Allelic Copy Number Variation in *FSCN2* Detected Using Allele-Specific Genotyping and Multiplex Real-Time PCRs

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Purpose. Allelic copy number variation (CNV) may alter the functional effects of a heterozygous mutation. The underlying mechanisms and their roles in hereditary diseases, however, are largely unknown. In the present study an *FSCN2* mutation was examined that has been reported, not only in patients with retinitis pigmentosa (RP), but also in the normal population.

Methods. Experiments were performed to investigate the gene and allele copy numbers of *FSCN2* in patients with RP who have the c.72delG mutation as well as healthy subjects with or without the mutation. A real-time PCR-based genotyping approach was established that used a real-time PCR assay to qualify the copy numbers of both the wild-type and mutant alleles of the *FSCN2* gene.

Results. Three patients with RP and three normal subjects had an equal ratio of the alleles. Of interest, another patient had an asymmetric allele ratio (4:1) of the copy number of the wild-type allele, compared with that of the mutant allele. These findings were further verified using quantitative assays. An allele-specific methylation assay demonstrated a random methylation pattern in the *FSCN2* gene.

Conclusions. The copy numbers of the *FSNC2* gene and of each allele in the mutant samples were quantified. The findings excluded the possibility that allelic CNV was associated with RP, suggesting that the c.72delG variant is not the primary cause of RP. It is not likely that the *FSCN2* gene is imprinted differentially. The real-time PCR-based genotyping method developed in this study is useful for investigations of allelic asymmetries within genomic regions with CNVs. (*Invest Ophthalmol Vis Sci.* 2008;49:3799–3805) DOI:10.1167/iovs.07-1656

Fascin 2, a member of the fascin family of actin filament-bundling proteins, is specifically expressed in the photoreceptors of the retina.[1] The coding gene, *FSCN2*, is located on chromosome 17, region q25, and encodes 493 amino acids. Wada et al.[2,3] identified a heterozygous c.72delG mutation, previously known as 208delG, in exon 1 of the *FSCN2* gene of patients with autosomal dominant retinitis pigmentosa (ADRP) or autosomal dominant macular degeneration (ADMD) by single-strand conformation polymorphism (SSCP). Mutational screening of the *FSCN2* gene in Italian, Spanish, or North American families with RP did not identify this mutation with methods based on direct PCR sequencing.[4–6] Sixteen nucleotide substitutions including a nonsense mutation were identified in the Spanish families with RP or macular degeneration, but the mutations did not cosegregate with the disease, suggesting that mutation of *FSCN2* is not sufficient to cause RP. More recently, Zhang et al.[7] screened patients with or without retinal degeneration for the c.72delG mutation, resulting in the identification of 8 of the 242 patients; again, the mutation did not cosegregate with retinal degeneration, suggesting that c.72delG is a nonfunctional mutation that is not associated with retinal degeneration. A mouse model carrying a targeted disruption of *FSCN2*, however, has been reported to show progressive photoreceptor degeneration, supporting the idea that haploinsufficiency of *FSCN2* leads to retinal degeneration.[8] More recently, investigators found that mutations in *PRPF31* lead to defective pre-mRNA splicing of the *FSCN2* transcript, indicating that *FSCN2* is an effector of *PRPF31* for retinal degeneration.[9]

Genetic screening of *FSCN2* generally uses PCR or SSCP analysis, together with direct DNA sequencing. These qualitative methods, which are capable of determining the presence or absence of the gene and the nucleotide change in the target amplicons, do not quantitatively evaluate the gene copy number. Copy number variation (CNV) has been increasingly recognized as an important genetic cause of some inherited diseases and complex disorders. CNVs in the *FSCN2* gene were recently discovered in normal populations using whole-genome array-based comparative genomic hybridization (array CGH) analysis.[10,11] Therefore, it is possible that gene or allele CNVs affect the disease phenotypes observed in patients or healthy subjects carrying the c.72delG mutation. For instance, allelic asymmetry with more mutant than wild-type alleles could lead to retinal degeneration.

Imprinting is a non-Mendelian form of inheritance characterized by monoallelic expression depending on the maternal or paternal origin. The imprinted genes always show a differentially methylated pattern. So far, at least 52 genes with parental imprinting have been identified in humans (http://www.geneimprint.org; maintained in the public domain by the Jirtle Laboratory, Duke University, Durham, NC) and no imprinted gene has been identified in patients with RP. It is also possible that imprinting status affects phenotype expression in the patients or normal subjects with the c.72delG mutation. To our knowledge, these possibilities have yet to be addressed.

