Whole-exome sequencing identifies \textit{ADAM10} mutations as a cause of reticulate acropigmentation of Kitamura, a clinical entity distinct from Dowling-Degos disease

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Reticulate acropigmentation of Kitamura (RAK) is a rare genetic disorder of cutaneous pigmentation with an autosomal dominant pattern of inheritance and a high penetration rate. The characteristic skin lesions are reticulate, slightly depressed pigmented macules mainly affecting the dorsa of the hands and feet, which first appear before puberty and subsequently expand to the proximal limb and the trunk. To identify mutations that cause RAK, we performed exome sequencing of four family members in a pedigree with RAK. Fifty-three SNV/Indels were considered as candidate mutations after some condition narrowing. We confirmed the mutation status in each candidate gene of four other members in the same pedigree to find the gene that matched the mutation status and phenotype of each member. A mutation in \textit{ADAM10} encoding a zinc metalloprotease, a disintegrin and metalloprotease domain-containing protein 10 (ADAM10), was identified in the RAK family. ADAM10 is known to be involved in the ectodomain shedding of various substrates in the skin. Sanger sequencing of four additional unrelated RAK patients revealed four additional \textit{ADAM10} mutations. We identified a total of three truncating mutations, a splice site mutation and a missense mutation in \textit{ADAM10}. We searched for mutations in the \textit{KRT5} gene, a causative gene for the similar pigmentation disorder Dowling-Degos disease (DDD), in all the patients and found no \textit{KRT5} mutation. These results reveal that mutations in \textit{ADAM10} are a cause of RAK and that RAK is an independent clinical entity distinct from DDD.

\textbf{INTRODUCTION}

Reticulate acropigmentation of Kitamura (RAK) (also called ‘acropigmentatio reticularis’) [MIM\#179850] is a rare genetic pigmentary disorder that was first reported in Japanese by Kitamura and Akamatsu in 1943 and first described in the European literature by Kitamura \textit{et al.} in 1953 (1). RAK generally shows an autosomal dominant pattern of inheritance with high penetrance, although some patients with sporadic RAK have been reported (2,3). One autosomal recessive case has been reported (4). To date, more than 130 cases have been reported, mainly in Japan (5). Since the first seven non-Japanese cases were reported in 1976 (6), cases have been reported in every ethnicity all over the world (3,7–10).

The typical clinical features are reticulate, slightly depressed, sharply demarcated brown macules without hypopigmentation, affecting the dorsa of the hands and feet (Fig. 1A, B, D) in the first or second decade of life (6). The disease onset is at the age of 20 years or younger in 76% of patients (5). The macules gradually darken and extend to the proximal regions of the extremities (6). The manifestations tend to progress until middle age, after which progression of the eruptions stops. The pigmentary
augmentation is found on the flexor aspects of the wrists, neck, patella and olecranon. Other features include breaks in the epidermal ridges on the palms and fingers, palmoplantar pits (Fig. 2C), occasionally plantar keratoderma (6), and partial alopecia (10).

Histopathologically, the brown macules show pigmentation in the tip of rete ridges with thinning of the epidermis, elongation and thinning of the rete ridges and slight hyperkeratosis without parakeratosis. Only a few inflammatory cell infiltrates and no incontinentia pigmenti are recognized in the dermis (Fig. 3A).

Cases of similar hereditary pigmentation disorders called Dowling-Degos disease (DDD) have been reported, mainly in European populations (11,12). Whether RAK and DDD are distinct clinical entities or variants of an identical disease has long been controversial. RAK and DDD have the similar hyperpigmented macules with a reticulate pattern, affecting the acral areas in the former and the flexures of the extremities in the latter (13). In the literature, several reports have suggested that RAK and DDD are identical disorders with different spectra (14,15). A few overlap cases of RAK and DDD have been reported (16,17). These cases support the idea that RAK and DDD are an identical disorder. Actually, the Online Mendelian Inheritance in Man (OMIM) database includes RAK comprehensively in the entity of DDD [MIM#179850]. However, the age at onset, the distribution, the order of appearance and the expanding patterns of skin manifestation are quite different between RAK and DDD. The association of keratotic lesions is another differentiation point (18).

As for DDD, genome-wide linkage analysis was performed on two German families and loss-of-function mutations in the KRT5 gene were identified as the causative genetic defect in 2006 (19). In contrast, the causative gene/molecule and the pathogenesis of RAK have not been clarified.

