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ORIGINAL ARTICLE

Mutations in the Desmoglein 4 Gene Are Associated with Monilethrix-like Congenital Hypotrichosis

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The gene encoding human desmoglein 4 (DSG4) was recently cloned, and a mutation in this gene has been reported in several consanguineous Pakistani families affected with localized autosomal recessive hypotrichosis (LAH). In addition, various mutations in the *Dsg4* gene have been identified in animal models of hypotrichosis that share a characteristic phenotype called "lanceolate hair". To date, the features of the hair-shaft anomaly in patients with LAH have not been well described. We report a Japanese patient affected with congenital hypotrichosis that was originally diagnosed as monilethrix because she had a hair-shaft abnormality that resembled moniliform hair. However, no mutations were found in the type II hair keratin genes, *hHb1*, *hHb3*, and *hHb6*, whose mutations cause monilethrix. Instead, we identified novel compound heterozygous mutations in the *DSG4* gene of our patient. On the maternal allele is a novel S192P transition within the extracellular cadherin II domain of DSG4; on the paternal allele is a novel 2039insT mutation leading to the generation of unstable transcripts. Here we present the observation that mutations in the *DSG4* gene can cause monilethrix-like congenital hypotrichosis. Based on our findings, we propose that LAH and monilethrix could overlap.

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INTRODUCTION

Hair is a highly keratinized tissue formed within the hair follicle. Growth of the hair originates in matrix cells located in the bulb region. In the matrix region, cells termed trichocytes actively proliferate, keeping their desmosomal structure. As they move upward to the precortex and the keratinizing zone, the switch from proliferation to differentiation occurs. During this process, hair keratins are gradually and sequentially expressed, resulting in the formation of the intermediate filament network and keratinization of the hair fiber. Hair keratins are a major structural component of the hair. They are divided into two groups: type I (acidic) and type II (basic to neutral). In humans, nine type I and six type II hair keratins have been identified (Rogers *et al.*, 1998, 2000), and their expression patterns in the hair have been characterized in detail (Langbein *et al.*, 1999, 2001). Winter et al. (1997) reported that mutations in two type II hair keratin genes, *hHb1* and *hHb6*, cause an autosomal dominant hypotrichosis, monilethrix (MIM 158,000). In addition, a mutation in another type II hair keratin gene, *hHb3*, has been identified in one family with monilethrix (van Steensel *et al.*, 2005). These hair keratins are expressed predominantly in the keratinizing zone of the hair fiber cortex (Langbein *et al.*, 2001), and their mutations are thus thought to lead to abnormal keratinization of the hair. The disease is characterized by a specific anomaly called moniliform hair, which consists of nodes and internodes (Ito *et al.*, 1984, 1990). Perifollicular hyperkeratosis is also a consistent feature of this disease. The expression of monilethrix is variable; in mild cases, moniliform hairs may be confined to the occiput; in severe cases, hairs of the entire scalp, eyebrows, eyelashes, and body can be affected. Nail deformities are sometimes observed, as well (Heydt, 1963).

In addition to hair keratins, recent advances in hair research have shown that mutations in genes encoding other structural components of the hair shaft cause some congenital fragile hair disorders. Among them, localized autosomal recessive hypotrichosis (LAH; MIM 607,903) is a new form of congenital hypotrichosis found in several consanguineous Pakistani families (Kljuic *et al.*, 2003). The patients suffer from hypotrichosis limited to the scalp, chest, arms, and legs (Kljuic *et al.*, 2003). The eyebrows and beard are less dense than normal, and the axillary hair, pubic hair, and eyelashes are normal (Kljuic *et al.*, 2003). In the affected areas, perifollicular papules caused by ingrown hairs are usually observed (Kljuic *et al.*, 2003). LAH has been mapped to chromosome 18q (Kljuic *et al.*, 2003; Rafique *et al.*, 2003). Subsequently, the *desmoglein 4* (*DSG4*) gene was cloned (Kljuic *et al.*, 2003; Whittock and Bower, 2003), and confirmed as the causative gene for LAH (Kljuic *et al.*, 2003). This gene consists of 16 exons, and encodes DSG4 protein, a new member of the cadherin superfamily (Kljuic *et al.*, 2003). So far, all Pakistani patients reported have had

