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Generalized Pure Cutaneous Rosai-Dorfman Disease: a Link Between Inflammation and Cancer Not Associated with Mitochondrial DNA and *SLC29A3* Gene Mutation?

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Abstract: Recently, we described a case of generalized pure cutaneous Rosai-Dorfman disease in a 43-year-old Asian man in *JAMA*. The lesions distributed on nearly all of the skin of the whole body, except for mucous sites. Molecular, immunophenotypic, and sequencing analyses seem to define it as a histiocytic-mesenchymal transition and intermediate proliferative histiocytosis not associated with mtDNA large deletion and pathogenic mutation, as well as the *SLC29A3* gene mutation. [*Discovery Medicine* 16(89):193-200, November 2013]

Introduction

In 1969, a distinct clinicopathologic entity named sinus histiocytosis with massive lymphadenopathy (SHML, also known as Rosai-Dorfman disease, RDD), mostly in cervical localization, was first described by Rosai and Dorfman (1969), and is now considered a non-malignant, self-limited, and inflammatory disorder (Paulli *et al.*, 1995) within the spectrum of histiocytic disorders of unknown origin (Sita *et al.*, 1996). Recently, we described a 43-year-old Asian man presenting with 6 months' development of multiple non-pruritic, nonpainful papules and nodules diagnosed as generalized pure cutaneous Rosai-Dorfman disease (CRDD) (Man and Zheng, 2013). Mutations and large deletions (>50 bp) in mitochondrial DNA (mtDNA) have been identified in many kinds of cancerous tissues (Brandon *et al.*, 2006). As RDD presents some overlapping characteristics with tumor, whether mtDNA mutations and large deletions could be involved in this disease is an interesting question. Herein we investigated whether the CRDD of this case is a malignant clonal disorder or an inflammatory histiocytic/phagocytic response triggered by aberrant cytokine.

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Materials and Methods

Samples

The study protocol was approved by the Institutional Ethics Committee of Second Affiliated Hospital, Zhejiang University School of Medicine. Written informed consent was signed and obtained from all participants. This research was carried out according to the principles of the Declaration of Helsinki. Skin and blood tissues were obtained from the proband with Rosai-Dorfman disease. Blood samples were also collected from 29 healthy controls for comparing with the patient. Genomic DNA was isolated from blood and/or skin samples.

Immunohistochemistry

The immunohistochemistry was performed as previously described (Man *et al.*, 2013). Briefly, the 4 μm paraffin tissue sections were deparaffinized and rehydrated, then were boiled for antigen unmasking in 10 mM sodium citrate buffer at a sub-boiling temperature. After that, the slides were incubated with 3% hydrogen per-

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oxide for 10 minutes (min) to block endogenous peroxidase activity and were further blocked by 10% goat serum. The sections were incubated overnight at 4°C with primary antibodies against CD68, CD1a, S100, vimentin, androgen receptor (AR), epidermal growth factor receptor (EGFR), HER-2, p53, p16, PTEN, fibronectin, β-catenin, N-Cadherin, and Bcl-2 (Santa Cruz Biotech, Santa Cruz, CA, USA), respectively. After washing three times, the sections were incubated with secondary antibody for 2 hours. DAB kit (Vector Laboratories, Burlingame, CA, USA) was used for staining.

PCR detection for large mtDNA deletions

In order to determine whether there are large mtDNA deletions in the skin tissue, four primer pairs which span frequent reported deletions (http://www.mitomap.org/MITOMAP) were used to amplify the targeted mtDNA regions of the patient and controls (Table 1). Considering that short PCR fragments will be preferentially amplified in the presence of large deletions, routine PCR conditions were adopted even though the primers span very long regions. PCR reactions were conducted in a total volume of 20 μL containing 1x PCR buffer (1.5 mmol/L $MgCl_2$), 0.5 units of TaKaRaLa Taq (TaKaRa Biotech, Dalian, China), 175 μmol/L of each dNTP, 0.15 μmol/L of each primer, and 50 ng of DNA template. After a pre-denaturation at 94°C for 5 min, 30 cycles consisting of 94°C for 30 seconds (s), 55°C for 30 s, and 72°C for 3 min were performed, followed by an extension at 72°C for 7 min. PCR products were evaluated in a 1.5% agarose gel.