In this study, in order to clarify the causative genetic defect of RAK, we performed whole-exome sequencing on a large Japanese pedigree, including a number of typical RAK patients; we found ADAM10 as a causative gene for RAK. Finally, we identified four other ADAM10 mutations in four RAK families and confirmed that RAK is a distinct clinical entity that, unlike DDD, results from ADAM10 mutations.

RESULTS

Whole-exome sequencing in a family with RAK

We performed whole-exome sequencing on three affected individuals showing typical clinical features of RAK [III-8 (Patient 1-3), IV-4 (Patient 1-1) and IV-5 (Patient 1-2)] and one unaffected person (III-7) in a large RAK pedigree, Family 1 (Fig. 1).

The RAK entity is autosomal dominant. Therefore, the patients had heterozygous disease-causing mutations. Exome sequencing covered 99.90% of the target region on the average of four samples, and the average sequence depth on target was 148.31 on the average of four samples.

We identified 698 SNV/Indels that all three affected persons had and that the unaffected person did not have. We were able to subsequently select 53 SNV/Indels on the basis of three criteria: the SNV/Indel was novel, the prevalence of SNV/Indels was less than 0.01 in 89 Japanese samples in the 1000 Genomes database and the sequence was preserved among vertebrates, excluding 645 SNV/Indels. The selected 53 SNV/Indels were considered to be candidate mutations. The results of whole-exome sequencing are summarized in Table 1.

Identification of the causative ADAM10 mutation in the RAK family

Sanger sequencing was performed as below to detect those SNV/Indels in four other family members whose genomic DNA was...
not analyzed by exome sequencing [One affected person (III-3, Patient 1-4) and three unaffected persons (IV-1, IV-2 and IV-3)]. Only one candidate mutation, the heterozygous ADAM10 insertion mutation c.424_425insCAGAG (p.Arg142fsX43), occurred in the patient but not in any of the three unaffected persons. Concerning any other candidates, the presence/absence of SNV/Indels did not match the presence/absence of the phenotype in the family members. Thus, we speculated that the mutation c.424_425insCAGAG (p.Arg142fsX43) in ADAM10 is a causative mutation in the family and that ADAM10 is a possible causative gene for RAK. We also found the base substitution c.415C>T at nine bases upstream of the insertion mutation. Next-generation sequencing confirmed that it is on the same allele.

**ADAM10 mutation search in four other unrelated families with RAK**

ADAM10 encodes a disintegrin and metalloprotease (ADAM) family member (20). ADAM10 is known to be expressed in the human epidermis (Fig. 4A and B) (21), in human melanoma cell lines (22) and in primary keratinocytes (23) (Fig. 4C). ADAM10-mediated E-cadherin release is known to modulate keratinocyte cohesion in eczematous dermatitis (21). Recently, it was reported that hairless mice carrying an Adam10 loss-of-function mutation showed freckle-like macules and mouse Adam10 was suggested to be an inhibitor of melanocyte expansion in adult skin (24).

Therefore, we performed mutation analysis of ADAM10 in five other RAK patients (Table 2) from four unrelated families by Sanger sequencing (Table 3). Including Family 1 for exome sequencing, a total of nine cases from five unrelated families were involved in this study. All the patients who participated in this study showed characteristic lesions on the hands and feet, and we detected the following five mutations: c.415C>T + c.424_425insCAGAG (p.Pro139Ser+p.Arg142fsX43) in Patient 1-1 and in three other affected members of Family 1, c.429T>A (p.Tyr143X) in Patient 2-1, c.1264delA (p.Thr422fsX19) in Patient 3-1, c.1511G>A which occurred at the 3′ end of exon 11 and was able to predict splice site mutation in Patients 4-1 and 4-2, and c.1571G>A (p.Cys524Tyr) in Patient 5-1 (Table 2, Fig. 5A–E). None of the five mutations was detected in the control genomic DNA samples from 102 unrelated healthy Japanese volunteers (204 alleles).

