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ORIGINAL ARTICLE

Optimal patch application time in the evaluation of skin irritation

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ABSTRACT

We investigated the optimum application for evaluating skin irritation response by using samples of irritants commonly used as additives in cosmetics and other common household products. We studied 47 volunteers (16 men and 31 women). We selected three types of surfactant, one moisturizer, one anti-infective agent and one oil solution. Using Finn chambers on Scanpor tape, we performed the patch test. A total of 0.015 mL of each sample was applied to the Finn chamber. For liquids, circular filter paper was soaked in 0.015 mL of the sample. Samples were placed on the upper back of participants, and closed for 4, 24 or 48 h. A patch application time of 24 h is sufficient to detect primary skin irritation from irritants in cosmetics and other common household products. In addition, we found that skin irritation reactions were strongest at 24 h after patch removal and that the reaction tended to be weaker at 48 h after patch removal. Patch testing to evaluate irritants should be performed by means of a 24-h patch test with a follow-up reading at 24 h after patch removal. An application time of 24 h places less of a burden on patients than a 48-h patch test.

Key words: 24-h patch test, Japanese standard for evaluating of skin irritation, new standard for evaluating of skin irritation, optimum application time, skin irritation response, sugai scoring.

INTRODUCTION

Human patch tests are often used to identify the substances (allergens) responsible for contact dermatitis.[1,2] Samples of various substances that may cause contact dermatitis are applied to a healthy area of the skin under a patch. They remain on for a period of time, usually 48 h, after which they are removed. Readings are taken at 1–2 h after and at 24 h after removal of the samples. An additional reading may be taken at 1 week to 10 days after application of the patch, not depending on the strength of the reactions at 72 h exhibited by the patient.

In standard clinical practice, chemicals and cosmetics are applied to the skin on a patch to determine responses to irritation, as well as allergic reactions. Medical institutions use an identical testing procedure to evaluate response to irritation and allergic response; there is no special patch test used only for evaluating skin irritation. There have been reports outside Japan of sodium lauryl sulfate (SLS) being used to evaluate skin irritation.[3,4] SLS has been used in closed patch tests to evaluate skin irritation, with application times of either 23–24 h[5–8] or 4 h.[9–14] In particular, closed patch tests with an application time of 4 h have been used to detect comparatively strong skin irritations, such as those involving skin irritants and substances corrosive to the skin in relation to regulations involving chemical substances.[9,10]

In the present study, we investigated the optimum application time for evaluating skin irritation response by using samples of irritants commonly used as additives in cosmetics and other common household products. The substances are known to cause an irritation response in people, although they are not strong irritants.

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METHODS

Participants

We studied 47 volunteers (16 men and 31 women), who all gave informed consent before participating in the study. The age of participants ranged 23–61 years, with a mean of 37.1 years.

The inclusion criteria were: healthy back skin (i.e. no dermatological conditions, inflammation, or desquamation caused by dryness), no oral or topical steroid use and no known allergies to patch test samples.

Patch test materials

Table 1 shows the samples used in the study. We selected three types of surfactant, one moisturizer, one anti-infective agent and one oil solution. It has been shown that although these substances are not strong irritants, they are suitable for observing irritation reactions in humans and animals. The concentration of samples was adjusted to a level that would likely cause irritation. Distilled water was used in the study as a solvent control, and white petrolatum and saline solution were used as negative controls. The identity of patch test samples was not revealed until the study was completed.

Patch test

Finn chambers (Epitest Ltd Oy., Tuusula, Finland) (five chambers per strip) on Scanpor tape (Alpharma AS, Norway) were used for the patch test. The size of Finn chamber was 11 mm and aluminum cup was 8 mm. A total of 0.015 mL of each sample was applied to the Finn chamber. For liquids, circular filter paper was soaked in 0.015 mL of the sample. Samples were placed on the upper back of participants, and closed for 4, 24 or 48 h (Fig. 1). Surgical tape was used as necessary to secure patch test units and to prevent peeling of adjacent skin.

