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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

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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.

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.

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^{TM}-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, repectively) 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 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					
1p11.2	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	FUCA1P
2q37.3	7 (39%)	240550837	240713841	163004	NDUFA10, OR6B2, OR6B3, OR5S1P
3q21.3	6 (33%)	131099654	131218467	118813	TRH
4q13.3	8 (44%)	74536267	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	FKBP6, NSUN5, TRIM50, FZD9, BAZ1B, BCL7B, TBL2, MLXIPL, VPS37D, WBSCR18, STX1A, ABHD11, CLDN3, CLDN4, WBSCR27, ELN, LIMK1, WBSCR1, LAT2, RFC2, CYLN2, GTF2IRD1, WBSCR23, GTF2I, PMS2L5, WBSCR16, NCF1, GTF2IP1
7q35	9 (50%)	143557352	143585922	28570	OR2A42, OR2A9P
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	68444177	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 $\log_2$ (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 $\log_2$ 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	
14q24.2	6 (33%)	71175267	71266409	91142	SIPA1L1
14q32.33	7 (39%)	105541523	105648959	107436	
15q25.2	9 (50%)	80539371	80776796	237425	RPS17
15q26.3	6 (33%)	99653473	99758817	105344	PCSK6*
17q12	6 (33%)	31081633	31171045	89412	RASL10B, GAS2L2, C17orf50, MMP28, TAF15
17q21.31	7 (39%)	39133290	39273024	139734	SOST, DUSP3, 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	115042	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					
4q35.2	10 (56%)	190963818	191006683	42865	
7q22.1	7 (39%)	100407386	100480418	73032	MUC12, MUC17
10q26.3	7 (39%)	134755203	134904547	149344	GPR123, KNDC1, UTF1, VENTX
14q32.33	8 (44%)	105821330	105907464	86134	
15q11.2	8 (44%)	19958100	20053879	95779	VSIG6
16p12.3	6 (33%)	16274784	16391850	117066	NOMO3, PKD1P1, PKD1P2
16p13.11	7 (39%)	14873336	14958740	85404	NOMO1, PKD1P3, NPIP
22q11.21	7 (39%)	17158480	18962782	1804302	GGT2, DGCR6, PRODH, DGCR5, DGCR2, TSSK7P, DGCR13, TSSK2, DGCR14*, GSCL, SLC25A1, CLTCL1, HIRA, MRPL40, UFD1L, CDC45L, CLDN5, SEPT5, GP1BB, TBX1, GNB1L, TXNRD2, COMT, ARVCF, C22orf25, DGCR8, RANBP1, ZDHHC8, RTN4R, DGCR6L

*=Verified by real-time PCR.

analyzed using real-time PCR. For relative quantification, the reactions were performed in a total volume of 50 μL, including 25 μL of 2× IQ™ SYBR® green supermix (Bio-Rad, Hercules, CA), 10 ng of DNA, and 10 pmol of each primer. Forty cycles of amplification, data acquisition, and data analysis were performed in an iCycler (Bio-Rad, Hercules, CA). Primers for four genes (*COL6A2, DGCR14, PCSK6,* and *SDHA*) were selected and the position of each clone was obtained from the UCSC genome database (May 2004). The primer sequences used were as follows: *COL6A2*, forward, 5'-TCCATGTCTCAAGCGTTCTG-3'; reverse, 5'-TGGGTTCTGCCCTATGACTC-3'; *DGCR14*, forward, 5'-GACAGTTCAGCAGGCTTTCC-3'; reverse, 5'-TGATCTTGGCTCACTGCAAC-3'; *PCSK6*, forward, 5'-GGCAACAAAAGAAAGCAAGC-3'; reverse, 5'-TCTCAACAGCTGGGAGAGGT-3'; *SDHA*, forward, 5'-CTGAGTGATGCCCTGCAGTA-3'; reverse, 5'-TTCCACGAGCAGAGGAAACT-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 C_T method [17]. C_T for each gene was determined using thermocycler software, and the average of three independent experiments was calculated. The copy number of the target gene was normalized to an endogenous reference, GAPDH, which showed no significant copy number change in each genome [18]. 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.

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, 8p, 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, 8p23.3, 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 14q32.33 and 15q11.2. Other regions with 33～39% frequency of DNA loss included 7q22.1, 10q26.3, 16p12.3, 16p13.11, and 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, examples which indicated higher p-value (＞50% frequency) are shown in the Tables

Table 3. Examples of *t* test analysis in specific alterative regions

Chromosome region	Log_2 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

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].