**Consequences of the five ADAM10 mutations detected in the RAK families**

The ADAM10 domain structure and mutation locations are shown in Figure 6. Three of the five mutations are truncating mutations. The three mutations c.415C>T + c.424_425insCAGAG (p.Pro139Ser+p.Arg142fsX43), c.429T>A (p.Tyr143X) and c.1264delA (p.Thr422fsX19) could be predicted to cause haploinsufficiency by nonsense-mediated mRNA decay. Otherwise, if truncated proteins exist in vivo, then the mutations c.415C>T + c.424_425insCAGAG (p.Pro139Ser+p.Arg142fsX43) and c.429T>A (p.Tyr143X) would lose the propeptide domain and the regions downstream. c.1264delA (p.Thr422fsX19) would lose the metalloproteinase domain and the regions downstream. These abolished regions are main functional sites, and serious dysfunction is thought to occur by those mutations.

Concerning the mutation c.1511G>A found in Family 4, in order to confirm aberrant splicing by the mutation, we performed PCR amplification of cDNA from the white blood cells of Patient 4-1 and Patient 4-2. However, no apparent aberrant splicing band was detected by agarose gel electrophoresis. We further performed direct sequencing of the PCR products and sequencing
Table 1. Clinical features and ADAM10 mutations of RAK patients in the present study

<table>
<thead>
<tr>
<th>Family no.</th>
<th>Patient no.</th>
<th>Age</th>
<th>Sex</th>
<th>Race</th>
<th>Onset</th>
<th>Distribution of skin manifestations at onset</th>
<th>Clinical course</th>
<th>Complications</th>
<th>Position</th>
<th>Nucleotide change†</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-1</td>
<td>34</td>
<td>Female</td>
<td>Japanese</td>
<td>5 years of age</td>
<td>Dorsa of the hands and feet</td>
<td>Progression to the limbs, the neck and the forehead</td>
<td>Atopic dermatitis</td>
<td>Exon 4</td>
<td>[c.415C&gt;T + c.424_425insCAGAG]</td>
<td>[p.Pro139Ser + p.Arg142fsX43]</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>33</td>
<td>Male</td>
<td>Japanese</td>
<td>10 years of age</td>
<td>Dorsa of the hands and feet</td>
<td>Progression to the limbs, the neck</td>
<td>N.P.</td>
<td>Exon 4</td>
<td>Same as above</td>
<td>Same as above</td>
</tr>
<tr>
<td></td>
<td>1-3</td>
<td>58</td>
<td>Female</td>
<td>Japanese</td>
<td>10 years of age</td>
<td>Dorsa of the hands and feet</td>
<td>Progression to the neck and external genitals, and the lesions have been thin since the sixth decade</td>
<td>N.P.</td>
<td>Exon 4</td>
<td>Same as above</td>
<td>Same as above</td>
</tr>
<tr>
<td></td>
<td>1-4</td>
<td>78</td>
<td>Female</td>
<td>Japanese</td>
<td>Childhood</td>
<td>Dorsa of the hands and feet</td>
<td>Stable, and the lesions have been thin since the sixth decade</td>
<td>N.P.</td>
<td>Exon 4</td>
<td>Same as above</td>
<td>Same as above</td>
</tr>
<tr>
<td>2</td>
<td>2-1</td>
<td>51</td>
<td>Female</td>
<td>Japanese</td>
<td>Childhood</td>
<td>Dorsa of the hands and feet</td>
<td>Stable</td>
<td>Meningioma</td>
<td>Exon 4</td>
<td>c.429T&gt;A</td>
<td>p.Tyr143X</td>
</tr>
<tr>
<td>3</td>
<td>3-1</td>
<td>20</td>
<td>Female</td>
<td>Japanese</td>
<td>Before 10 years of age</td>
<td>Dorsa of hands and the cheek</td>
<td>Progression to the carpal region and the patella</td>
<td>N.P.</td>
<td>Exon 10</td>
<td>c.1264delA</td>
<td>p.Thr422fsX19</td>
</tr>
<tr>
<td>4</td>
<td>4-1</td>
<td>36</td>
<td>Female</td>
<td>Japanese</td>
<td>Around 10 years of age</td>
<td>Dorsa of the hands and feet, the olecranon and the patella</td>
<td>Progression to the cubital and popliteal fossa, the axilla and the neck</td>
<td>N.P.</td>
<td>Exon 11</td>
<td>c.1511G&gt;A</td>
<td>Splice site mutation</td>
</tr>
<tr>
<td></td>
<td>4-2</td>
<td>66</td>
<td>Male</td>
<td>Japanese</td>
<td>6 years of age</td>
<td>Dorsa of the hands</td>
<td>Progression to the olecranon, the patella, the cubital and popliteal fossa, the axilla, the external genitals and the neck. The eruption became thin</td>
<td>N.P.</td>
<td>Exon 11</td>
<td>Same as above</td>
<td>Same as above</td>
</tr>
<tr>
<td>5</td>
<td>5-1</td>
<td>34</td>
<td>Male</td>
<td>Japanese</td>
<td>10 years of age</td>
<td>Dorsa of the hands and feet, and the neck</td>
<td>Stable</td>
<td>N.P.</td>
<td>Exon 12</td>
<td>c.1571G&gt;A</td>
<td>p.Cys524Tyr</td>
</tr>
</tbody>
</table>
of the clones from the PCR products using the Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA, USA), although no aberrant splice variants were detected. Finally, relative quantification of ADAM10 gene expression was done with the 96-plate LightCycler 480 System II (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's manual. The ADAM10 expression level adjusted by β-tubulin expression in the white blood cells from Patient 4-1 and Patient 4-2 were approximately half that of normal healthy control (Fig. 7), probably due to mRNA decay of aberrantly spliced products. In addition, in silico analysis using the SD-score algorithm (25), which represents a common logarithm of the frequency of a specific 5′ splice site in the human genome on the website, suggested that the mutation c.1511G>A causes aberrant splicing. From these results, we concluded this mutation is pathogenic. The splice site mutation c.1511G>A is expected to cause haploinsufficiency by nonsense-mediated mRNA decay as shown above (Fig. 7).

