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Genome-wide association study identifies *CDH13* as a susceptibility gene for rhododendrol-induced leukoderma

Running title: GWAS for chemical vitiligo

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Abstract

Racemic RS-4-(4-hydroxyphenyl)-2-butanol (rhododendrol; trade name: Rhododenol [RD]), which is used in topical skin-lightening cosmetics, was unexpectedly reported in Japan to induce leukoderma or vitiligo called RD-induced leukoderma (RIL) after repeated application. To our knowledge, no studies have investigated chemical-induced vitiligo pathogenesis on a genome-wide scale. Here, we conducted a genome-wide association study (GWAS) for 147 cases and 112 controls. *CDH13*, encoding a GPI-anchored protein called T-cadherin (T-cad), was identified as the strongest RIL susceptibility gene. RD sensitivity was remarkably increased by T-cad knockdown in cultured normal human melanocytes. Furthermore, we confirmed tyrosinase upregulation and downregulation of the anti-apoptotic molecules (BCL-2 and BCL-XL), suggesting that T-cad is associated with RD via tyrosinase or apoptotic pathway regulation. Finally, MBEH sensitivity also tended to increase with T-cad knockdown, suggesting that the T-cad could be a candidate susceptibility gene for RIL and

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other chemical-induced vitiligo forms. This is the first GWAS for chemical-induced vitiligo, and it could be a useful model for studying the disease's genetic aspects.

Significance

Up to 20,000 users of rhododendrol (RD, a naturally occurring phenolic compound) containing cosmetics have developed leukoderma or vitiligo due to a disorder called RD-induced leukoderma (RIL). Unexpectedly, RIL is a useful model for investigating chemical-induced vitiligo pathogenesis. Here, we conducted the first genome-wide association study of this disease. *CDH13*, encoding T-cadherin, was identified the strongest susceptibility gene. Regulation of tyrosinase and anti-apoptotic molecules were suggested as the key T-cadherin functions in RIL pathogenesis.

Keywords:

GWAS, phenol, rhododendrol, T-cadherin, vitiligo

INTRODUCTION

Chemical leukoderma or chemical-induced vitiligo is characterized by acquired skin depigmentation induced by repeated exposure to particular chemical agents. Occasionally, these chemicals appear to induce melanocyte autoimmunity (Boissy & Manga, 2004; Ghosh & Mukhopadhyay, 2009; Harris, 2017). To date, various chemical compounds have been reported to be responsible for leukoderma or vitiligo, including phenols, catechols, sulfhydryls, mercurials, arsenics, cinnamic aldehyde, azelaic acid, and other chemicals (Boissy & Manga, 2004; Harris, 2017). Among them, phenolic compounds like monobenzyl ether of hydroquinone (MBEH) and 4-tertbutylpyridine (4-TBP), alkaline or phenyl-phenol are representative chemicals associated with the disorder (James, Mayes, & Stevenson, 1977; Stevenson, 1981).

In 2013, racemic RS-4-(4-hydroxyphenyl)-2-butanol (rhododendrol; trade name: Rhododenol [RD]), a naturally occurring phenolic compound and a melanin synthesis inhibitor used

in topical skin-lightening cosmetics, was unexpectedly reported from Japan to induce leukoderma called RD-induced leukoderma (RIL) after repeated applications (Nishigori et al., 2015; Tokura et al., 2015). Approximately 2% of the estimated 800,000 RD-containing cosmetic users have developed leukoderma (Sasaki et al., 2014). The majority of the patients with RIL experienced depigmentation only at the cosmetic-exposed site. However, 14% of them developed vitiligo in non-exposed areas, indicating autoimmunity induction against melanocytes (A. Ito et al., 2015). The mechanisms underlying this disorder have been investigated using biochemical, cytological, immunological, and histopathological studies (Abe et al., 2016; Fujiyama et al., 2015; S. Ito, Gerwat, et al., 2014; S. Ito, Ojika, Yamashita, & Wakamatsu, 2014; Kasamatsu et al., 2014; Nishioka et al., 2015; Okamura et al., 2015; Sasaki et al., 2014; Tanemura et al., 2015; Yang et al., 2015). RIL also happens to be a useful model for investigating chemical-induced leukoderma/vitiligo pathogenesis. While a number of susceptibility genes for non-segmental vitiligo have been identified using genome wide association studies (GWAS), the representatives of which are associated with the innate and adaptive immune systems, apoptotic and cytotoxic pathways, and melanocyte functions (Jin et al., 2016; Shen et al., 2016; Spritz, 2012), no study has focused on genetic aspect of chemical induced vitiligo. Considering the incidence rate of the RIL (98% of the users have not developed leukoderma) as well as other chemical induced vitiligo, it is reasonable to speculate there are unignorable genetic factors associated with this disease entity. In this study, we performed a GWAS in Japanese patients with the RIL and identified *CDH13* as the strongest susceptibility gene, the impact of which on the RIL was supported by functional analysis.