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Abbreviations: bp, base pairs; DSG4, Dsg4, desmoglein 4; EC, extracellular cadherin; lah, lanceolate hair; LAH, localized autosomal recessive hypotrichosis; RT, reverse transcriptase; SEM, scanning electron microscopy

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an identical homozygous 5-kb deletion that includes exons 5–8 of the *DSG4* gene (Kljuic *et al.*, 2003; Moss *et al.*, 2004; Rafiq *et al.*, 2004). In addition, various mutations have been found in the *Dsg4* gene of the lanceolate hair (*lah*) mouse (Kljuic *et al.*, 2003), the *lah*^{J} mouse (Kljuic *et al.*, 2003), the *lah* rat (Jahoda *et al.*, 2004), the Iffa Credo hairless rat (Bazzi *et al.*, 2004), and the spontaneously hypertensive rat (Meyer *et al.*, 2004). These are all animal models of congenital hypotrichosis that share a characteristic hair-shaft anomaly in which a broken end shows a lance-head shape – thus, ''lanceolate hair''. Histological studies have shown the formation of a bulbous ''bleb'' in the hair shaft within the hair follicle of both LAH patients and the model animals (Montagutelli *et al.*, 1996; Sundberg *et al.*, 2000; Kljuic *et al.*, 2003; Bazzi *et al.*, 2004; Jahoda *et al.*, 2004; Meyer *et al.*, 2004). So far, there is no detailed information about the hair-shaft features of patients with LAH. Here, we report the presence of novel compound heterozygous mutations in the *DSG4* gene of a Japanese patient affected with congenital hypotrichosis, and analyze the hair shafts by scanning electron microscopy (SEM).

RESULTS

SEM observation

We first observed the patient's hair shafts when she was 7 months old. We found no anomalies in the hairs by light microscopy. SEM, however, revealed that the thickness of the hair shaft was slightly, but obviously, inconsistent in some parts, resulting in nodes and internodes (Figure 1a and b). The nodes seemed to be of normal thickness, but the internodes were abnormally thin (Figure 1a and b). The internodes did not show a constant periodicity. The breaks in the hair shafts always occurred at internodes (Figure 1b). At higher magnification, longitudinal ridges or flutes were observed at the internodes (Figure 1c), but not the nodes (Figure 1d). Furthermore, some distal portions of hairs were tapered and kept their cuticular scales (Figure 1e), whereas others showed roughly broken bundles of cortical fibers (Figure 1f). At the age of two, the patient had long terminal hairs, which looked normal even by SEM (data not shown). Nevertheless, short broken hairs were also present, which showed the same anomalies as described above (data not shown). These features resemble those of moniliform hair specific to a congenital fragile hair disorder, monilethrix.

Analysis of hair keratin genes

Given our SEM findings, we thought that our patient represented a sporadic case of monilethrix. We therefore analyzed the patient's genomic DNA for three basic hair keratin genes, *hHb1*, *hHb3*, and *hHb6*, in which mutations have been shown to cause monilethrix. No mutations, however, were found in these genes.

Identification of two different mutations in the *DSG4* gene

Next, we analyzed the *DSG4* gene, which was recently reported as the causative gene for LAH (Kljuic *et al.*, 2003). We identified two different mutations in the *DSG4* gene of the patient's genomic DNA.

The first mutation was a heterozygous T→C transition (574T→C) in exon 6 of the *DSG4* gene, resulting in an S192P substitution at the protein level (Figure 2a). This nucleotide