Of the five mutations that we found in this study, only one mutation, c.1571G>A (p.Cys524Tyr), was an amino acid substitution mutation (Table 2, Fig. 5E). The amino acid substituted by the mutation is in the disintegrin domain of ADAM10 and is a highly conserved residue among diverse species, including mosquito, drosophila, zebrafish, chicken, rat, mouse, cow, wolf, monkey and chimpanzee (Fig. 8A). Furthermore, the cysteine residue at 524 is conserved among 19 human ADAM family members (Fig. 8B). As described above, the missense mutation was not found in 102 healthy Japanese control subjects (204 alleles). Thus, we consider the mutation c.1571G>A (p.Cys524Tyr) to be pathologic.

Exclusion of KRT5 mutations in the RAK families

As we mentioned above, whether RAK and the similar hereditary pigmentation disorder DDD are independent clinical entities or variants of one identical disease has been controversial. Loss-of-function mutations in the KRT5 gene have been identified as the causative genetic defect in DDD (19). Thus, we searched for mutations in the KRT5 gene in all the patients in the present study, and we found no KRT5 mutation in them (data not shown), thereby excluding the possibility of DDD.

DISCUSSION

Summarizing the results of the present causative mutation search in RAK, five ADAM10 mutations—three truncating, one splice site and one missense—were detected in nine RAK patients from five unrelated families. We clearly demonstrated that ADAM10 loss-of-function mutations are genetic defects underlying RAK. We observed no apparent differences in RAK phenotypes, including initial distribution and extending patterns of the skin lesions and age of onset among the patients with the present five different ADAM10 mutations (Table 2). Thus, at least in the present series of patients, there are no obvious genotype/phenotype correlations in RAK.

ADAMs (A disintegrin and metalloproteases) are a new gene family of transmembrane and secreted proteins that belong to the zinc protease superfamily (20,26). The ADAM family consists of more than 40 members (26). ADAM10 is involved in the ectodomain shedding of various substrates in the skin, including adhesion molecules such as L1 cell adhesion molecule (L1-CAM), CD44, E-cadherin, N-cadherin, IL-6R and CD30 (27).

ADAM10 haploinsufficiency was recently reported to cause freckle-like macules in a hairless mouse strain (24). This mouse strain shows freckle-like pigmentation not only on the dorsal aspect of the hands and foot, but also diffuse pigmentation on the trunk in adults. The pathomechanisms of pigmented macules and long-term maintenance of pigmentation patterns in adult mice are not well understood. Interestingly, ADAM10 haploinsufficiency alone, without the homozygous Hairless mutation, cannot cause these macules. Both the ADAM10 mutation and the homozygous Hairless gene mutation are necessary for the phenotype expression in this mouse strain. In contrast, the present human RAK patients show neither hair abnormalities nor alopecia. We confirmed that no member of Family 1 has any mutation in the Hairless gene.