MATERIALS AND METHODS

Study participants

A total of 155 RIL patients who had been exposed to RD-containing cosmetics for two weeks to five years participated in this study. As a control, we chose 117 individuals who used the cosmetics but did not develop leukoderma. Written informed consent and saliva samples were obtained from all participants under protocols approved by the Ethics Committee of Yamagata University Faculty of Medicine. The study protocol was designed in accordance with the ethical principles that have their origin in the Declaration of Helsinki.

SNP genotyping and data cleaning

A total of 272 subjects (155 patients with the RIL and 117 controls) were genotyped with the

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HumanOmni2.5-8 v1.1 BeadChip (Omni2.5) in accordance with the manufacturer's instructions. We first conducted identity by descent (IBD) estimation and principal component analysis (PCA) to avoid including familial relatives and to check outliers and potential population substructure, respectively. As a result, eight patients and five controls were excluded. The following thresholds for SNP quality control were used for data cleaning: SNP call rate $\geq 95\%$, bi-allelic, minor allele frequency $\geq 5\%$, and HWE P-value ≥ 0.001 in 259 subjects. A total of 1,057,565 SNPs on autosomal chromosomes passed our filters.

Statistical analysis

Each SNP's association with RIL was assessed using logistic regression analysis adjusted for age, total amount of RD-containing cosmetics used, and values of first to fourth principal components. In the analysis, SNP genotype, an independent variable, was encoded using the number of minor alleles for each subject, i.e. 0, 1, or 2 (i.e., additive model). SNPs on chromosome X were not evaluated. Statistical analyses were performed using the SNP & Variation Suite 7 software (Golden Helix, Bozeman, MT, USA). The Manhattan plot was constructed using the qqman package in the R programming language. The regional association plot was created using web-interface LocusZoom (<http://csg.sph.umich.edu/locuszoom>).

Chemicals and antibodies

RD was supplied by Kanebo Cosmetics Inc. (Tokyo, Japan). MBEH and 4-TBP were purchased from MilliporeSigma (Burlington, MA, USA). Primary antibodies for western blotting analysis were rabbit polyclonal *anti*-T-cadherin antibody (1:1000; ABT121, MilliporeSigma), rabbit monoclonal *anti*-BCL-2 antibody (1:1000; 4223S, Cell Signaling Technology, Danvers, MA, USA), rabbit monoclonal *anti*-BCL-XL antibody (1:1000, 2764S, Cell Signaling Technology), mouse monoclonal *anti*-tyrosinase antibody (1:5000; ab738, abcam, Cambridge, MA, USA), rabbit polyclonal *anti*-GADPH antibody (1:2000; sc-25778, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and

mouse monoclonal *anti*- β -actin antibody (1:30000; A1978, MilliporeSigma).

