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ORIGINAL ARTICLE

Functional splicing assay of *DSPP* mutations in hereditary dentin defects

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OBJECTIVE: Dentin sialophosphoprotein (*DSPP*) gene mutations have been identified in isolated hereditary dentin defects; however, the genotype–phenotype correlations are poorly understood. We performed *in vitro* splicing assays to test the hypothesis that *DSPP* mutations in splice junctions as well as proposed missense/nonsense mutations experimentally result in aberrant pre-mRNA splicing.
MATERIALS AND METHODS: The genomic fragment of the human *DSPP* gene was cloned into the pSPL3 splicing vector, and previously reported as well as informative *de novo* mutations were then introduced by PCR mutagenesis. The COS-7 cells were transfected with each plasmid vector, and total RNA was isolated. RT-PCR result was analyzed, and the band intensity of the product was calibrated using ImageJ.
RESULTS: The predictions by others of exon 3 skipping in specific *DSPP* mutations have been validated and a cryptic splicing donor site has been identified. However, the degree of mutational effect on pre-mRNA splicing varied considerably depending on the changed nucleotide.
CONCLUSIONS: The predictions of exon 3 skipping in specific *DSPP* mutations have been validated, and a cryptic splicing donor site has been identified. Our data may provide insight into the contribution of *DSPP* mutations in the pathogenesis and genotype–phenotype correlations of hereditary dentin defects.
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Keywords: dentin sialophosphoprotein; dentinogenesis imperfecta; dentin; dentin dysplasia; splicing assay

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Introduction

Dentin is one of the major tissues in the tooth and supports the enamel. Tooth enamel is the hardest tissue in the human body and does not contain cellular components. Without proper dentin support, enamel tends to easily wear off or be prone to fracture. Alteration in dentin structure and composition can occur genetically, and these alterations (hereditary dentin defects) are categorized into dentinogenesis imperfecta (DGI) and dentin dysplasia (DD). DGI is divided into three types and DD into two types according to clinical and radiological characteristics (Shields *et al*, 1973).

The dentin sialophosphoprotein (*DSPP*) gene, located on chromosome 4q21, encodes the major non-collagenous protein of the dentin matrix and is one of several candidate genes for hereditary dentin defects (MacDougall *et al*, 1997). So far, mutations have been identified only in this gene, and the mutations caused non-syndromic hereditary dentin defects (DGI type II, III and DD type II). After removal of the 15 amino acid signal peptide, the N-terminus of the DSPP is encoded by the Ile from the end of exon 2 and then Pro and Val from exon 3, making the highly conserved tripeptide, IPV. The role of this conserved IPV domain has yet to be determined, but involvement in the signal peptide cleavage or subsequent protein processing has been suggested (McKnight *et al*, 2008b).

Mutations in the *DSPP* gene can be grouped into the three classes (McKnight *et al*, 2008b): mutations in the signal peptide (Rajpar *et al*, 2002; Malmgren *et al*, 2004), mutations affecting the IPV domain (Xiao *et al*, 2001; Zhang *et al*, 2001, 2007; Kim *et al*, 2004, 2005; Holappa *et al*, 2006; Song *et al*, 2006; Hart and Hart, 2007; Lee *et al*, 2008, 2009, 2011b; McKnight *et al*, 2008b; Kida *et al*, 2009; Bai *et al*, 2010), and net -1 frameshift mutations (McKnight *et al*, 2008a,b; Song *et al*, 2008; Lee *et al*, 2011a; Nieminen *et al*, 2011).

Some mutations in the 5′ end of the *DSPP* gene, including missense mutations affecting the IPV domain

and the single reported nonsense mutation, have been predicted to cause aberrant pre-mRNA splicing (McKnight *et al*, 2008b). However, experimental evidence exists for only two mutations: c.52-6T > G resulting in DD type II (Lee *et al*, 2008) and c.53T > A resulting in a severe form of DGI type II (resembling DGI type III) (Lee *et al*, 2009). We investigated the effects of both reported and additional possible *DSPP* mutations affecting the IPV domain on pre-mRNA splicing to better understand the molecular genetic pathogenesis of aberrant dentin biomineralization.

