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ASSOCIATION OF THE JUN DIMERIZATION PROTEIN 2 GENE WITH INTRACRANIAL ANEURYSMS IN JAPANESE AND KOREAN COHORTS AS COMPARED TO A DUTCH COHORT

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Abstract—In a previous study a linkage region for association to IA patients was found on chromosome 14q22. In this study, we report the findings of a positional candidate gene, Jun dimerization Protein 2 (*JDP2*), and single nucleotide polymorphisms (SNP) of that gene that are associated with intracranial aneurysms in different ethnic populations. We screened the linkage region around chromosome 14q22 and narrowed it down to *JDP2*. We then genotyped case and control groups of three different ethnic populations: 403 Japanese intracranial aneurysm (IA) cases and 412 controls, 181 Korean IA cases and 181 controls, 379 Dutch cases and 642 Dutch controls. Genotyping was performed using polymerase chain reaction and direct sequencing technology. The allele distribution of three SNPs (two intronic: rs741846; *P*=0.0041 and rs175646; *P*=0.0014, and one in the untranslated region: rs8215; *P*=0.019) and their genotype distribution showed significant association in the Japanese IA patients. The allelic and genotypic frequency of one intronic SNP (rs175646; *P*=0.0135 and *P*=0.0137, respectively) and the genotypic frequency for the SNP in the UTR region (rs8215; *P*=0.049) was also significantly different between cases and controls of the Korean cohort. There was no difference in allelic or genotypic frequencies in the Dutch population. These SNPs in *JDP2* are associated with intracranial aneurysms, suggesting that variation in or near *JDP2* play a role in susceptibility to IAs in East Asian populations.

Key words: intracranial aneurysm, single nucleotide polymorphism, Jun dimerization protein 2, candidate gene.

Genetic studies have yet to show the exact role of genetics in intracranial aneurysm (IA) formation. Several genome-wide linkage and candidate gene analyses have been performed using patient cohorts of different ethnic background. Among these, many studies report results of different associated candidate genes and linkage regions (Yamada et al., 2004; Nahed et al., 2005; Ruigrok et al., 2005; Bilguvar et al., 2008). In a previous genome-wide linkage analysis with affected Japanese sibpairs, we identified a susceptibility locus on 14q22 (Onda et al., 2001). We performed a comprehensive genetic association study after screening around chromosome 14q22 using single nucleotide polymorphisms (SNP). After narrowing down the region we intensified our screening and focused on the gene JDP2 (on chromosome 14q24) and tried to verify our findings in two different ethnic populations.

EXPERIMENTAL PROCEDURES

Intracranial aneurysm subjects

The Ethics Committee of the Tokyo Women's Medical University, Chonbuk University and University of Utrecht approved the study protocol and all of the study participants gave informed consent before inclusion. The DNA samples for the association study and genotyping were from 403 Japanese intracranial aneurysm cases and 412 controls. The Japanese IA patients included both familial IA patients (178 patients that have first- or second-degree relatives with an IA) and sporadic IA patients (225 patients, age of onset below 60 years of age). The Korean cohort consisted of 181 ruptured intracranial aneurysm cases that were verified during surgery and 181 unrelated controls. We included 390 Dutch Caucasian patients prospectively collected from January 1999 to January 2006 with ruptured and unruptured IA admitted to the University Medical Centre Utrecht and 642 ethnically matched Dutch Caucasian controls.

The affected subjects harboring at least one intracranial aneurysm were diagnosed by either 3D-CT angiography, MR angiography, conventional angiography or surgical findings. The unrelated controls were outpatients with diseases other than intracranial aneurysm, accordingly of Japanese, Korean and Dutch ethnicity.

The collection of Japanese samples was performed in multiple neurosurgical services certified by the Japan Neurosurgical Society. Korean samples were collected at Chonbuk University. The Dutch samples were collected at the University of Utrecht.

DNA extraction and genotyping

Genomic DNA was prepared from samples of whole blood by standard methods. The genotyping of the SNP was performed

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Abbreviations: AP-1, activator protein 1; BATF, basic leucine zipper transcription factor; IA, intracranial aneurysm; JDP2, Jun dimerization protein 2; JNK, c-Jun N-terminal kinase; MMPs, matrix metalloproteinases; SNP, single nucleotide polymorphisms.

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with Taqman SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA) using the 7900HT Sequence Detection system (Applied Biosystems). The tables containing the SNP assay identification numbers can be found online. The primers and probes were designed by Applied Biosystems assay on demand service for allelic discrimination with the 5′ nuclease assay and fluorogenic probes. The material required was the TaqMan universal PCR Master mix, 40× assay mix and Genomic DNA diluted in dH20. The 40× Assay mix consists of unlabeled PCR primers and TaqMan probes labeled with 6-carboxyfluorescein (FAM) and VIC™ dye. The primers and probes for the three positively associated SNPs are available on request. The PCR was performed as follows: after enzyme activation for 10 min at 95 °C, 40 two-step cycles were performed for 15 s denaturation at 92 °C followed by 1 min annealing/elongation at 60 °C.