In this study, we used a real-time PCR-based genotyping approach to analyze the allelic ratio of *FSCN2*, and performed a reproducible real-time PCR assay (*Taq*man; Applied Biosystems, Inc. [ABI], Foster City, CA) in both patients with RP and normal subjects. We found no difference in the allelic ratios of the patients with RP and the healthy individuals. Of interest, allelic asymmetry present in a RP patient and our results indi-

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TABLE 1. Primer and Probe Sets Used for Genotyping and Real-Time PCR Assays

Primer	Primer Sequence	Probe Sequence and Fluorescence and/or Remarks	
FSCN2-P1	TCCAGTTTGGCCTCGTCAAC TCCTGGGTCGGGTTCCA	For wild-type allele: 5-VIC-CGCTACCTGACAGCTG-MGB-3	For genotyping and multiplex real-time PCRs
FSCN2-P2	TGGCCTCGTCAACGACACT GTGCCGAGGCATTGACCTT	For mutant allele: 5-FAM-CGCTACCTACAGCTGAGA-MGB-3	For genotyping and simplex real-time PCRs
FSCN2-P3	CTGAAGATGCCGACGAACG CCTTGAAGCCGAAGCTCTCA	5-VIC-AAGATCCAGTTTGGCCTC-MGB-3	For multiplex real-time PCRs
RNaseP	GCGGAGGGAAGCTCATCAG TCAGACCTTCCCAAGGGACAT	5-FAM-CACGAGCTGAGTGCG-MGB-3	Internal control for real-time PCRs
FSCN2-P4	CCAGCCTCAAGAGGAAGCAG GTCCTCTTCTGCCGACAGGTA	For SYBR GREEN real-time PCRs	
FSCN2-P5	GCTCTTTGATCTGGAGGAGAGTCA CACAGAGACGTAGCGGTGGTT	For SYBR GREEN real-time PCRs	
Ps-f	CCAGGTGCTGAAGATCCAG	Common forward primer, Tm: 56°C	
Ps-1r	GAACTCCAGCGTGTAGCAG	Reverse primer 1, product: 622 bp	
Ps-2r	TCCAGATCAAAGAGCTCATCC	Reverse primer 2, product: 744 bp	
Ps-3r	CGCACAGACACGATACCAC	Reverse primer 3, product: 909 bp	
Ps-4r	AACGCACAGACACGATACC	Reverse primer 4, product: 911 bp	
Ps-5r	TGAACGCACAGACACGATAC	Reverse primer 5, product: 913 bp	
Ps-6r	CATGTGAACGCACAGACAC	Reverse primer 6, product: 917 bp	
Ps-7r	AGTGGCCACACATGTCC	Reverse primer 7, product: 960 bp	
Ps-8r	GCTGAGAAAGGCCAGGAAC	Reverse primer 8, product: 1059 bp	
MSP-f	GTCGTGAGTATTTAGAGGGCGT	Specifically anneal to upstream/exon 1 region in methylated allele	
MSP-r	ACAACCGTACCTTATCCTAAATCG		
USP-f	GGGTTGTGAGTATTTAGAGGGTGT	Specifically anneal to upstream/exon 1 region in unmethylated allele	
USP-r	CAACCATACCTTATCCTAAATCAAA		
M3-f	TTATGAGGAAAAGGGGATTAATTTT	Amplify a CpG-rich region (−368 to −168)	
M3-r	CAACCTAAACAACTCTTAAAAAACCC		

cate the presence of multiple copy numbers of the *FSCN2* gene in subjects from the normal population. In addition, methylation analysis was performed to address the imprinting possibility.

METHODS

Patients and Healthy Subjects

In another study, we examined three patients with RP (P0037, P0075, and P0100) carrying a c.72delG mutation in *FSCN2* and 115 healthy subjects of which two (N-MT and N-ST) were shown to have the same mutation.[12] We reexamined these samples by testing them for the *FSCN2* copy number. In addition, 32 patients clinically suspected of having X-linked RP who did not have any identified mutations in their *RPGR* and *RP2* genes[13] and 54 Japanese volunteers with apparently normal retinas as determined by ophthalmic examinations were recruited for this study. The study was performed in accordance with the Declaration of Helsinki, and the protocols were approved by the institutional ethics committee.

Screening for the *FSCN2* Mutation

Genomic DNA was extracted from peripheral blood samples (QIAamp DNA Blood Mini Kits; Qiagen, Hilden, Germany). Amplification of the genomic region-spanning exon 1 of *FSCN2* and flanking upstream or intronic regions was performed using PCRs with appropriate primers as described in a previous study.[2] The amplified products were sequenced according to standard protocols (model 3130; ABI). Sequencing data were assembled automatically (SeqMan module of the Lasergene program; DNAStar, Madison, WI).