As mentioned above, there is a controversy over whether RAK and DDD are two distinct clinical entities or just on a spectrum of a single disease. Although RAK and DDD show similar reticular pigmentation, there are a number of differentiating points between RAK and DDD both clinically and histopathologically.

The skin manifestations of DDD develop after adolescence and mainly on the flexures and the trunk. In contrast, the skin manifestations of RAK develop before puberty, with the pigmented macules first appearing mainly on dorsal aspect of the hands and feet and subsequently spreading to the proximal regions from the distal sites of the limb and to the trunk. To differentiate between these two diseases, the order of appearance and expanding patterns of skin lesions are very important.

Concerning the nature of skin lesions in RAK and DDD, the eruptions of RAK are uniformly light brown, whereas pigmentation in DDD shows color variation from brown to black (28). Furthermore, abnormal epithelial proliferation involving mainly the pilosebaceous apparatus has been recognized as hidradenitis...
suppurativa, multiple keratoacanthoma, pitted perioral acneiform scars, comedo-like hyperkeratotic dark follicular papules and epidermal and trichilemmal cysts in DDD (11,19,29,30). These abnormalities in the epidermis are probably associated with the keratin 5 gene (KRT5) mutations underlying DDD. In contrast, no RAK patient associated with such abnormal epithelial proliferation has been reported in the literature or observed in our series of patients. Pruritus in the affected areas is also a characteristic feature of DDD (31), but it is not seen in RAK.

Histopathologically, DDD skin lesions caused by KRT5 gene mutation exhibit more intensive epidermal changes than RAK lesions. Thinning of suprapapillary epithelium and elongation of rete ridge with basal hyperpigmentation are recognized in skin lesions of both RAK and DDD patients. However, DDD skin lesions characteristically show a filiform or antler-like pattern of epidermal downgrowth involving the follicular infundibulum as well as the epidermis (31). In contrast, the rete ridge elongation in RAK is less prominent than that in DDD and does not show a filiform or antler-like pattern. In addition, the rete ridge elongation in RAK lesions is seen only in the interfollicular epidermis, but not in the follicular infundibulum.

Changes in melanocytes and melanosomes seem much more prominent in RAK than in DDD. Increased numbers of melanocytes in the epidermis and melanosome complexes and giant melanosomes are seen in melanocytes and keratinocytes in the lesional epidermis of RAK (32–34), although these changes are not observed in DDD lesions (12,35). Perivascular lymphocytic infiltrates or dermal melanosis were observed in the papillary dermis with dermal fibrosis in DDD patients (31). No inflammatory cell infiltrates or dermal melanosis is seen in the dermis of RAK patients.

From these data, we think that RAK and DDD have quite different clinical and histopathological characteristics, and we can speculate that the difference is due to the different causative genes, ADAM10 versus KRT5, for the two diseases.

In conclusion, we clarified that ADAM10 loss-of-function mutations underlie RAK. The present findings clearly indicate that RAK is a genetically distinct clinical entity independent from DDD and put an end to the long-term controversy over whether RAK and DDD are identical disorders.

### MATERIALS AND METHODS

#### Ethics statement

This study was performed according to the principles expressed in *The Declaration of Helsinki*. The study was approved by the
Ethics Review Committee of Nagoya University School of Medicine. Written informed consent was obtained from the participants.

Whole-exome sequencing and data analysis

Genomic DNA was extracted from whole blood using QIAamp DNA blood maxi kit (Qiagen, Valencia, CA, USA), sheared into approximately 150–200 bp fragments and used to make a library for multiplexed paired-end sequencing (Illumina, San Diego, CA, USA). The constructed library was hybridized to biotinylated cRNA oligonucleotide baits from the SureSelect Human All Exon V4 Kit (Agilent Technologies, Santa Clara, CA, USA) for exome capture. Targeted sequences were purified by magnetic beads, amplified and sequenced on an Illumina HiSeq2000 platform in paired-end 101 bp configuration.

Adapter sequences were removed by Cutadapt (v1.2.1). After quality control, reads were mapped to the reference human genome (hg19) using Burrows-Wheeler Aligner (BWA) (ver.0.6.2). Mapping results were corrected using Picard (ver.1.73) for removing duplicates and a genome analysis tool kit (GATK, ver.1.5-32) for local alignment and quality score recalibration. SNV and Indel calls were performed with multi-sample calling using GATK and were filtered to coordinates with Variant Quality Score Recalibration (VQSR) passed and variant call quality score \( \geq 30 \). Annotations of SNVs and Indels were based on dbSNP135, CCDS (NCBI, November 2011), RefSeq (UCSC Genome Browser, November 2011), Encode (UCSC Genome Browser, ver. 7) and the 1000 Genomes database (October 2011).