Complete mtDNA genome sequencing

The entire mtDNA genome of the patient's skin tissue

Table 1. Primers for Detecting Large mtDNA Deletions.

Primer Pair	Sequence (5'-3')	Coverage
L29	GGTCTATCACCCTATTAACCACGCA	6910 bp
H6899	CTGCAGCAGATCATTTC	
L8215	ACAGTT TCATGCCCATCGTC	7871 bp
H16048	GTCAATACTTGGGTGGTACC	
L6337	CCTGGAGCCTCCGTAGACCT	9749 bp
H16048	GTCAATACTTGGGTGGTACC	
L5781	AGCCCCGGCAGGTTTGAAGC	8444 bp
H14186	TGGTTGAACATTGTTTGTTGG	

Table 2. Primers for Amplifying and Sequencing the Coding Region of the *SLC29A3* Gene.

Primer Pair	Sequence (5'-3')	Reference
5'UTR-F	GTGTTTGCAAACGGGGAA	This study
exon 1-R	GGAGGGAGGTCGCTGAG	Morgan *et al*., 2010
exon 2-F	CCCAGCCTTGGTTTCTACTC	Morgan *et al*., 2010
exon 2-R	CTGAAATCTCCATCTTCCCC	Morgan *et al*., 2010
exon 3-F	GGCCCTGTCTCTGCTCG	Morgan *et al*., 2010
exon 3-R	GGGAGTAGAGGAGGCAGGAG	Morgan *et al*., 2010
exon 4-F	CAGCCCACACAGGAGCC	Morgan *et al*., 2010
exon 4-R	CTCATCTCTGGCTCCCTCC	Morgan *et al*., 2010
exon 5-F	CGCAGCCACTCCTCCTC	Morgan *et al*., 2010
exon 5-R	AAAAGAAGCATGGCTATAACTGG	Morgan *et al*., 2010
exon 6-F	GCTGTGCTGACTCAGATCCC	Morgan *et al*., 2010
3'UTR-R	GAATATCTGCCTTGCACGGA	This study

Note: Primers were named by the exon number followed by "F" or "R" to designate the forward/upstream or reverse/downstream, respectively.

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was amplified and sequenced by using our previously described methods (Bi *et al.*, 2010; Wang *et al.*, 2008). Sequences were handled and analyzed with the DNASTAR software package (DNASTAR Inc., Madison, WI, USA). Sequence variations were scored relative to the revised Cambridge Reference Sequence (rCRS) (Andrews *et al.*, 1999) and were presented in a tree profile, together with one reported mtDNA AP009462.1 (Kazuno *et al.*, 2005) that closely matched the skin tissue sample, following the same phylogenetic approach described in our recent studies (Bi *et al.*, 2011; Ji *et al.*, 2008; Yao *et al.*, 2006).

Quantification of the mtDNA copy number

The standard curve method of real-time PCR described previously (Bi *et al*., 2010), which quantitates unknown samples by using a set of relative standards (a serial dilution of PCR product), was employed to determine the mtDNA copy number in the blood and skin tissues of the patient and blood tissues of 29 healthy controls without any large mtDNA deletion. Primer pair L394 (5′-CACCAGCCTAACCAGATTTC-3′) / H475 (5′-GGGTTGTATTGATGAGATTAGT-3′) was used to measure mtDNA copy; primer pair HBB502f (5′-CTATGGGACGCTTGATGT-3′) / HBB614r (5′-GCAATCATTCGTCTGTTT-3′) was used to amplify β-globin gene for normalization (Bi *et al.*, 2010). The relative mtDNA copy number was determined as the ratio of mtDNA to nuclear DNA. The amplification assays were performed with SYBR Premix Ex Taq II kit (TaKaRa Biotech) according to the manufacturer's manual on the MyiQ2 system (BioRad Laboratories, Hercules, CA, USA).