Cell culture and siRNA transfection

Normal human epidermal melanocytes (NHEMs) derived from neonatal foreskins were cultured at 37°C in a 5% CO₂ atmosphere as previously described (Kondo et al., 2016). Briefly, Cells were maintained in melanocyte growth medium composed of MCDB153HAA medium (Research Institute for the Functional Peptides Co., Ltd, Yamagata, Japan), Human Melanocyte Growth Supplement (HMGS; Thermo Fisher Scientific, Fremont, CA, USA), and 0.1 mM CaCl₂ (FUJIFILM Wako Pure Chemical Co., Osaka, Japan). For the siRNA transfection experiments, NHEM was seeded in 6-, 24-, or 96-well plates at a density of 2.0×10^5 cells/ml (2.5, 0.5, or 0.1 ml/well, respectively). A day after the plating, the cells were transiently transfected with 25 nM of Stealth RNAi™ siRNA, including three different sequences targeting human *CDH13* mRNA (HSS101704, HSS101705, and HSS101706; Thermo Fisher Scientific) or negative control siRNA (Thermo Fisher Scientific) using Lipofectamine RNAiMAX (Thermo Fisher Scientific). Cells treated with *CDH13* siRNAs or negative control siRNA were exposed to RD 144 h after transfection at the concentration of 0 to 10mM for 24 h. siRNA-treated NHEM were also exposed to MBEH and 4-TBP in the same manner at the concentration of 0 to 0.75mM, and 0 to 1mM, respectively.

Western blotting analysis

Cells were rinsed twice with HEPES buffer, and lysed with lysis buffer containing 2% sodium dodecyl sulfate and 100 mM Tris-HCl (pH 6.8). After centrifugation of the homogenate at $13,500 \times g$ for 10 min at 4°C, the supernatants were used for immunoblot analysis. Twenty micrograms of protein were separated by SDS-PAGE, and transferred onto a polyvinylidene difluoride membrane. Membranes were incubated with each primary antibody in 5% skim milk in TBST buffer. The membranes were then washed with TBST buffer, followed by incubation with appropriate secondary IgG antibodies for 1h at room temperature. Immune complexes were detected using Supersignal West

Dura Chemiluminescent Substrate (Thermo Fisher Scientific). Signal intensities of immunoreacted bands were quantitated using ImageJ® software (National Institute of Health, Bethesda, MD, USA), followed by being analyzed with student's t test.

Cell viability assay

To evaluate cytotoxicity, NHEM treated with each siRNA was cultured in the presence of various RD concentrations in 24- or 96-well plates for 24 h. Then, Cell Count Reagent SF (07553-44; Nacalaitesque, Kyoto, Japan) was used for the assay according to the manufacturer's instructions.

RESULTS

GWAS identifies *CDH13* as the strongest RIL susceptibility gene

The results of the GWAS are presented in Figure 1 (Manhattan plots) and Table 1 (a list of nearby genes/loci of SNPs which revealed P values of less than 1.0×10^{-5}). The SNP showing the lowest P-value was rs1364301 ($P = 1.1 \times 10^{-7}$, OR = 4.59), located in *CDH13* (encoding T-cadherin: T-cad, also called H-cadherin or cadherin-13; arrow in Figure 1a and b). We identified some other interesting susceptibility gene candidates. *PPP2R5C* encodes one of the protein phosphatase 2A components associated with cell proliferation, apoptosis, and Wnt signaling (Liu et al., 2015; Thompson & Williams, 2018). The *RAB4* gene product is involved in E-cadherin recycling, which is associated with vitiligo pathogenesis (Kachhap et al., 2007; Wagner et al., 2015) (Table 1).

Knockdown of T-cad in NHEM alters sensitivity to RD

To investigate the contribution of *CDH13* to RIL development, we knocked down T-cad using specific *CDH13* siRNAs transfected into NHEM followed by performing cell proliferation assay after loading various RD concentrations. The proper knockdown efficacy of T-cad was confirmed 144 h after transfecting *CDH13*-specific siRNAs at the protein level ($P = 0.0119$; Figure 2a). We treated each NHEM with RD 144 h after the siRNA transfection. As a result, NHEM treated with *CDH13* siRNAs showed significantly higher sensitivity to RD than the NHEM treated with negative control siRNA (Figure 2b). The increased RD sensitivity after T-cad knockdown was also confirmed in another NHEM cell line from a different donor (Supplementary Figure 1).