DNA Subcloning

The PCR product, including c.72delG from the DNA sample of 0075, was cloned into a plasmid vector (pCR4-TOPO; Invitrogen, Carlsbad, CA) according to the provided protocols. Plasmids were purified (Wizard Plus Minipreps DNA Purification System; Promega, Madison, WI), then digested with *Eco*RI and electrophoresed on agarose gels for verification or sequenced as described earlier. Vector DNA representing the mutant or the wild-type sequence was used as authentic control samples to generate a mixture of serial allelic ratios from 4:1 to 1:4. Each mixture was then diluted to 1:10, $1:10^2$, $1:10^3$, and $1:10^4$ with TE buffer.

Allele-Specific Genotyping

The allele-specific probe (Table 1) for either the wild-type or mutant allele was designed and synthesized by ABI Japan (Tokyo, Japan). Primers corresponding to the probes (Table 1) were designed on computer (Primer Express 3.0; ABI). Genotyping was performed with real-time PCR (*Taq*Man StepOne; ABI), according to the recommended protocol. In brief, 5 μL of genotyping master mix (*Taq*Man; ABI) was mixed with forward and reverse primers (900 nM), two probes (200 nM *Taq*Man) for each allele, and the DNA sample. The mixture of the vector DNA, including either the mutant allele or the wild-type allele, was prepared as the standard (4:1, 3:1, 2:1, 1:1, 1:2, 1:3, and 1:4). A thermal profile was set up as one prereading cycle of 60°C for 30 seconds and a hot-start cycle of 95°C for 10 minutes, to activate the enzyme, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, and 70°C for 30 seconds, and a postreading cycle of 60°C for 30 seconds. Data were automatically analyzed (StepOne program; ABI). All tests were repeated independently at least twice.

Real-Time PCR Assay

Quantitative detection of the *FSCN2* wild-type or mutant allele was performed using real-time PCR-based methods. *RNaseP*, a single-copy gene, was used as an endogenous control in this study. Before the quantitative PCRs, a standard curve for each primer and probe set was determined by using a set of dilutions of the standard DNA (1:10). Multiplex PCRs were undertaken using 20-μL reactions: 10 μL PCR master mix (*Taq*Man Universal; ABI) with uracil *N*-glycosylase, 450 nM *RNaseP* primers, 900 nM *FSCN2* primers, 125 nM *RNaseP* probe, 250 nM *FSCN2* probe, and 1 μL of the DNA sample. The PCR procedure followed the default setting for the systems with 1 cycle of 50°C for 2