Materials and methods

Cloning of the human DSPP *gene and mutagenesis*
A PCR amplification was performed using a normal control DNA to include exons two, three and four of the *DSPP* gene (amplicon size: 2767 bp, sense: 5′-GGGGGCGGCCGCGGGCAAATGCTTACACATCA-3′, antisense: 5′-AGGATCCATGATTTCACATAAGAC-3′). The amplicon was purified and cloned into the pSPL3 splicing vector following digestion with *Not*I and *Bam*HI restriction endonucleases. We introduced previously reported as well as informative *de novo* mutations for the completeness of the assay using PCR mutagenesis. The sequences were confirmed by direct sequencing (Table 1).

In silico *splicing assay*
We performed *in silico* splicing assays using SplicePort (http://spliceport.cs.umd.edu/SplicingAnalyser2.html) and NNSPLICE version 0.9 (http://www.fruitfly.org/seq_tools/splice.html). Predicted values for the wild-type sequence and the changed scores for each mutated sequence are listed in Table 1.

In vitro *splicing assay*
The COS-7 cells (5×10^5) were seeded in a 35-mm culture dish and incubated at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Gibco-BRL, Carlsbad, CA, USA) and antibiotic-antimycotic liquid (Gibco-BRL). On the following day, the culture medium was exchanged to remove antibiotics, and each plasmid DNA (4 μg of wild-type or 17 mutant pSPL3 vectors) was transfected using the conventional calcium phosphate method. After 6 h, the culture medium was replaced with complete medium containing antibiotics. Forty-eight hours after transfection, total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was made from 4 μg RNA using one-step 5× RT-PCR premix (ElpisBio, Taejeon, Korea).

Image analysis
RT-PCR amplification (wild-type amplicon size: 666 bp; sense primer: 5′-TAGAGTCGACCCAGCACCAT-3′, antisense primer: 5′-CCTCGTTTCTACAGGAATTCTCA-3′) was performed using HiPi PCR premix (ElpisBio). Amplification products were resolved on a 4% polyacrylamide gel and stained with 0.1% ethidium bromide. The gel image was photographed, and band intensity was analyzed using ImageJ (NIH, Bethesda, MD, USA). Band intensity was normalized to the size of the amplification product (wild type: 666 bp; exon 3 deletion: 582 bp; alternative splicing: 592 bp). The experiments were duplicated, and bands were excised from the gel, purified, and characterized by DNA sequencing.

Results

The *in vitro* splicing assay revealed that several mutations cause defective pre-mRNA splicing, as predicted by others using *in silico* splicing assay program (McKnight *et al*, 2008b) (Figure 1, Table 1). As predicted, disease-associated nucleotide changes in the third position at the end of exon 2 (c.49C > A or T resulting in ITV and ISV, respectively) did not cause changes in splicing. Furthermore, changes in the second or third bases of exon 3 (c.53T > A, C or G and c.54 T > A, C or G resulting in the disease-causing missense mutation IPD, the hypothetical but unreported mutations IPA, IPG, or retained IPV, respectively) also behaved as expected and did not influence normal pre-mRNA splicing. Mutations in the polypyrimidine tract (c.52-6T > G) and splicing acceptor site (c.52-3C > G or A) affected pre-mRNA splicing, causing skipping of exon 3 (Kim *et al*, 2004; Holappa *et al*, 2006; Lee *et al*, 2008). Nucleotide changes in the first nucleotide of exon 3 (c.52G > T, A or C) also showed defective splicing; however, the effect varied depending on the changed nucleotide. The change from G to A (c.52G > A) had little effect on pre-mRNA splicing. Mutations near the donor site of intron 3 also affected pre-mRNA splicing. Mutations of the first conserved nucleotide of the donor site (c.135 + 1G > A or T) has long been predicted to cause exon 3 skipping (Xiao *et al*, 2001; McKnight *et al*, 2008b), and we have experimentally shown this to be true. Moreover, the nonsense mutation (c.133C > T) at the third nucleotide position from the end of exon 3 also affected pre-mRNA splicing. These changes not only largely deleted exon 3, but also triggered a cryptic splicing donor site usage not previously predicted. The cryptic donor site is expected to result in frameshift mutation, introducing premature termination codon in exon 4. The percentage of each transcript is listed in Table 1. Sequencing results are shown in Figure 2.