T-cad in malignant melanoma reportedly functions as a pro-apoptotic tumor suppressor that antagonizes AKT/CREB/AP-1/FoxO3a signaling accompanied by the regulation of the anti-apoptotic molecules like BCL-2 and BCL-XL (Bosserhoff et al., 2014). On the other hand, it has been shown that RD serves as a good substrate for mushroom tyrosinase (TYR), and RD cytotoxicity in NHEM is remarkably associated with TYR activity (Kasamatsu et al., 2014; Sasaki et al., 2014). We investigated BCL-2, BCL-XL, and TYR expression in the NHEM after T-cad knockdown. We found that BCL-2 expression was significantly decreased by T-cad knockdown ($P = 0.0049$), and BCL-XL expression also revealed same tendency ($P = 0.0292$; Figure 2c). On the other hand, it was surprising that TYR expression was increased after T-cad knockdown ($P = 0.0451$; Figure 2c). These results suggested that T-cad has an important role in protecting melanocytes from RD exposure by regulating apoptotic pathways and TYR activity.

Finally, we assessed the impact of the T-cad knockdown in NHEM on the sensitivity toward the other representative vitiligo-inducing chemical agents MBEH and 4-TBP. We did not observe apparent changes in 4-TBP sensitivity (Figure 3a and Supplementary Figure 2a). However, MBEH sensitivity tended to increase after T-cad knockdown (Figure 3b and Supplementary Figure 2b), suggesting that the T-cad can be a candidate susceptibility gene for RIL and other forms of chemical-induced vitiligo.

DISCUSSION

Here, we identified *CDH13* as the strongest RIL susceptibility gene. T-cad is an atypical member of the cadherin superfamily because it lacks the transmembrane and cytoplasmic domains and is anchored to cell membranes through glycosylphosphatidylinositol (GPI) anchors. It is generally accepted that T-cad is a signaling receptor involved in transduction of extracellular cues inside the cell rather than an adhesion molecule (Rubina et al., 2015). Like in melanoma cell lines (Bosserhoff et al., 2014), we demonstrated the downregulation of anti-apoptotic molecules, such as BCL-2 and BCL-XL, after T-cad knockdown in NHEM. Melanocyte cell deaths via endoplasmic reticulum stress accumulation or apoptotic pathway activation have been suggested to cause RIL (Abe et al., 2016; Sasaki et al., 2014; Yang et al., 2015). From these results, we speculated T-cad protects

RD-induced melanocyte-killing by regulating the apoptotic pathway. On the other hand, TYR upregulation by knocking down T-cad was very informative. We previously reported that mRNA expression of phosphatidylinositol glycan K gene (*PIGK*), which plays an essential role in protein-GPI anchoring, was significantly inversely correlated with TYR activities in NHEM (Okamura et al., 2017). GPI acts as a membrane anchor and a post-translational modifier for more than 150 proteins (GPI-anchored proteins; GPI-APs). We speculated that regulation of protein-GPI anchoring via *PIGK* might affect the expression or function of some GPI-APs associated with TYR activity. However, there has been little study investigating GPI-AP roles in melanocytes. We incidentally identified a GPI-AP associated with TYR expression, supporting our previous speculation.

Although we could not show clear evidence that T-cad contributes to the sensitivity of other chemical agents in NHEM, the increased tendency of MBEH sensitivity by T-cad knockdown was notable. Both MBEH and 4-TBP are representative 4-substituted phenols, which have direct toxicity in melanocytes. However, the mechanism of melanin inhibition and melanocyte death induced by these two chemical agents have been shown to be different. A recent study showed that 4-TBP has much weaker activity in TYR inhibition than MBEH. While MBEH is converted into a reactive quinone that binds to thiol groups like glutathione (GSH), 4-TBP does not form quinone. Thus, 4-TBP does not facilitate GSH depletion in melanocytes (Kammeyer et al., 2019). On the other hand, RD is a good substrate for TYR, and it is converted into RD-quinone which bound to GSH (S. Ito, Ojika, et al., 2014). It has been also shown that GSH maintenance has a crucial role in RD-induced melanocyte death *in vitro* (Kondo et al., 2016). From these studies, it can be predicted that the mechanism of RIL resembles leukoderma induced by MBEH rather than by 4-TBP, which is reflected in our results.