Mutation screening for the SLC29A3 *gene*

As Rosai-Dorfman disease has been reported to be associated with mutations in the *SLC29A3* gene (Morgan *et al.*, 2010), the entire coding region and part of 5′UTR and 3′UTR regions of the *SLC29A3* gene in the patient's blood and skin tissues were amplified and sequenced by using the method described by Morgan *et al*. (2010), with some modifications. All PCR primers were listed in Table 2. PCR amplification was performed in a total volume of 30 μL mixture containing 1x TransTaqHiFi buffer I (TransGen Biotech, Beijing, China) [for exon 1, we used high GC buffer I (TaKaRa Biotech)], 1.5 units of HiFiTaq, 175 μmol/L of each dNTP, 0.15 μmol/L of each primer, and 50 ng of DNA template. A total of 35 thermal cycles after an initial denaturation cycle at 94°C for 5 min was performed under the following conditions: first 10 cycles consisting of 94°C for 30 s, 62°C for 30 s (for exon 1, 58°C for 30 s), and 72°C for 30 s; the remaining 25 cycles consisting of 94°C for 30 s, 56°C for 30 s (for exon 1, 50°C for 30 s), and 72°C for 30 s, then ended with a final extension at 72°C for 7 min. PCR products were sequenced by using the sense and/or antisense PCR primers (Table 2). Sequencing results were handled with the DNASTAR software package. Sequence muta-

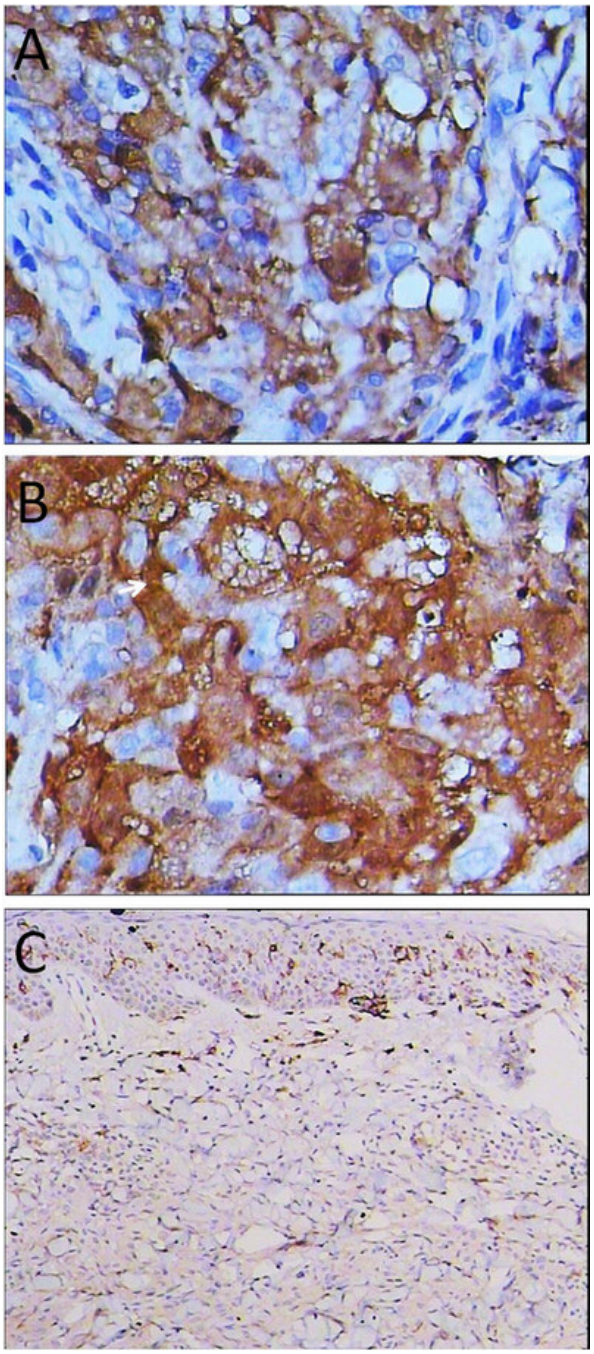

Figure 1. Immunostaining of CD68, S100, and CD1a. Positive staining for (A) CD68 and (B) S100, and negative for (C) CD1a of lesional sections (×400).

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tions/variations were scored relative to the reference sequence NG_017066.1 from GenBank and the genomic sequence retrieved from the UCSC Genome Browser Home (http://genome.ucsc.edu).