Discussion

Splicing prediction algorithms are robust and have successfully defined normal exon–intron boundaries in many genome projects. These programs are frequently used to explain how mutations sometimes cause disease by predicting exon skipping or intron retention events. In general, the predictions made using computer algorithms were confirmed in this study; however, certain gene sequences appear to better fit specific prediction algorithms. The mutation (c.53T > A), which was previously reported to not affect pre-mRNA splicing (Lee *et al*, 2009), was confirmed in this study; however, a change in the prediction value from one program (0.91 → 0.48) may indicate a defective splicing event,

Table 1 Splicing analysis of mutations in DSP region

		Prediction (SplicePort/ NNSPLICE)[b]							
	cDNA[a]	*I2 donor*	*I2 acceptor*	*I3 donor*	*Prediction*[c]	*mRNA splicing*	*Protein*[d]	*IPV domain*	*Phenotype (References)*
E2	Wildtype	0.90/0.98	0.91/0.97	0.94/0.89		Normal	Wildtype	IPV	
	c.49C > A	0.87/0.98			Normal	Normal	p.P17T	ITV	DGI-II (Xiao *et al*, 2001)
	c.49C > T	0.81/0.98			Normal	Normal	p.P17S	ISV	DGI-II (Hart and Hart, 2007; Zhang *et al*, 2007)
I2	c.52-6T > G		0.36/0.74			Normal + X3 del (44:56)	Wildtype + p.V18_Q45del (44:56)	IPV + IPD	DD-II (Lee *et al*, 2008)
	c.52-3C > G		−0.86/N		X3del	X3del	p.V18_Q45del	IPD	DGI-III (Kim *et al*, 2004)
	c.52-3C > A		−0.04/0.60		X3del	Normal + X3del (20:80)	Wildtype + p.V18_Q45del (20:80)	IPV + IPD	DGI-II (Holappa *et al*, 2006)
E3	c.52G > T		−0.02/0.85		X3del or Normal	Normal + X3del (43:57)	p.V18F + p.V18_Q45del (43:57)	IPF + IPD	DGI-II, DGI-III (Xiao *et al*, 2001; Kim *et al*, 2005; Holappa *et al*, 2006; Song *et al*, 2006)
	c.52G > A		0.47/0.94			Normal + X3del (92:8)	p.V18I + p.V18_Q45del (92:8)	IPI + IPD	
	c.52G > C		0.23/0.85			Normal + X3del (70:30)	p.V18L + p.V18_Q45del (70:30)	IPL + IPD	
	c.53T > A		0.48/0.91			Normal	p.V18D	IPD	DGI-III (Kida *et al*, 2009; Lee *et al*, 2009)
	c.53T > C		0.47/0.93			Normal	p.V18A	IPA	
	c.53T > G		0.34/0.93			Normal	p.V18G	IPG	
	c.54T > A		0.79/0.97			Normal	Wildtype	IPV	
	c.54T > C		0.67/0.97			Normal	Wildtype	IPV	
	c.54T > G		0.76/0.97			Normal	Wildtype	IPV	
	c.133C > T			0.39/N	X3del or Normal	X3del + Normal + alt. (69:15:16)	p.V18_Q45del + NMD (69:31)	IPD	DGI-II (Zhang *et al*, 2001; Song *et al*, 2006)
I3	c.135 + 1G > A			−3.00/N	X3del	X3del + alt. (80:20)	p.V18_Q45del + NMD (80:20)	IPD	DGI-II (Xiao *et al*, 2001)
	c.135 + 1G > T			−3.51/N	X3del	X3del + alt. (81:19)	p.V18_Q45del + NMD (81:19)	IPD	DGI-II (McKnight *et al*, 2008b)

[a]Numbering assumes the A of the ATG start codon as nucleotide 1 based on reference sequence NM_014208.3.
[b]Prediction program (SplicePort = http://spliceport.cs.umd.edu//SplicingAnalyser2.html, NNSPLICE = http://www.fruitfly.org/seq_tools/splice.html).
[c]Prediction by McKnight *et al* (2008b).
[d]Mutational effect on the protein product is predicted from the result of the splicing assay.
E, Exon; I, Intron; N, not predicted as a splice site; Alt., alternative splicing; X3del, skipping of exon 3; NMD, degraded by nonsense mediated decay; DD, dentin dysplasia; DGI, dentinogenesis imperfecta.