In this study, we did not identify any SNPs overlapping with susceptible SNPs detected by previous GWAS studies for vitiligo (Jin et al., 2016). However, one of the SNPs in this study was located in internal region of *HLA-B* and *HLA-C* (rs9380237; Table 1). In addition, Gene Ontology

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Enrichment Analysis (<http://geneontology.org/page/go-enrichment-analysis>) for 15 genes/loci listed in Table 1 revealed that gene products associated with antigen presentation or antigen processing via MHC class I molecules were enriched (data not shown), suggesting that adaptive autoimmunity against melanocytes might have some roles in the pathogenesis of RIL as well as autoimmune vitiligo.

There are several limitations for this study. First of all, the sample size is much smaller than other usual GWAS. It is because of the small number of entire patients with RIL; furthermore, it was very hard to recruit the controls who used RD-containing cosmetics but not developed leukoderma. In addition, replication analysis could not be conducted since new patients with RIL were not expected. Insufficient power due to the small sample size and the lack of replication analysis may result in high risk of false-positive/negative susceptibility gene discovery. Instead, we compensated for the limitation by the functional studies. The limitation in our functional study was that we did not evaluate the direct impact of the SNP (rs1364301) on the regulation of *CDH13*. Also, we could not investigate the differential expressions of *CDH13* or downstream *TYR*, *BCL-2*, and *BCL-XL* between patients and controls. Further analysis will be expected in the future. Nevertheless, our first GWAS for chemical-induced leukoderma/vitiligo could be a good model for investigating the genetic aspect of this disease entity.

DATA AVAILABILITY

The datasets presented in the current study are available from the corresponding authors upon request.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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FIGURE LEGENDS

Figure 1. GWAS results. (a) The Manhattan plot of the study. rs1364301 ($P = 1.1 \times 10^{-7}$; arrow) located in *CDH13* was the SNP with the lowest P -value in the study. P value of each SNP is shown on the $-\log_{10}$ scale. (b) Regional association plot for the *CDH13* locus. The color of each plotted SNP shows its r^2 with the rs1364301 (purple diamond). Estimated recombination rates, which were based on the JPT samples from the HapMap project, were plotted in light blue.

Figure 2. Sensitivity toward RD (exposed 24 h) in normal human epidermal melanocyte (NHEM) treated with *CDH13* siRNAs. (a) The proper knockdown efficacy of *CDH13* at the protein level was observed 144 h after transfection with *CDH13*-specific siRNAs ($P = 0.0119$). (b) NHEM treated with *CDH13* siRNAs showed significantly higher sensitivity to RD than NHEM treated with negative control siRNA (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; P-values were calculated using student's t test). (c) T-cad knockdown decreased BCL-2 and BCL-XL expression ($P = 0.0049$ and 0.0292 , respectively), but it increased TYR expression ($P = 0.0451$). All experiments have been conducted in triplicate at least three times.

Figure 3. Sensitivities against 4-TBP and MBEH (exposed 24 h each) in NHEM treated with *CDH13* siRNAs. (a) T-cad knockdown NHEM did not show apparent 4-TBP sensitivity changes. (b) However, increasing MBEH sensitivity tendency was observed after the knockdown. All experiments have been conducted in triplicate at least three times (see Supplementary Figure 2).

Figure S1. (a, b) Sensitivity to RD were also increased in another NHEM cell line from a different donor after T-cad knockdown (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; P-values were calculated using student's t test).

Figure S2. Results of two other independent experiments investigating the sensitivities against 4-TBP and MBEH in NHEM treated with *CDH13* siRNAs. (a) In consistent with Figure3a, we could not identify clear 4-TBP sensitivity changes by T-cad knockdown. (b) On the other hand, increased tendencies of MBEH sensitivity by T-cad knockdown were also confirmed.

Figure 1

a

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Figure 1

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Figure 2

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Figure 2

c

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Figure 3

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