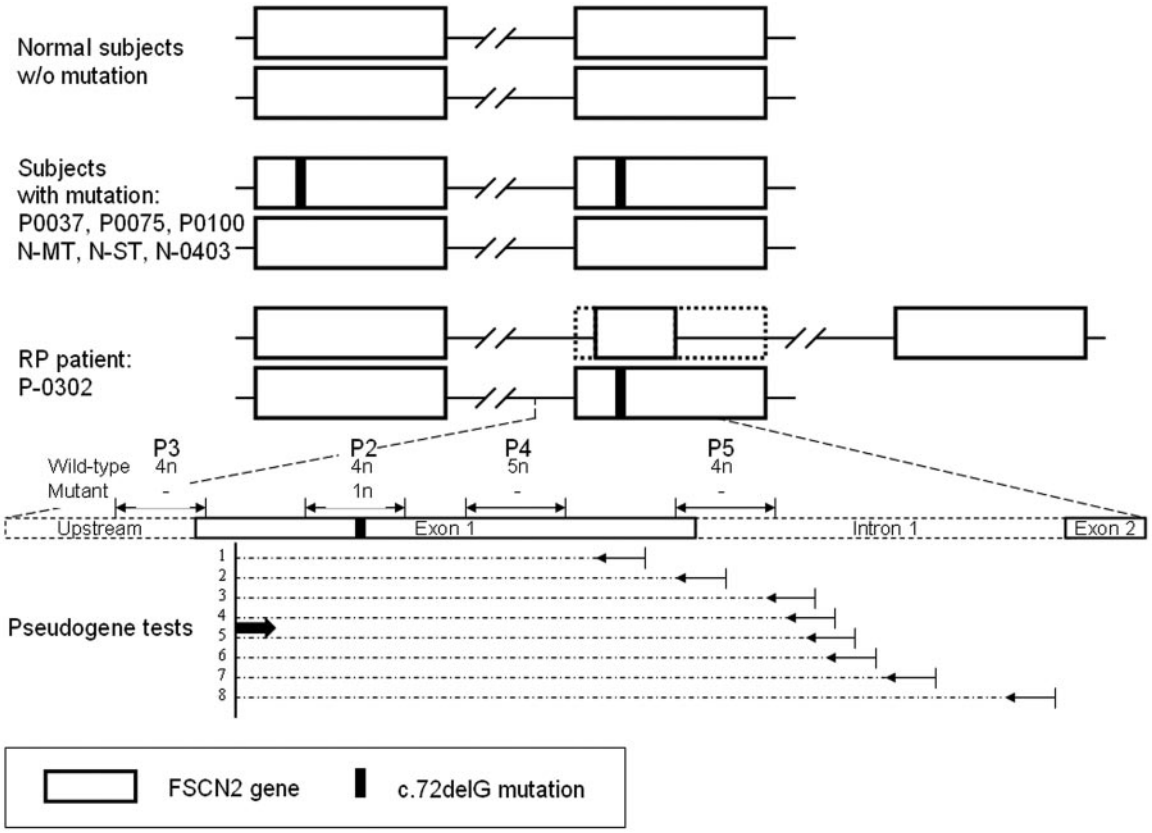

FIGURE 1. A schematic representation of the *FSCN2* genotypes and the locations of the primers used to test for pseudogenes. The putative *FSCN2* genotypes of the patients with RP (P0037, P0075, P0100, and P0302) and healthy subjects (N-MT, N-ST, and N-0403) carrying the c.72delG mutation, as well as those of the phenotypically and genotypically normal subjects. Note that P0302 had an unusual CNV and a putative shortened copy of the gene with breakpoints in the 5′ and 3′ ends of exon 1. *Bottom*: the locations of the primer sets used to test for the presence of a pseudogene.

minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A no-template control was included in each assay. Simplex real-time PCRs were performed by using separate tubes, including the *FSCN2* FAM-probe or *RNaseP* FAM-probe, to quantify the copy number of the mutant *FSCN2* allele. To quantify the copy number of the region downstream of the mutation in 0302 and a normal control (primers P4 and P5), genomic DNA regions were assayed using SYBR green master mix (ABI). Primers and probes used for multiplex or simplex real-time PCRs are shown in Table 1.

Data Analysis

A comparative C_T method of quantitation ($\Delta\Delta Ct$) was used according to the manufacturer's instructions (StepOne Real-Time PCR system; ABI). The $\Delta\Delta Ct$ was calculated separately for *FSCN2* and *RNaseP* for each sample. The $\Delta\Delta Ct$ for *FSCN2* and the $\Delta\Delta Ct$ for *RNaseP* can then be reported as a simple ratio (the $\Delta\Delta Ct$ *FSCN2* value divided by the $\Delta\Delta Ct$ *RNaseP* value), from which the copy number of *FSCN2* was calculated. The PCR amplification efficiencies for *RNaseP*, wild-type *FSCN2*, and mutant *FSCN2* were evaluated separately using standard curves.

Pseudogene Analysis

To exclude the possibility that the normal human genome contains an *FSCN2* pseudogene, we designed a series of primers to amplify the coding region of exon 1 (Table 1, Fig. 1), and the PCR products were sequenced as described earlier. In addition, the genomic sequence of the *FSCN2* gene was analyzed using BLASTN homology searches (www.ncbi.nlm.nih.gov/BLAST/, provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). Transcripts or genomic sequences that showed both significant homology and similar lengths were chosen as possible pseudogenes.

Methylation Analysis

A bisulfite DNA modification was performed (EZ DNA Methylation-Gold Kit; Zymo Research Corp. Orange, CA). Modified DNA was amplified with primers that anneal either methylated- or unmethylated alleles (Table 1). The MSP and USP primers flank the upstream region and part of exon 1 where the mutation is located. An additional primer set (M3) was used to amplify a CpG-rich repeat in the promoter region (Fig. 2). PCR conditions were as follows: initial denaturation at 94°C for 3 minutes, followed by 35 cycles of 94°C, 30 seconds; 58°C, 30 seconds, 72°C, 30 seconds and a final extension at 72°C for 3 minutes. PCR products were purified and were cloned into the pCR4-TOPO vector (Invitrogen, CA). Twenty clones were picked and analyzed by DNA sequencing. The methylation patterns of the promoter region were analyzed with the CpGviewer program.[14]

RESULTS

A Mutation in the *FSCN2* Gene

In our previous large-scale screening for mutations among patients with simplex (sporadic) or multiplex (non-Mendelian or unknown inheritance) RP by using dHPLC-sequencing methods, we identified the *FSCN2* c.72delG mutation in 3 unrelated patients with RP (P0037, P0075, and P0100) and 2 (N-MT and