Sanger sequencing

Standard PCR amplification procedures were employed with high-fidelity polymerase, Platinum Taq DNA Polymerase (Invitrogen) and 37.5 ng genomic DNA as the template in 50 \( \mu \)l volume. The thermal conditions were the following: 94°C for 5 min, followed by 30 cycles at 94°C for 15 s, 65°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were sequenced directly to identify a mutation by Sanger sequencing with Applied Biosystems 3730 DNA Analyzer.

Cell culture and protein extraction

Normal human epidermal melanocytes (NHEM), normal human epidermal keratinocytes (NHEK) and normal human dermal
fibroblasts (NHDF) (Kurabo, Osaka, Japan) were maintained at 37°C with 10% CO2 in Medium 154 with HKGS kit, Medium 254 with HMGS kit and Medium 106 with LSGS kit, following the manufacturer’s respective protocols. Cells were harvested and lysed using the RIPA Lysis Buffer System (Santa Cruz Biotechnology, Dallas, TX, USA) supplemented with Protease Inhibitor Cocktail for use with mammalian cell (Nacalai Tesque, Kyoto, Japan). Supernatant of samples was collected by centrifugation at 15 000 g for 5 min at 4°C.

**Western blots**

Concentrations of the proteins extracted from NHEM, NHEK and NHDF were determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer’s instructions. For each sample, 20 μg of sample was resuspended in Lane Maker Reducing Sample Buffer (Thermo Scientific, Rockford, IL, USA), boiled for 5 min at 96°C and resolved by SDS–PAGE on 4–20% Mini-Protean TGX Gel (Bio-Rad). Separated proteins were transferred onto nitrocellulose membrane by Mini Trans-Blot Cell (Bio-Rad) that was blocked for 45 min with StartingBlock Blocking Buffer (Thermo Scientific). The membrane was incubated overnight at 4°C with anti-ADAM10 antibody (Abcam, Miami, FL, USA) used at 1:500 and anti-β-actin antibody (Sigma, Saint Luis, MO, USA) used at 1:2000. Blots were developed using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) and were exposed to Amersham Hyperfilm ECL (GE Healthcare, Pittsburgh, PA, USA).

**RNA isolation from the patients’ white blood cells, reverse transcription and quantitative real-time PCR (QPCR)**

Total RNA from the patients’ white blood cells was extracted with RiboPure Kit according to the manufacturer’s instructions. One microgram of RNA was reverse-transcribed using the Taqman Reverse Transcript Kit (Applied Biosystems). Real-time PCR was performed with LightCycler 480 System II 96 plate (Roche Diagnostics) in a final volume of 20 μl containing 2 × LightCycler 480 Probes Master (Roche Diagnostics), 40 ng cDNA as the template and 20x TaqMan Gene Expression Assay for ADAM10 and β-actin gene (Applied Biosystems), respectively. The thermal conditions were the following: 95°C for 10 min, followed by 45 cycles of 95°C for 10 s and 60°C for 30 s with a final cooling at 50°C for 30 s. Endpoint fluorescence was measured for each sample well. QPCR data analysis was performed using Lightcycler 480 software, release 1.5.0 (Roche Diagnostics). The amount of the ADAM10 gene relative to β-actin gene was determined using a second derivative maximum method, and the results were expressed as a ratio of ADAM10 : β-actin. A 45-cycle threshold was set, beyond which the gene was considered undetectable.

**Immunohistochemistry**

Immunohistochemistry was performed as described previously (36) using anti-MART-1 (Melan-A) antibody (1:100, Dako, Glostrup, Denmark) and anti-ADAM10 antibody (1:100, Abcam) as the primary antibody.
WEB RESOURCES

The URLs for data presented herein are as follows.
The Genome Analysis Toolkit (GATK): http://www.broadinstitute.org/gatk/
UCSC Genome Browser hg19: http://hgdownload.cse.ucsc.edu/goldenpath/hg19/chromosomes/.
UCSC Genome Browser hg19: http://hgdownload.cse.ucsc.edu/goldenpath/hg19/chromosomes/.

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Conflict of Interest statement. None declared.

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