Results

Immunohistochemical results

The immunohistochemical labeling demonstrated that the histocytoid cells were strongly positive for CD68 (Figure 1A) and S100 (Figure 1B), but were nonreactive for CD1a (Figure 1C). A panel of mesenchymal and tumor markers were used to identify the origin of the pathogenic cells of the disease. The immunohistochemical studies produced positive intracellular staining of Vimentin, N-Cadherin, fibronectin, β-catenin, HER-2, Slug andp53, p16, and PTEN in histiocytes and multinucleated cells or Touton giant cells, but negative staining for AR, EGFR, and Bcl-2 (Figure 2).

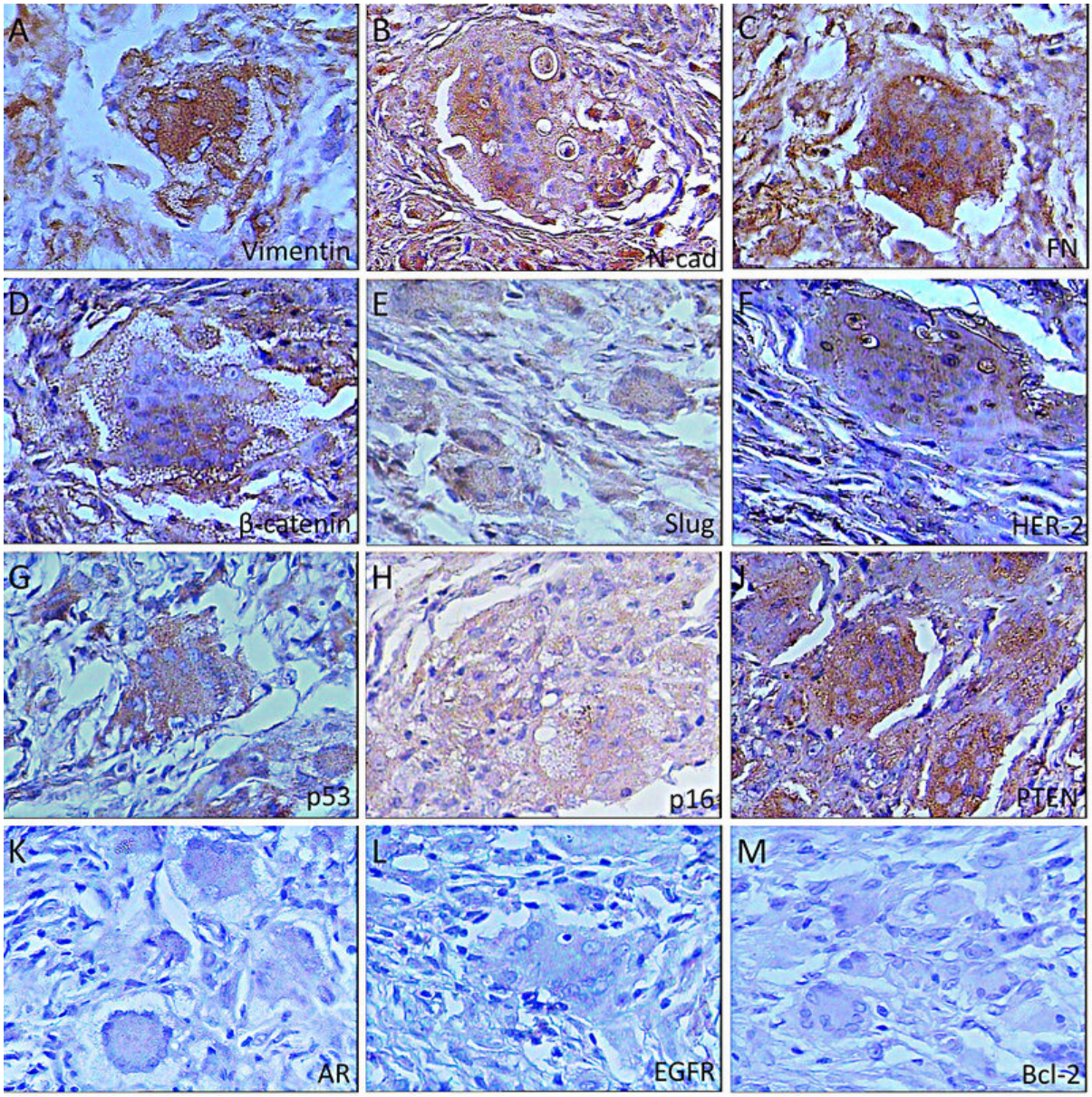

Figure 2. Immunohistochemical results. Intense immunohistochemical positivity of (A) Vimentin, (B) N-cadherin, (C) Fibronectin, (D) β-catenin, (E) Slug, (F) HER-2, (G) p53, (H) p16, and (J) PTEN, and negativity of (K) AR, (L) EGFR, and (M) Bcl-2. Original magnification, ×400.