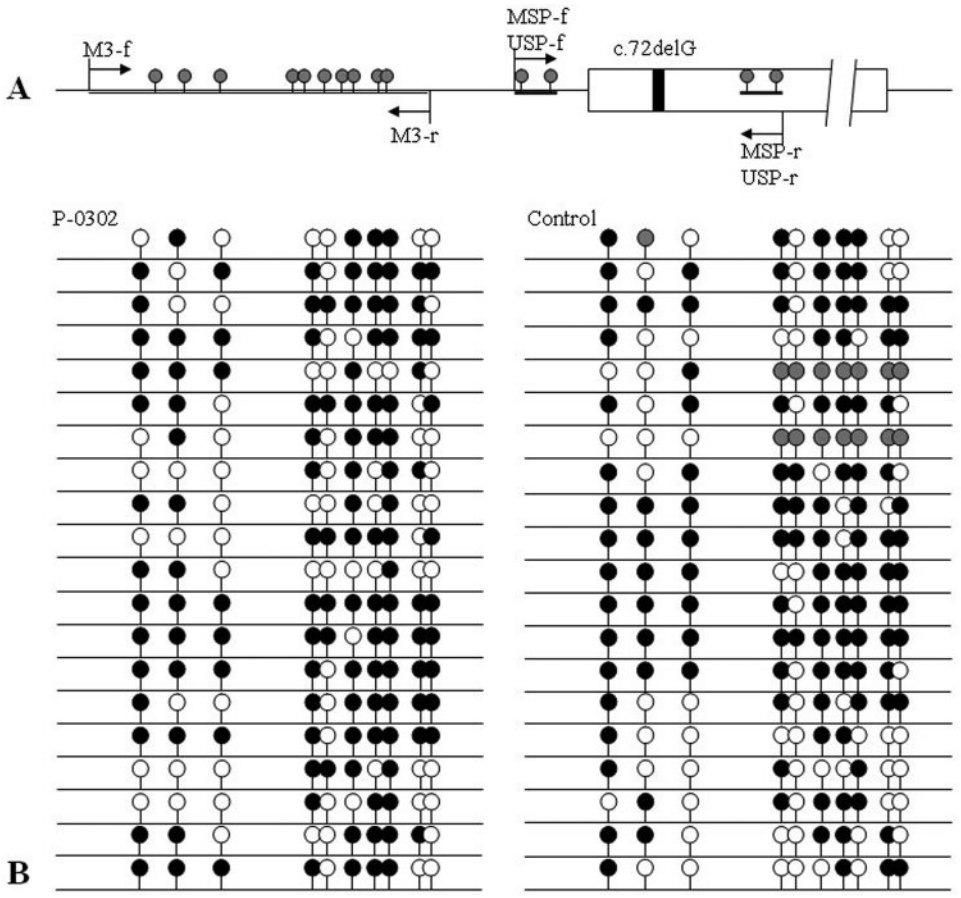

FIGURE 2. Methylation analysis of *FSCN2* promoter and exon 1 regions. (**A**) Schematic representation of analyzed *FSCN2* exon 1 and upstream region using MSP, USP, or M3 primers. *Gray circles*: the CpG-dinucleotides. The M3 primer targets an upstream region flanking 10 CpGs. (**B**) Scaled "lollipops" show output of M3 subcloning data. The example is the data of the tested patient (P0302) and a control sample. *Open circle*: unmethylated CpG; *black filled circle*: methylated CpG; *gray filled circle*: unclear or unreadable sequence.

N-ST) of the 115 healthy subjects.[11] In the present study, an additional 54 volunteers with normal retinas based on funduscopic examinations were recruited, among which one subject (N-0403) was found to be heterozygous for this *FSCN2* mutation. Among the 32 patients with clinically suspected X-linked RP, a severely affected isolated patient (P0302) with high myopia was also found to have this *FSCN2* mutation.

Allele-Specific Genotyping

Prepared mixtures containing a series of ratios of mutant and wild-type sequence plasmids were tested as scaling standards for allele-specific genotyping (Fig. 3A). DNA samples from patients with RP and normal control subjects, together with no-sample negative control samples, were subjected to genotyping tests. Allelic discrimination plot analysis using the PCR system software (StepOne; ABI) showed the separation of each allele ratio; normal controls without the c.72delG mutation were clustered near the horizontal axis, whereas the samples from the three patients and two normal controls heterozygous for the mutation were centered in the 1:1 allele ratio area (Fig. 3B). Of interest, P0302 demonstrated a 4:1 ratio of wild-type to mutant alleles (Fig. 3B).

Real-Time PCR Quantification

To test the allelic asymmetry of the *FSCN2* mutation, we performed an allelic quantification test. The copy numbers of both the wild-type and mutant alleles were analyzed in the three patients with RP and four normal control subjects who had been shown to carry the c.72delG mutation. As shown in Figure 4A, the patients and three healthy control subjects showed 1:1 allele ratios, whereas P0302 showed a 4:1 allele ratio (wild-type allele to mutant allele). These results are consistent with the findings from the genotyping assay, suggesting that allelic asymmetries are present, albeit not commonly, in individuals carrying the c.72delG mutation.