In an initial screening process we genotyped 148 IA cases and 190 controls of Japanese cohort using 100 commercially available Taqman Assay Probes located around the previously described linkage region of D14S74 (1.2 Mbp: rs224174 – rs3917187), 50 upstream of the marker *D14S74* and 50 downstream. In an earlier publication our institute was able to calculate a maximum logarithm of odds score of 2.31 at this marker location which is 73.3 cM away from the petit-arm-terminal end (pter) of chromosome 14 (Onda et al., 2001).

The results showed association for the Japanese IA cases for SNPs located within the *BATF* (basic leucine zipper transcription factor, ATF-like) and *JDP2* (Jun dimerization protein 2) genes. Taking these findings into consideration we continued genotyping 22 of the 96 screened SNPs located around the *JDP2* and *BATF* genes by increasing the Japanese case and control size to a total of 403 intracranial aneurysm cases and 412 controls (rs741847 – rs12436885). This additional genotyping revealed one SNP of *JDP2* that was significantly associated with Japanese IA (rs741846). Using the HapMap database we determined the LD blocks within *JDP2*, and picked further SNPs located within the block containing the associated SNP. A total of 10 SNPs of *JDP2* were finally genotyped using the increased sample size (total list of rs numbers upon request).

Statistical analysis

Genotype and allele frequencies were compared between groups, and allelic association with IA was evaluated by chisquare statistic. The allele frequency distribution at each polymorphism locus was tested against the Hardy–Weinberg-Equilibrium under the Mendelian biallelic expectation by performing the chi-square-test. Multiple polymorphism marker – disease association with haplotype, the assessment of linkage disequilibrium and all other statistics were performed with commercially available software (SNPalyze, version 5.03 pro; Dynacom, Yokohama, Japan). A probability value of less than 0.05 was set to be significant.

Multipoint nonparametric linkage analysis was performed by a maximum-likelihood method implemented in the GENEHUNTER program (Kruglyak et al., 1996). The maximum limit of detection score was calculated by the method of possible triangle constrains. All sibpairs from sibships containing two or more affected individuals were counted, and the unweighted option was used.

Primary culture of umbilical artery smooth muscle cells (UASMCs)

We obtained 52 umbilical cords during delivery at the Department of Obstetrics of Tokyo Women's Medical University and Kosei General Hospital. All of the participants gave written informed consent, and the study was performed under the approval of the Ethical Committee of Tokyo Women's Medical University and Kosei General Hospital. Both umbilical arteries were excised from the cords, and cut into small pieces. Umbilical artery smooth muscle cells were separated using Hanks buffer containing 2 mg/mL collagenase and cultured in HuMedia-SG medium (Kurabo, Osaka, Japan) supplemented with epithelial growth factor (0.5 ng/mL), basic fibroblast growth factor (2 ng/mL), insulin (5 μg/mL), antibiotics, and 5% fetal bovine serum (FBS).

Quantitative RT-PCR analysis

We extracted total RNA from UASMCs using TRIzol reagent according to the manufacturer's instruction (Invitrogen, Tokyo, Japan). The total RNA (500 ng) was used as a template in first-strand cDNA synthesis with the SuperScript III First-Strand Synthesis System (Invitrogen) and random hexamers. The mRNA expression levels of *JDP2* (assay ID: Hs01650501_m1) and beta-2-microglobulin (*B2M*, Hs00187842_m1) as an endogenous reference, were quantified by a real-time PCR method (Taqman Chemistry) on a sequence detection system (ABI Prism 7900HT; Applied Biosystems) in a total reaction volume of 10 μl containing 1×Master Mix without UNG, the Taqman assay and cDNA PCR conditions were 95 °C for 10 min, and then 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Genotyping of the SNP rs175646 in UASMCs

To obtain genotype-defined UASMC specimens, isolated DNAs from UASMCs were genotyped for the rs175646 SNP using sense primer: 5′-AGAGCAGGCTGTCTGAGGAG-3′ and antisense 5′-GCATGCTTTTCCCTGTTCTC-3′.

Detection of splicing variant of JDP2

The first-strand cDNA from the genotype-defined UASMC was used for examining a splicing variant of *JDP2*.

The primers used are available upon request. The PCR settings were according to a standard protocol. The PCR products were subjected to an agarose gel electrophoresis (1%), followed by staining with ethidium bromide.

RESULTS

In the Japanese IA patient group the percentage of women was 62.8%, in the control group it was 35%. Whereas the mean age in the patient group was 53.8 and 64.1 years in the control group. 74.4% of the patients had suffered from subarachnoid hemorrhage (SAH).

The Korean cases had a female ratio of 72.7% and 42.8% in the controls. Mean age of the cases was 55.3 and 61.5 years in the controls. 97% of the IA cases had suffered from SAH.

The percentage of women was 69.5% in the Dutch patient group and 44.3% in the Dutch control group. The mean age was 57.6 years in the patients and 49.4 years in the controls. In the patient group 83.7% of the patients had ruptured IA with a mean age at the time of rupture of the IA of 50.7 years.

Tables 1 and 2 show that the allele and genotype distribution of three SNPs presented significant association with Japanese IA patients. Two SNPs (rs741846 and rs175646) are in an intron region and one (rs8215) in the gene ending untranslated region of the *JDP2* gene. All three SNPs were in the same LD block. In order to verify the results in different ethnic populations we genotyped the three significantly associated SNPs in a Korean and Dutch population. The rs175646 SNP showed significant allelic and genotypic association in the Korean population (see Tables 1 and 2), whereas there was also a weak significant