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No large deletion was observed in the patient's skin tissue

The amplification results for large mtDNA deletions by using four different primer pairs which targeted those frequently reported deletions were presented in Figure 3. Although there were several small PCR fragments for control samples and the patient's blood, these bands were confirmed to be non-specific PCR products by sequencing (data not shown). There was no expected fragment from the patient's skin and blood tissues indicating the presence of mtDNA with deletion of >50 bp.

Analysis for the entire mtDNA genome showed no deletion or potentially pathogenic mutation in the patient's skin tissue

Sequence variations in the entire mtDNA sequence of the patient's skin tissue, together with a closely matched sequence AP009462.1 from a published source (Kazuno *et al.*, 2005), were presented in an mtDNA phylogenetic tree (Figure 4). The patient's mtDNA belonged to haplogroup D4 according to the current global mtDNA tree (van Oven and Kayser, 2009). No mtDNA deletion or potentially pathogenic mutation was observed in the patient's skin tissue.

The mtDNA copy numbers in the patient's blood and skin tissues were within the range of normal controls

To further detect whether there was any abnormality of mtDNA content in the patient, we quantified the relative mtDNA copy number of the patient's blood and skin tissues, and compared it to that of healthy controls. As shown in Figure 5, the mtDNA copy number of the patient was within the range of healthy controls, with the skin tissue having a slightly higher mtDNA copy number.

No pathogenic mutation in the SLC29A3 *gene was observed in the patient's blood and skin tissue*

In order to investigate whether the patient with Rosai-Dorfman disease had any mutations in the *SLC29A3* gene, we sequenced the entire coding region and part of 5′UTR and 3′UTR regions of the *SLC29A3* gene by using genomic DNA isolated from the patient's blood and skin tissues. Compared with the reference sequence NG_017066.1, no mutation/variation was observed in the sequenced regions.

Discussion

Although skin involvement is a frequent extranodal site of RDD, pure cutaneous Rosai-Dorfman disease without node involvement is very rare and appears to be more common in the Asian population (Brenn *et al.*, 2002; Lu *et al.*, 2004).

Our case presented with numerous papules and nodules that nearly cover the whole body, except for mucous sites. There were massive multinucleated giant cells expressing S-100 protein and CD68, the most useful immunohistologic markers for RDD histiocytes (Foucar *et al.*, 1990; Paulli *et al.*, 1992), but negative for CD1a. The immunophenotype of the cells demonstrates features of monocyte- and/or macrophage-differentiated cells, not Langerhans-differentiated cells.