To test the specificity of the primer/probe set for the mutant allele, we performed real-time PCR assays of normal samples without the mutation. All the tested samples did not show a signal for the mutant allele (data not shown).

To investigate the gene copy number of *FSCN2* in the healthy population, we screened 54 phenotypically and genotypically normal samples and seven samples from phenotypically normal subjects or patients with RP known to carry the mutation using the P2 and P3 primer/probe sets (Table 1). The results showed that all the individuals carried four copies of the *FSCN2* gene. One patient (P0302) who carried the mutation was found to have four copies of the wild-type allele and one copy of the mutant allele when the P2 set was used, whereas they had only four copies of the wild-type allele with the P3 set, indicating the presence of a shorter copy of the gene with a forward breakpoint between the P2 and P3 regions (Fig. 1). To locate the terminal breakpoint, the P4 and P5 primers (Table 1) were used to examine the corresponding regions in P0302 and a normal control subject. As shown in Figures 1 and 4B, P0302 had more copies of the P4 region compared with the normal control sample, whereas no difference in the copy numbers of the P5 regions were observed. This result indicates that the terminal breakpoint was located between the P4 and P5 regions.

Pseudogene Tests

BLASTN analysis did not identify any transcripts or genomic sequences with both a significant homology score and a similar length. Although pseudogenes have DNA sequences similar to

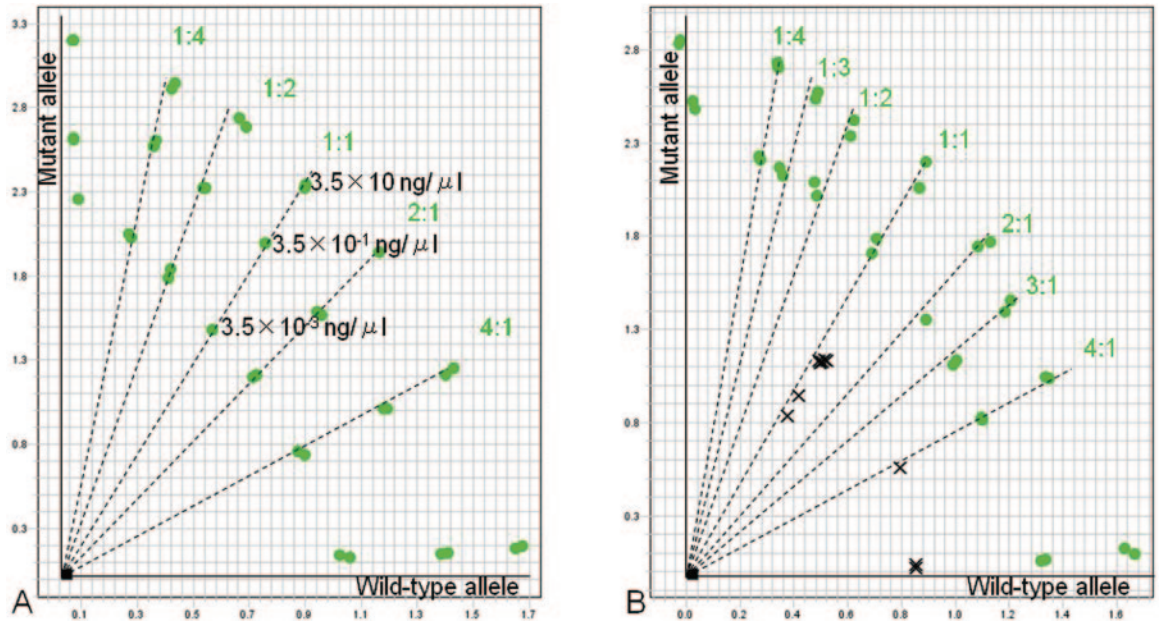

FIGURE 3. Raw data from the genotyping tests. (**A**, *left*) Data representing the different concentrations of the plasmid DNAs used as standard samples. (**B**, *right*) The allelic ratios of the patients or the normal subjects with or without the mutation. The cross marks indicate the tested samples. Note one sample (P0302) showed a 4:1 wild-type to mutant ratio result, distinguishing it from the other samples.

those of their functional counterparts, most of them have multiple sequence variations located throughout the gene. We performed PCRs with a forward primer specific for the 5′ end of exon 1 and eight different reverse primers specific for sequences in exon 1 or intron 1. If the c.72delG allele was located in an unknown pseudogene, some of the amplified products would harbor the mutation together with other neighboring sequence variations. All the amplified products, however, contained a unique heterozygous single-base deletion, suggesting that c.72delG is not located in a pseudogene.