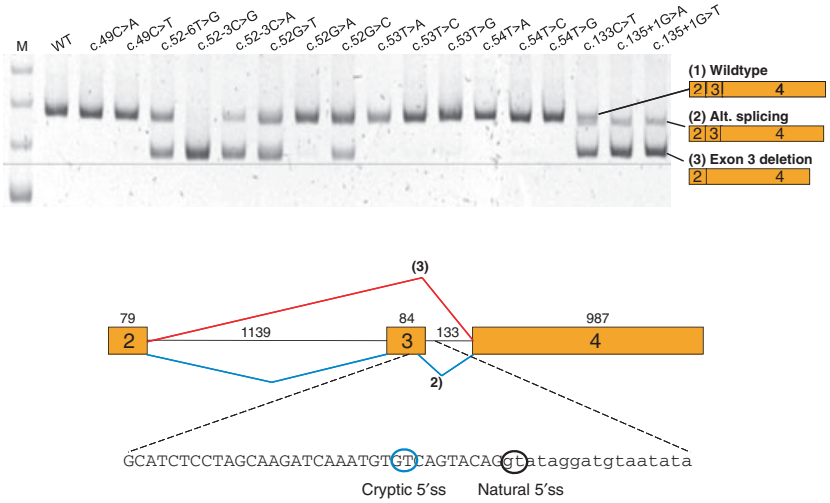

Figure 1 Splicing assay of nucleotide changes in the DSP domain of the human *DSPP* gene. (Upper) Polyacrylamide gel image of RT-PCR product. Changed nucleotides are shown above the image (M; marker, WT; wild type). (1) Indicates normal transcript. (2) Indicates alternative splicing product using a cryptic 5′ splicing donor site (5′ ss). (3) Indicates exon 3-deleted transcript. (Lower) Gene diagram showing altered splice products (exon 3-deleted transcript is shown by a red line and alternative splicing product using a cryptic 5′ splicing donor site is shown by blue lines). The nucleotide sequences of the exon 3 and intron 3 boundary are shown (exon sequences in upper case and intron sequences in lower case). The natural 5′ splicing donor site is indicated by a black circle and the cryptic 5′ splicing donor site is indicated by a blue circle. The orange box indicates an exon; the exon number is shown in the box. Lines between exons indicate introns. Exon and intron length are shown above the boxes and lines

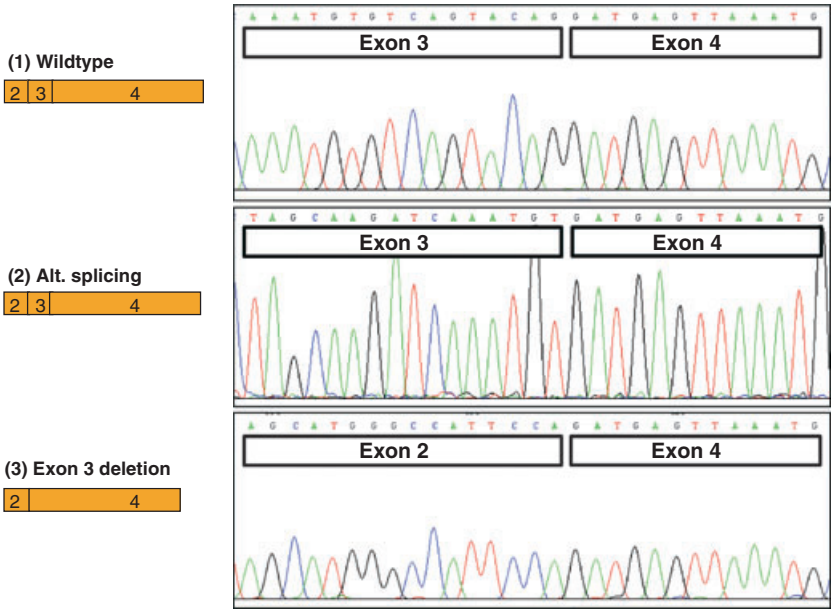

Figure 2 Chromatograms of RT-PCR products. (1) Wild-type sequence shows normal splicing, (2) Alternative splicing using a cryptic 5′ splicing donor site, (3) Exon 3-deleted sequence

while the other (0.97 → 0.91) does not. And none of the programs predicted the alternative splicing donor site found in this study. We recommend that the predicted values be interpreted with caution; multiple prediction programs appear needed to predict mutational effects on pre-mRNA splicing events and such predictions be verified by experimental results.