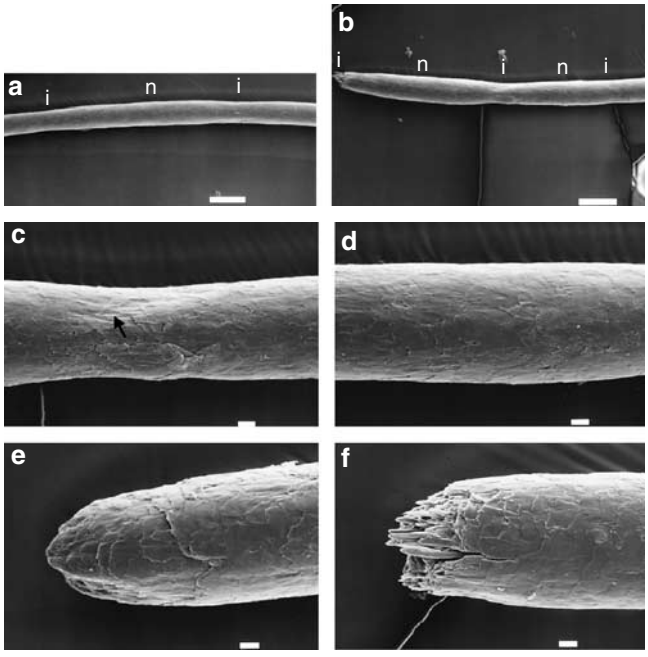

Figure 1. SEM observation. (**a**, **b**) The hair formed nodes (n) and internodes (i), and (**b**) always broke at internodes. (**c**) Longitudinal ridges or flutes (arrow) were observed at internodes, (**d**) but not at nodes. (**e**) The broken ends of hairs tapered, or (**f**) showed bundles of cortical fibers. Bars: (**a**, **b**) 100 μm and (**c**–**f**) 10 μm.

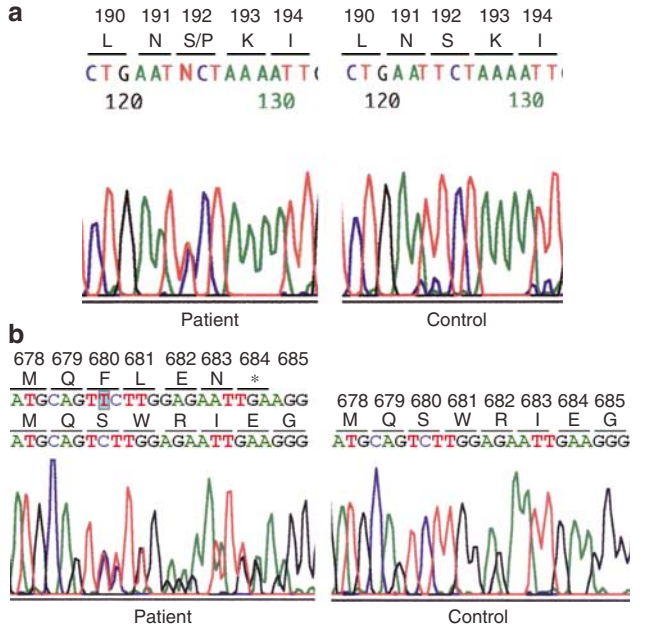

Figure 2. Compound heterozygous mutations in the patient's *DSG4* gene. (**a**) A heterozygous 574T→C (S192P) mutation was identified in exon 6 of the maternal *DSG4* gene. (**b**) A heterozygous 2039insT mutation was detected in exon 13 of the paternal *DSG4* gene.

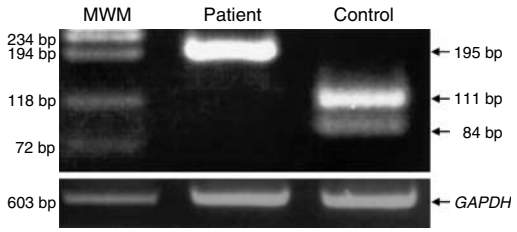

Figure 3. RT-PCR analysis of the *DSG4* transcripts. The RT-PCR product between exons 5 and 6 of the *DSG4* (195 bp) gene was digested with *Eco*RI enzyme. The uncut single fragment (195 bp) was detected in the patient, whereas two distinct fragments (111 and 84 bp) digested by the enzyme were observed in a control individual. *GAPDH* messenger RNA (597 bp) was amplified as a control. MWM, molecular weight markers.

substitution abolished an *Eco*RI restriction enzyme site, which was used to reveal that not only the patient but also her mother had the 574T→C mutation heterogeneously, but neither her father nor 100 control individuals had this transition (data not shown).