Bisulfite Sequencing of Cloned DNA Fragments

Bisulfite-treated DNAs from patients P0302, P0100 and a normal subject, N-ST, and another normal control subject (without the mutation) were amplified by using the three primer sets. The MSP and USP primers specifically anneal to the methylated and unmethylated alleles respectively while the M3 primer amplify both alleles. Among the 20 clones derived from the MSP or USP products, we did not find a deviated ratio of the methylation pattern in all tested samples (Table 2). In P0302, approximately 20% of the methylated clones and 25% of the unmethylated clones were found to have the mutation, which is consistent with the 4:1 allelic CNV and indicates a random methylation pattern of either mutant or wild-type allele. Furthermore, no preferential methylation pattern was observed in both the patients and healthy individuals in the M3 clones (Fig. 2).

DISCUSSION

One of our purposes in this study was to investigate whether the *FSCN2* gene is responsible for retinal degeneration. To

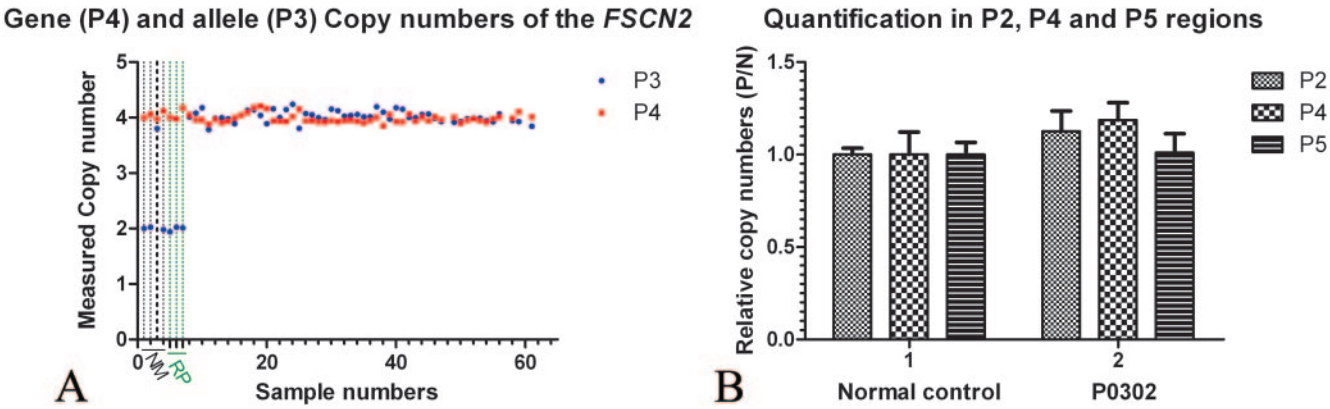

FIGURE 4. Copy number of *FSCN2* in each diploid genome. (**A**) Patients 1–4: normal subjects (NM); patients 5–7: patients with RP (RP). Subjects 8 to 61 are phenotypically and genotypically normal subjects. Note that P0302, who carried the mutation, had two copies of the wild-type allele in the P3 region without the mutation and four copies of the wild-type allele in the P4 region, which contained the mutation. (**B**) P0302 had more copies in the P2 and P4 regions than was observed in the normal control, but no difference in the P5 region was observed. The copy number of the normal control was set as 1, and P0302 showed a 1.187-fold increase of P4 and a 1.125-fold increase of P2, which suggests a multiplying factor of 5/4.

Table 2. Bisulfite Sequencing Results of the Subcloning Products

Sample	Methylated Allele (MSP)		Unmethylated Allele (USP)	
	Mutant (%)	Wild-Type (%)	Mutant (%)	Wild-Type (%)
P-0302	4 (20%)	16 (80%)	4 (25%)*	12 (75%)*
P-0100	6 (33%)*	12 (67%)*	11 (55%)	9 (45%)
N-0403	12 (60%)	8 (40%)	8 (42%)*	11 (58%)*
Control	0 (0%)	20 (100%)	0 (0%)	20 (100%)

* Sequencing analysis for some clones (1–4) failed because of unreadable chromatograph or subcloning failure.

date, both frameshift and nonsense sequence changes have been identified in normal control subjects as well as in families with members who have RP.[2,5] These variants were located in the coding region and probably created loss-of-function alleles in the carriers. A loss-of-function mouse model heterozygous for the c.72delG mutation has been reported to show progressive photoreceptor degeneration.[8] The investigators, however, found no clinical cosegregation of the mutation with the disease.[5] In a previous report, a nonsense mutation in RP1 gene, *R1933X*, which is predicted to create a truncation of the extreme C-terminal, was identified in both the RP patients and a healthy individual.[15] The authors suggested that it is a nonpathologic truncating mutation. Another group reported the same mutation in three Japanese families with ADRP.[16] Differing from this mutation, *FSCN2* c.72delG is a mutation located in the 3′ end of the gene and is predicted to create both 119 abnormal amino acids and an early termination of protein at the codon 143 (24Lfs119ter143).