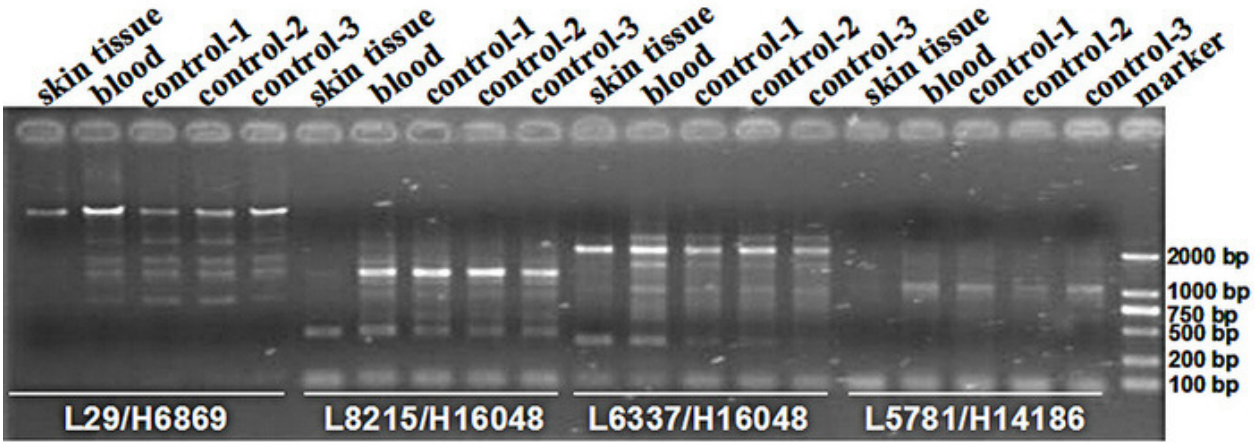

Figure 3. Screening for mtDNA large deletions. Four different long mtDNA regions, which cover frequently reported mtDNA large deletions, were amplified by using the blood DNA samples from three normal controls and DNA samples of the patient's blood and skin tissues. The small bands in control samples and the patient's blood were confirmed to be non-specific PCR products by sequencing. The detailed information regarding primer pairs L29/H6869, L8215/H16048, L6337/H16048, and L5781/H14186 was listed in Table 1. PCR products were separated by 1.5% agarose gel.

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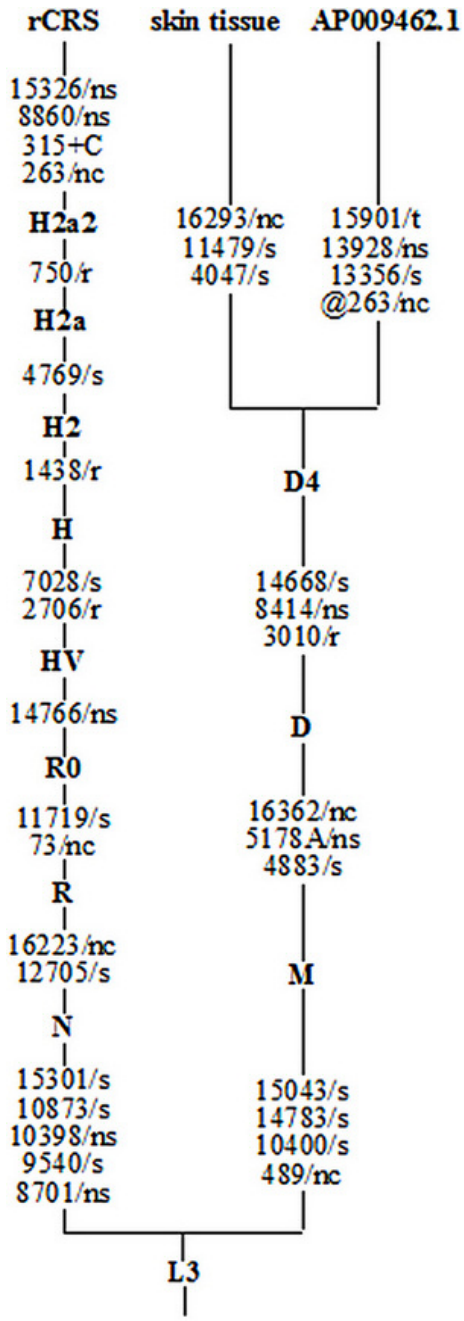

Figure 4. Classification tree of complete mtDNA sequence of the patient's skin tissue and one near-matched mtDNA sequence (AP009462.1) from published resources (Kazuno *et al.*, 2005), plus the revised Cambridge reference sequence (rCRS) (Andrews *et al.*, 1999). Synonymous and nonsynonymous mutations are denoted by "s" and "ns," respectively. "r" indicates the mutation/variant in the rRNA genes; "t" indicates the mutation/variant in the tRNA genes; "nc" indicates the mutation/variant in the non-coding region. Insertion is labeled by "+." Suffix A means transversion. Back mutation is underlined and marked "@."

To date, most investigators have speculated that the histiocytes of RDD probably derive from circulating monocytes and are a sort of distinctive activated histiocytes with coexpression of partial immunophenotypic characteristics of both monocyte/macrophage system and dendritic/Langerhans cell family (Chu and LeBoit, 1992). In this case, immunohistochemical studies with Vimentin, N-Cadherin, fibronectin, β-catenin, and Slug, which are molecular markers for epithelial-mesenchymal transition (EMT) (Kalluri and Weinberg, 2009; Zeisberg and Neilson, 2009), produced strong positive results in the dermal histiocytes and multinucleated giant cells. These features led us to conclude that histiocytes in generalized CRDD may undergo a transition from histiocyte to mesenchymal cells. The histiocytes in this case may be explained as a novel entity with features of both macrophage and mesenchymal traits, which may be named as histiocytic-mesenchymal transition (HMT).