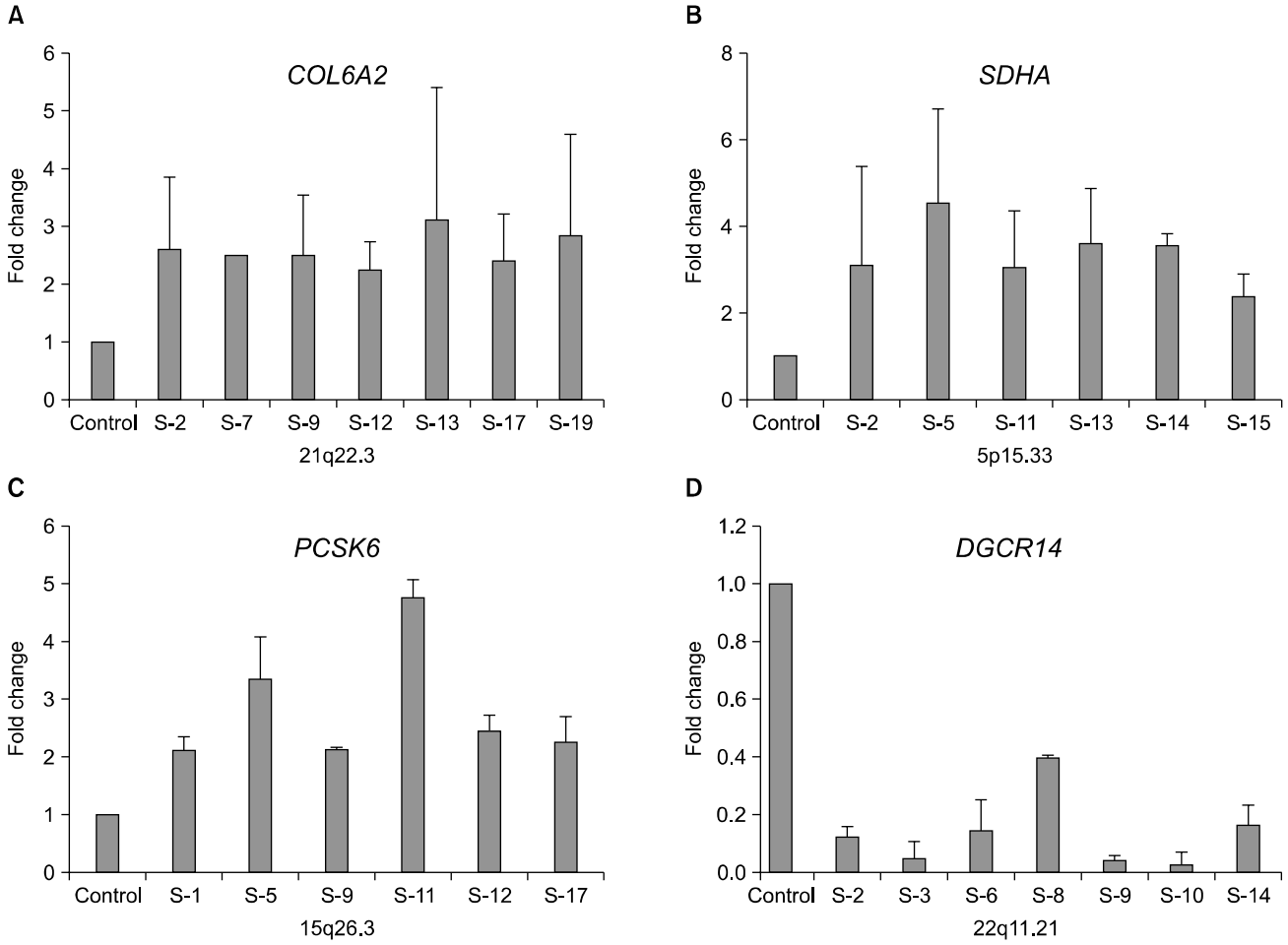

Fig. 1. Relative fold differences selected from 4 genes in which the most frequent gains and losses detected were in the 5p, 15q, 21q, and 22q regions. Each sample is depicted (x axis), and the fold difference of the N-value was delineated in real-time PCR (y axis). A threshold level of 2 indicates significant 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

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 7p, 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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