frequent gains and losses detected were in the 5p, 15q, 21q, and 22q regions. Each sample is depicted (x axis), and the fold difference of the N-value was delineated in real-time PCR (y axis). A threshold level of 2 indicates significant DNA gain (A∼C), and 0.5 indicates significant DNA loss (D). At the chromosomal 21q22.3 location, COL6A2 (A), the fold change of the sample was 2.25- to 3.14-fold (seven samples) versus 1-fold for the reference sample. The fold difference for the SDHA (B) was 2.4- to 4.57-fold (six samples). For the PCSK6 (C), in the chromosomal 15q26.3 region, the fold change of the sample was 2.11- to 4.76-fold (six samples) with a gain at the location. Finally, DGCR14 (D) had a 0.08~0.4-fold change (seven samples) with a loss at the location (p<0.05).

Current research is aimed at determining the phenotypic consequences of specific copy number abnormalities in patients with ADs [21]. Cytogenetic analyses have revealed that numerous somatic genetic changes in the pathogenesis of human diseases are involved in region-specific gains or losses of DNA copy number; however, such changes have not yet been reported in most ADs. Including classical cytogenetics, fluorescence in situ hybridization (FISH), Southern blot analysis, quantitative PCR based assays, and CGH have been used to detect copy number changes in human diseases, but these techniques have several limitations [22]. Therefore, we attempted to use array CGH. Unlike chromosomal CGH, array CGH highly improves resolution and provides quantitative information of the level of chromosomal gain or loss [16,23]. BAC array CGH has been used in several diseases but has not been applied to ADs.

In this report, the DNA copy number alterations frequently identified in 18 patients with AD were analyzed using array based CGH. Many regions throughout the entire genome were altered. The DNA copy number alterations observed in the
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ADs were not random; they involved particular regions of the genome and most usually involved parts of or the entire DNA. DNA gains were found in regions 1p, 1q, 2p, 2q, 3q, 4q, 5p, 6p, 7q, 8p, 8q, 10p, 10q, 11q, 12p, 12q, 13q, 14q, 15q, 17q, 19p, and 21q, and losses were found in 4q, 7q, 10q, 14q, 15q, 16p, and 22q (Table 2). DNA gains were more frequently observed than DNA losses.

Real-time PCR can be used to validate and quantify the genomic changes identified by BAC array CGH [17]. Of the more than 30% frequency clones in array CGH results, those considered to have vascular related aberrations in the chromosome were selected for real-time PCR. Frequently, gains of chromosomal DNA at COL6A2 were detected by CGH in patients with ADs. The collagen, type VI, alpha-2 (COL6A2) gene encodes 1 of the 3 subunits that comprise the full collagen VI protein. Collagen VI is expressed in developing human hearts [24]. The finding that collagen VI is a structural protein supports that collagen VI is a candidate gene involved in the development of congenital heart disease in Down syndrome. The most frequent locus alteration was a gain at the 15q26.3 region containing the PCSK6 gene. PCSK6 (PACE4) is expressed at high levels in the anterior pituitary, central nervous system, the developing olfactory bulb, heart, and liver [25]. Moreover, SDHA is Complex II of the mitochondrial respiratory chain, also known as succinate dehydrogenase or succinate ubiquinone oxidoreductase, and it consists of four nuclear-encoded polypeptides. The specific function of this gene has not yet been determined. Bourgeron et al. identified a homozygous mutation in the SDHA gene in two siblings with a mitochondrial complex II deficiency presenting as Leigh’s syndrome. Their study results demonstrated that the flavoprotein subunit gene is duplicated in the human genome and located on chromosome 5p15 [26]. Chromosome arm 22q, which includes the COMT and DGCR14 genes, was frequently under-represented. Chromosome 22q11.21 includes the catechol O-methyltransferase (COMT) gene, and its deletion syndrome is a common microdeletion syndrome associated with a markedly elevated risk of several diseases [27]. In addition to this region, DiGeorge syndrome critical region gene 14 (DGCR14) has been associated with an elevated risk of several diseases. A group of developmental disorders, including DiGeorge syndrome (DGS), velocardiofacial syndrome (VCFS), conotruncal anomaly face syndrome, and some familial or sporadic conotruncal cardiac defects have been associated with microdeletion of 22q11.2 [28]. The four altered regions (5p15.33, 15q26.3, 21q22.3 and 22q11.21) that showed frequent genomic alteration were confirmed by real-time PCR.

In summary, array based CGH analysis of 18 patients with AD showed that DNA copy number alterations are common in AD. Real-time PCR confirmed that array CGH properly detected gains in AD related genes. Aortic dissection is believed to develop through a chronic degeneration of the aortic wall associated with aging, atherosclerosis, or hypertension. But its etiology remains unresolved [20]. In this study, we were able to delineate discrete regions of DNA copy number alterations in several regions of the chromosomes that are likely to harbor some relevant genes.

CONCLUSION

These data support the utility of array CGH for the identification of genomic alterations in AD. Although our results suggest a new aspect of genomic alteration in patients with ADs, our study was limited by a small sample number (n=18) and the fact that it only included individuals of Korean descent. Therefore, further studies should be carried out using CGH techniques to validate our results. Information on chromosomal variability can be generated by comparing the results of array CGH with information from the Human Structural Variation Database and increasing patient numbers.

This study is the first to use array CGH to search for candidate regions in Korean aortic dissection patients, these findings might contribute to the understanding of important chromosomal regions and the identification of candidate genes, and may be used to investigate the pathogenesis of aortic dissections.

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