The second mutation was a heterozygous insertion of T (2039insT) in exon 13 of the *DSG4* gene (Figure 2b). This frameshift led to the generation of a premature stop codon just downstream of the mutation (Figure 2b). For screening, we performed mismatch-allele-specific PCR. We modified the forward primer to introduce an *Eco*RI restriction enzyme site only in the 2039insT mutant allele. The results showed that both the patient and her father had the 2039insT mutation heterogeneously, whereas her mother and 100 control individuals were homozygotes for the wild-type sequence (data not shown).

Stable *DSG4* transcripts were generated only from the maternal allele

To analyze whether *DSG4* transcripts were expressed stably in the patient's skin, reverse transcriptase (RT)-PCR was performed for the region between exons 5 and 6 of the *DSG4* gene. The PCR products (195 base pairs (bp)) were digested with *Eco*RI, because the 574T→C mutant allele lacked this enzyme site and both the wild-type and 2039insT mutant alleles possessed it. Agarose gel electrophoresis of the digested products showed the presence of two fragments (111 and 84 bp) in a control individual (Figure 3). In the patient, however, only a single fragment (195 bp) was observed (Figure 3), indicating that the *DSG4* transcripts derived from the 574T→C mutant allele were stably expressed in the patient's skin; those from the 2039insT mutant allele could not be detected in this experiment.

DISCUSSION

In this study, careful observation by SEM revealed that the hair shafts of our patient shared common features with moniliform hair, which is a specific anomaly of monilethrix (Ito *et al.*, 1984, 1990). Although previous studies have shown that typical moniliform hairs can be easily observed even by light microscopy owing to the great difference in thickness between nodes and internodes (Ito *et al.*, 1984), in some monilethrix patients with a mutation in the *hHb1* gene, moniliform hairs are detectable only by SEM, like the hair-shaft anomalies of our patient (Winter *et al.*, 1998). Monilethrix is usually transmitted as an autosomal dominant trait, but sporadic cases have also been reported (Korge *et al.*, 1998; Pearce *et al.*, 1999; Winter *et al.*, 1999). Based on our patient's moniliform hairs with perifollicular papules, we originally suspected that she suffered from monilethrix, probably owing to a spontaneous mutation in a hair keratin gene. We did not, however, detect any mutations in the three hair keratin genes, *hHb1*, *hHb3*, and *hHb6*.

We then became aware of a report in which an LAH patient showed clinical features similar to those of our patient (Moss *et al.*, 2004), and we noticed that the distal portion of our patient's hair looked not only like moniliform hair but also like lah (Figure 1b and e), which prompted us to analyze her *DSG4* gene. We identified novel compound heterozygous mutations, 574T→C and 2039insT, in the *DSG4* gene of our patient. Of these, the 2039insT on the paternal allele resulted in a premature stop codon at codon 684 (Figure 2b). In hair samples from the patient, transcripts from the 2039insT allele were not amplified by RT-PCR, whereas those from the 574T→C allele were clearly detected (Figure 3). This result indicates that the messenger RNA from the 2039insT allele was largely degraded by nonsense-mediated messenger RNA decay (Maquat, 1996; Frischmeyer and Dietz, 1999), which led to the loss of heterozygosity at the protein level and only the DSG4 proteins from the 574T→C allele were generated in our patient. Here it should be noted that haploinsufficiency is not the mechanism of the disease because the patient's unaffected father has the 2039insT mutation heterogeneously. We consider that our patient would be in the same condition as being homozygotes for the 574T→C mutant allele, which was transmitted from her mother and caused a nonconservative S192P substitution at the protein level (Figure 2a). Multiple amino-acid sequence alignments showed that the serine at this position is completely conserved among all human DSGs, as well as the Dsg4 of mice and rats (Figure 4). The DSG4 protein has four extracellular cadherin (EC) repeats (ECI–ECIV) in its N-terminus (Figure 5). The S192P mutation was within the ECII of DSG4 (Figure 5). To date, all the mutations reported in the *DSG4* gene of LAH patients and model animals with lah have been in the extracellular domain, which would affect the adhesion between adjacent cells (Figure 5). In particular, both Y196S in the *lah* mouse (Kljuic *et al.*, 2003) and E228V in the *lah* rat (Jahoda *et al.*, 2004) are nonconservative amino-acid changes in the ECII (Figure 5). These data support the idea that S192P is not a polymorphism but a pathogenic mutation. Previous studies have demonstrated that DSG4 is expressed abundantly in all layers of the hair shaft (Kljuic *et al.*, 2003). Furthermore, based on the expression analyses of Dsg4 and hair keratins in the *lah*^{J} mouse and Iffa Credo hairless rat, mutant Dsg4 proteins are thought to cause perturbations in the switch from the proliferation to differentiation of trichocytes, resulting in abnormal and premature keratinization of the hair fiber (Kljuic *et al.*, 2003; Bazzi *et al.*, 2004). We speculate that aberrant DSG4 protein with the S192P mutation caused the