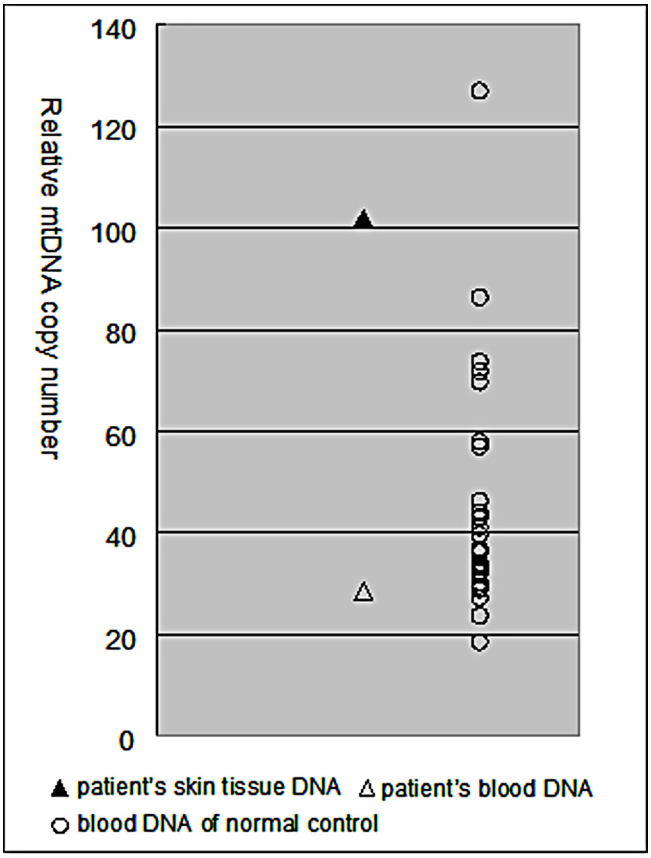

Figure 5. The relative mtDNA copy number of the patient with Rosai-Dorfman disease and 29 healthy controls. Both DNA samples from the blood and skin tissues of the patient were investigated, and were compared to the blood DNA samples of 29 normal controls.

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EMT is a key developmental program that is often activated during cancer invasion and metastasis (Mani *et al.*, 2008). The significance of HMT in generalized CRDD is not determined. However, we hypothesized that HMT might be associated with invasion-metastasis. To confirm this hypothesis, the expression of p53, p16, PTEN EGFR, HER-2, AR, and Bcl-2 was examined by immunohistochemistry. The positive staining of tumor suppressor proteins p53, p16, and PTEN, and negative staining for tumor proteins EGFR, AR, and Bcl-2 confirmed that this generalized CRDD case was a benign proliferative disorder, which is in agreement with previously published pathological results (McClain *et al.*, 2004; Sita *et al.*, 1996). However, the strong expression of the HER-2 oncoprotein indicates a tumor specificity of the histiocytes, which is in accordance with the HMT in this case. Given the tumor suppressor actions of p53, p16, and PTEN, it seems unlikely that all of these would be functional in a truly malignant cell. Therefore, the generalized CRDD may represent a unique intermediate phenotype between benign and malignant disease, or a transition/link between inflammation and cancer.

In addition, we genotyped the whole mtDNA sequences and screened mutations of the *SLC29A3* gene recently reported in RDD (Morgan *et al.*, 2010). However, we found no mtDNA large deletions and/or pathogenic mutations in the skin tissue of the patient. The mtDNA content in both blood and skin tissues of the patient was also within the range of normal controls. Therefore, it is unlikely that the skin disease of the patient was caused by abnormality of mtDNA. Meanwhile, sequencing of the entire coding region of the *SLC29A3* gene also yielded no evidence that the Rosai-Dorman disease in this patient was caused by mutations in the *SLC29A3* gene.

Acknowledgment

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Disclosure

The authors report no conflicts of interest.

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