CNV has recently been identified as a cause of global genetic variation in the human genome. CNVs have been estimated to occur in approximately 12% of the genome,[17] and have been reported to be associated with various diseases and variations in drug efficacies.[18,19] Over 2000 CNVs have been identified to date, and both increased and decreased CNVs covering the *FSCN2* gene in chromosome 17 have been reported recently.[10,11] This finding led us to hypothesize that CNVs of *FSCN2* may be associated with retinal degeneration. Furthermore, copy number alterations of the wild-type or mutant allele may underlie the differences between affected patients and healthy subjects who carry the c.72delG variant. In this study, we examined both the gene and the allele copy numbers of *FSCN2* in patients with RP and healthy subjects. We did not find a CNV between the RP patients and the normal subjects. It should be noted that we did not investigate the cosegregation of the mutation and the disease in the families, because the cases were either isolated, or the DNA samples from the other family members were not available. Therefore, whether the mutation is associated with the disease in these family cases is unknown.

In agreement with our hypothesis, allelic asymmetry was observed in a severely affected patient with RP who showed an unusual allelic variation, with four copies of the wild-type allele and one copy of the mutant allele. To our knowledge, this type of variation has not been described previously. The other 54 normal individuals showed the same copy numbers, suggesting that normal Japanese individuals carry at least four copies of the *FSCN2* gene. Wong et al.[10] have described 22 subjects with decreased CNVs spanning the *FSCN2* gene among 95 normal subjects using array CGH. In the group of normal individuals, however, no decreased CNVs were identified with the real-time PCR assay. This bias indicates the type and frequency of CNVs depend on the ethnicity of the examined population.

It is important to quantify the allelic copy numbers in CNVs to understand how they contribute to genetic disorders, because CNVs may alter the functional effects of heterozygous mutations. In the present study, we established an efficient genotyping method based on real-time PCRs, to investigate the allelic ratio of a heterozygous sequence change. As mentioned, three RP patients and three normal subjects carrying the c.72delG mutation were found to have a 1:1 ratio of the alleles. One patient, however, was found to have a 4:1 wild-type to mutant allelic ratio. These results were further confirmed following the quantification of each allele by real-time PCR assays. Our results suggest that the allele CNV identified in this study is unlikely to be associated with the pathogenicity of the disease. The current method based on conventional, reliable assays, however, may facilitate investigations into the allelic ratios of specific regions containing CNVs and their roles in other genomic disorders. Although the chromosomal array CGH is extensively used for the genome-wide detection of CNVs,[10,11] it uses a relatively large amount of genomic DNA and may fail to detect relatively small CNVs as well as slight changes in allelic ratios. In contrast, the quantitative real-time PCR-based method we used in the present study is more sensitive and specific. Padiath et al.[19] and Hosono et al.[20] recently developed a SNP-based invader assay to detect allele asymmetries within CNV regions. It is believed that both of these new methods will be useful for studying the detailed allelic functions associated with individual disease susceptibilities.

We had to exclude the possibility of an *FSCN2* pseudogene in the human genome. The mammalian genome contains pseudogenes that correspond to several functional genes, including such commonly used internal controls as β-actin, *GAPDH*, and cyclophilin. There are three main types of pseudogenes: processed (or retransposed) pseudogenes, nonprocessed (or duplicated) pseudogenes, and unitary pseudogenes. Because most of the known pseudogenes have several sequence disparities compared with the functional gene, we amplified the coding exon 1 by using multiple flanking primers to test whether the c.72delG mutation is located in a pseudogene. Sequencing of all the PCR products consistently detected only the current sequence variant, strongly suggesting that there were no relevant pseudogenes. Furthermore, direct PCR sequencing of exons 2 to 5 with flanking primers did not identify any other sequence alterations. BLASTN analysis of the current human genome sequence database did not detect any sequences that were highly similar to *FSCN2*. Together, these findings make it unlikely that an *FSCN2* pseudogene in the human genome influenced our results. It must be noted, however, that we could not exclude the presence of a duplicate pseudogene that had all the same characteristics as the *FSCN2* gene. Furthermore, bisulfite sequencing analysis of the DNAs from patients or normal subjects failed to find a preferential methylation in either allele. Based on this result, we conclude that it is not likely that the *FSCN2* gene is imprinted with monoallelic expression in the patients or healthy subjects examined in the present study.

In brief, our analysis of CNVs as well as variations in the number of alleles with or without the c.72delG mutation in the *FSCN2* gene among Japanese subjects suggests that *FSCN2* is unlikely to be the pathogenic genetic cause of retinal degeneration. We believe the method established in this study, which is based on a real-time PCR assay, is useful for identifying allelic and genomic CNVs that may underlie various genomic disorders.

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