Table 1. Patch test materials

No	Sample	Concentration (%)	CAS No
1	Sodium lauryl sulfate (SLS)	0.5	151-21-3
2		0.3	
3		0.1	
4	Propylene glycol (PG)	50.0	57-55-6
5		30.0	
6	Sodium laurate (SL)	2.0	629-25-4
7		1.0	
8	Isopropyl myristate (IPM)	100.0	110-27-0
9	Benzalkonium chloride (BC)	0.1	8001-54-5
10		0.05	
11	PolyOxietylene (IO) oleyl ether	10.0	8009-03-8(NF)
12	(Oleth-10)	5.0	
13	White petrolatum (WP) solvent	as is	8009-03-8(NF)
14	Distilled water (DW) solvent	as is	
15	Saline negative control	as is	

CAS: Chemical Abstracts Service.

Assessment of participants

Participants were assessed by dermatologists at the Department of Dermatology, Toho University Omori Medical Center; Department of Dermatology, Hyogo College of Medicine; and Department of Dermatology, Fujita Health University School of Medicine. Follow-up readings were performed 2, 24 and 48 h after patch removal. Skin response was evaluated according to two sets of criteria: the new criteria for skin irritation of the Skin Irritation Research Group of the Japanese Society for Contact Dermatitis[15,16] and the standard Japanese criteria (Table 2).[2]

Approval was obtained from the Ethics Committee of the Japanese Society for Dermatoallergology and Contact Dermatitis before commencing the study.

Statistical analysis

The χ^2-test was used in the comparison of the incidence of the new criteria for skin irritation and the standard Japanese criteria after 4, 24 and 48 h. $P < 0.05$ was considered to be significant.

RESULTS

Figure 2 and Table 3 show the results of the patch test at different application times, according to the new criteria for skin

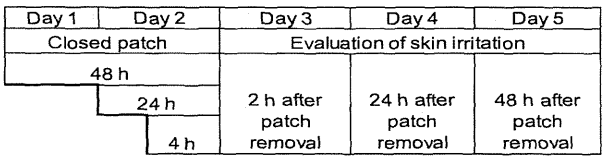

Figure 1. The test schedule.

Table 2. The new criteria for skin irritation of the skin irritation research group of the Japanese society for contact dermatitis

New standard for evaluating skin irritation*	Score	Japanese Standard†	Sugai Scoring‡
No reaction	0	—	0
Perceptible erythema	1	− or±	0 or 0.5
Weak-to-moderate erythema	2	±	0.5
Distinct erythema	3	+	1
Erythema with popular or edematous reaction	4	++	2
Erythema with vesicular reaction	5	+++	3
Corrosive reaction (bullae formationnecrosis)	6	++++	4

*Kawai[15].
†Kawamura[2].
‡Sugai[20].