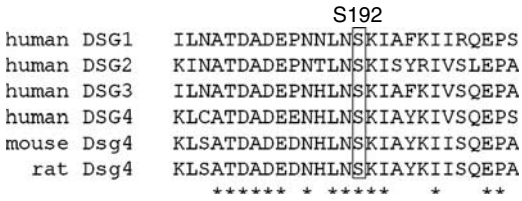

Figure 4. Amino-acid sequence alignment of human DSG1–4 and mouse/rat Dsg4. Amino-acid residues completely conserved among these proteins are indicated by asterisks. The serine residue at position 192 is boxed.

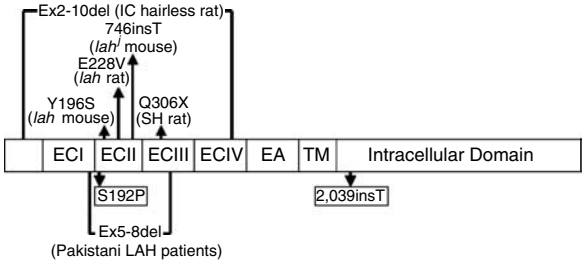

Figure 5. Domain organization of DSG4, and pathogenic mutations identified in human LAH patients and model animals. ECI–ECIV, extracellular cadherin repeat domains; EA, extracellular anchoring domain; TM, transmembrane domain. The S192P and 2039insT mutations in our patient are boxed.

abnormal expression of hair keratins, especially in the hair fiber cortex, resulting in a phenotype similar to that of monilethrix in our patient.

The clinical course of LAH in most Pakistani patients is relatively severe, as it tends to result in the nearly complete absence of scalp hairs (Kljuic *et al.*, 2003; Moss *et al.*, 2004; Rafiq *et al.*, 2004). In contrast, the hair symptoms of our patient are improving with age (Figure 6b). The difference in the disease severity between our patient and the Pakistani patients may be owing to the different mutations. Although the involvement of the ECII is shared, the deletion in DSG4 of the Pakistani patients contains a part of the ECI (Figure 5), which is an important component for cell adhesion in other DSGs (Shapiro *et al.*, 1995; Shimizu *et al.*, 2005). In addition, environmental factors may be involved. Further studies are required to reveal the genotype–phenotype correlation of LAH.

Monilethrix is usually an autosomal dominant disease. In some cases, however, autosomal recessive transmission has been suggested (Hanhart, 1955; Salamon and Schnyder, 1962). Furthermore, there are reports of monilethrix without mutations in the *hHb1*, *hHb3*, and *hHb6* genes (Korge *et al.*, 1999; Horev *et al.*, 2003). Our data indicate that some of these cases might have been caused by mutations in the *DSG4* gene. Based on our findings that our patient with monilethrix-like hair morphology possesses mutations in the *DSG4* gene, we consider that LAH and monilethrix should not be regarded independently. Here we propose the notion that LAH and monilethrix could overlap. Thus, we believe that both diseases are a possible diagnosis for our patient. In future, we hope that various mutations in the *DSG4* gene will be identified in patients with congenital hypotrichosis, which