This study has experimentally verified the predictions of others that many mutations in the DNA encoding the amino terminus of DSPP, including a nonsense mutation and several missense mutations, affect pre-mRNA splicing. The nonsense mutation (c.133C > T) resulted in normal and alternative (using the cryptic 5′ donor site) splicing in about 30% of total transcripts, and these transcripts would be degraded by the nonsense-mediated decay system (Shyu *et al*, 2008). The mutational effect of this nonsense mutation appears to be exon 3-deleted DSPP changing IPV to IPD. The mutation (c.52G > T) in the first position of exon 3 has also been suggested to influence pre-mRNA splicing on the basis of an *in silico* prediction (0.91 → −0.02) (McKnight *et al*, 2008b), while change in prediction values made by the other programs in this study appears insignificant (0.97 → 0.85). Aberrant pre-mRNA splicing events

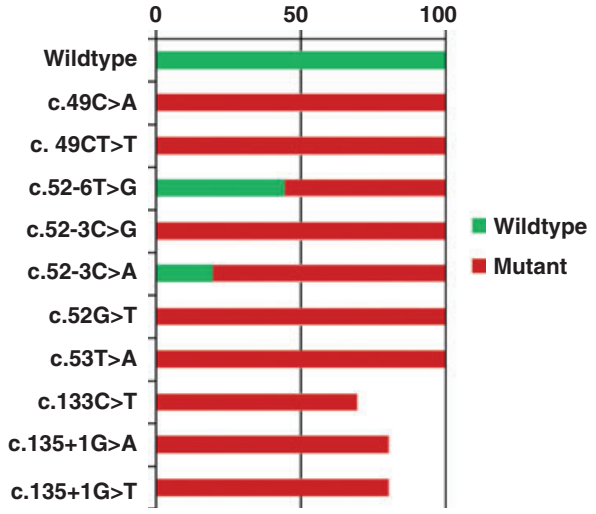

Figure 3 Predicted protein products presented as a bar graph. Ratios were obtained from Table 1, and a value of 100 was assigned to the entire amount of DSPP produced from the mutated allele. The term 'mutant' refers to missense or exon 3-deleted mutations resulting in a defective IPV domain

resulting in deletion of exon 3 were confirmed in this study.

One of the interesting findings is that while a mutation in the polypyrimidine tract (c.52-6T > G) is related to DD type II (Lee *et al*, 2008), a mutation in the splicing acceptor site (c.52-3C > A) causes DGI type II (Holappa *et al*, 2006). Here, we show that the only difference between these two mutations is the ratio of the normal and exon 3-deleted transcripts. The c.52-6T > G mutation would produce about 50% wild-type DSPP from the mutated allele, whereas the c.52-3C > A mutation would result in less than 20% wild-type DSPP (Figure 3). Furthermore, other mutations that cause DGI type II or III would produce no wild-type DSPP. Thus, the degree of reduction in the amount of wild-type DSPP may explain the phenotypic difference between DGI type II (severe form) and DD type II (mild form).

However, when another mutation (c.16T > G, p.Y6D) that causes DD type II (Rajpar *et al*, 2002) is considered, a reduction in the amount of wild-type DSPP appears insufficient in explaining the phenotypic difference. Mutant DSPP protein is expected to have a dominant negative effect on cell function and/or dentin matrix mineralization. Our study also showed that the amount of mutant DSPP in DD type II was much smaller than in DGI type II. The c.16T > G mutation was shown to produce almost no DSPP protein from the mutated allele because of defective signal peptide (Rajpar *et al*, 2002). Taken together, the genotype–phenotype correlation appears to be influenced not only by the degree of reduction in the amount of wild-type DSPP but also by the amount of mutant DSPP protein.

All mutations tested in this study resulted in a change of the proposed IPV domain (McKnight *et al*, 2008b). It is not known whether this IPV domain is essential for signal peptide cleavage, or whether it has other functional roles, such as protein folding, post-translational modification, or protein–protein interaction. Further studies are required to investigate whether changes in the IPV domain affect signal peptide cleavage and to functionally characterize DSPP, which may lead to a better understanding of the normal and pathologic basis of dentin biomineralization.

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Author contributions

Kyung-Eun Lee, Sook-Kyung Lee, and Seung-Eun Jung performed the experiments. Zang Hee Lee and Jung-Wook Kim designed the study and prepared the manuscript.

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