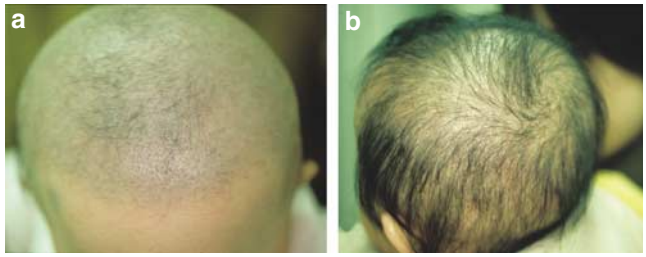

Figure 6. Clinical appearance of the patient. (**a**) At the age of 7 months, the patient's scalp hairs were extremely sparse, and diffuse perifollicular papules were observed. (**b**) By the age of 2 years, the long terminal hairs had gradually increased.

should provide insight into the function of DSG4, the pathogenesis of LAH and monilethrix, the mechanism of hair fiber keratinization, the genotype–phenotype correlation, and other related issues.

MATERIALS AND METHODS

Subject

A 7-month-old Japanese girl was brought to our hospital because of sparse and fragile scalp hairs since birth. Her scalp hairs were short, dry, brittle, and lusterless, and most of them emerged from keratotic follicular papules and broke easily (Figure 6a). Her eyebrows were also sparse, whereas her eyelashes were spared. Her nails and teeth were normal, and she did not have any other complications. She has been treated with an ointment containing 20% urea. As she has grown, her hair symptoms have gradually improved. At the age of 2 years, long terminal hairs had increasingly appeared, but short and thin hairs as well as perifollicular papules remained (Figure 6b). Clinical examination confirmed that neither parent was affected, nor was there any consanguinity. A skin biopsy from the patient could not be performed because her parents declined permission.

Scanning electron microscopy

Hair samples from the patient were examined by SEM (JSM-840) after being coated with gold.

Source of DNA

After informed consent and approval from the ethics commission of Niigata University were obtained, peripheral leukocyte DNA was prepared from the patient and her parents, using standard protocols. Genomic DNA was also extracted from 100 unrelated healthy Japanese individuals as controls. The study was conducted in accordance with the Declaration of Helsinki Principles.

Analysis of the *hHb1, hHb3, hHb6,* and *DSG4* genes

Using the patient's DNA, all exons of the *hHb1*, *hHb3*, *hHb6*, and *DSG4* genes with adjacent sequences of exon–intron borders were amplified by PCR. The primers for the *hHb1*, *hHb3*, and *hHb6* genes were designed as reported previously (van Steensel *et al.*, 2005), and those for the *DSG4* gene are indicated in Table S1. PCR was performed using Advantage™ 2 DNA polymerase (Clontech, Tokyo, Japan). The amplification conditions for each PCR reaction were 96°C for 3 minutes, followed by 30 cycles of 96°C for 30 seconds, the indicated annealing temperature for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 7 minutes. The amplified

PCR fragments were analyzed on 1.5% agarose gels. After gel extraction of the fragments, direct fluorescent chain-termination DNA cycle sequencing was performed (Big Dye DNA sequencing kit, Applied Biosystems, Foster City, CA). The DNA sequences were analyzed on an ABI373 DNA sequencer (Applied Biosystems).

RT-PCR

Total RNA was isolated from three plucked hairs obtained from the patient and a control individual using the Isogen Kit (Nippongene, Tokyo, Japan) according to the manufacturer's recommendations. The RNA was reverse transcribed with random primers and the Super-Script™ II RT (Invitrogen, Carlsbad, CA). The cDNAs were then amplified by nested PCR with Advantage™ 2 DNA polymerase (Clontech). The primer pairs used for the first PCR were 5′-AGGCCTCT TGAGCTTAGAGT-3′ (upstream) and 5′-TACTGTGTTGCTCTCTGTC C-3′ (downstream). Those used for nested PCR were 5′-GCTCCAGTC TTTTCGCAAAGTGTA-3′ (upstream) and 5′-GACTTCTCCAGTGTACC TATTCAG-3′ (downstream). The first PCR products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and a 1 out of 50 volume of each product was used for the nested PCR reaction. The amplification conditions for each PCR reaction were 96°C for 3 minutes, followed by 30 cycles of 96°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 7 minutes. The nested PCR products were digested by *Eco*RI restriction enzyme at 37°C overnight and run on 3.0% agarose gels. *GAPDH* messenger RNA was amplified as a control.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Table S1. Primer pairs for amplification of the *DSG4* gene.

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