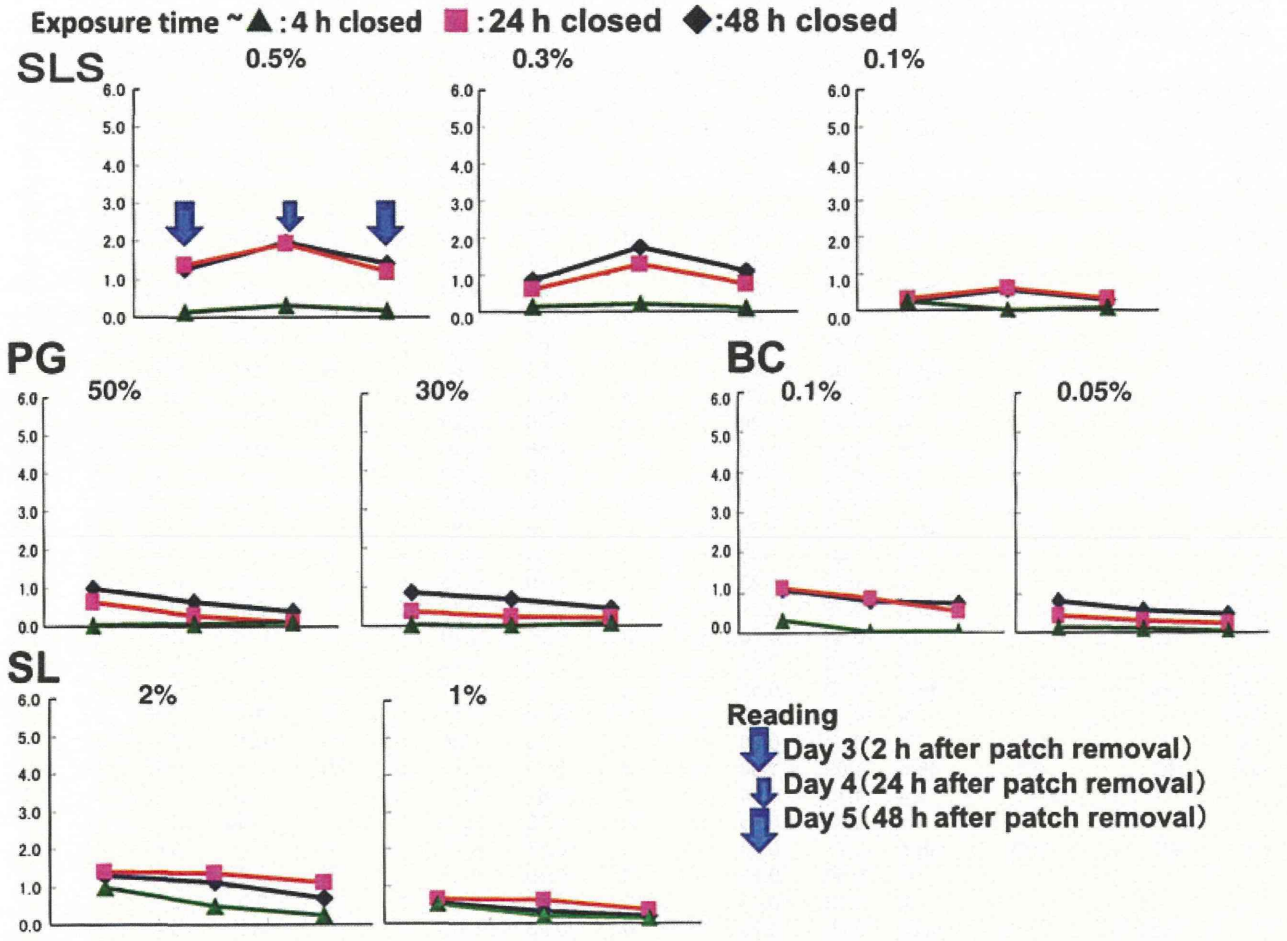

Figure 2. New standard. BC, benzalkonium chloride; PG, Propylene glycol; SL, sodium laurate; SLS, sodium lauryl sulfate.

irritation Similarly, Figure 3 and Table 4 show the results of the patch test at different application times, according to the standard Japanese criteria. The incidences of skin reactions after 4 h of application were the lowest of all three follow-up time points. This was true using both sets of evaluation criteria. For application times of 24 and 48 h, the incidence of skin reactions was usually higher at a follow-up time of 24 h after patch removal. After 4 h of application, follow-up time was not associated with the incidence of skin reactions.

At 24 h after patch removal, there was no significant association between the incidence of skin reaction and application time (4, 24 or 48 h) for white petrolatum or saline solution (negative controls) or for distilled water (a solvent control according to the new criteria for evaluating skin irritation); however, there was a significant association between the incidence of skin reaction and application time for all samples except the isopropyl myristate (IPM) sample, for which the incidence of reaction was low at all three application time points. There was no significant difference in the incidence of skin reaction after applications of 24 and 48 h, except for the 30% propylene glycol (PG) aqueous solution. Regarding the incidence of skin reaction as determined by the standard Japanese criteria, most results were identical to those obtained using the new criteria for all three application times, although there was a significant difference in the incidence of skin reactions after 24 and 48 h of application for the 30% PG aqueous solution, the SL (1%) aqueous solution, and oleth-10 (10%; χ^2-test, $P < 0.05$).

According to the new criteria, the mean irritation score for SLS was similarly high after 24 and 48 h of application, at each concentration of SLS. However, the scores after only 4 h of application were markedly lower. For all concentrations of PG, the mean irritation score increased with increasing application time. For all concentrations of SLS there was little difference in mean irritation score as a result of application time at 2 h after patch removal. For an application time of 4 h, the mean irritation score tended to decrease with time. However, after 24 and 48 h of application, the decrease was comparatively slight. Mean irritation score was low at all application times for IPM . For benzalkonium chloride, the mean irritation scores were similarly high after 24 and 48 h of application, as was the case with SLS. However, the mean irritation score after 4 h of application was markedly lower. Trends for the mean Sugai irritation

Table 3. The summary of results in new standard

				Evaluation 2 h after				Evaluation 24 h after				Evaluation 48 h after			
Chem	Sample	Conc. (%)	hour	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min
C01	SLS	0.5	48 h	1.26	1.09	3	0	1.98	1.22	4	0	1.43	1.08	3	0
C01			24	1.36	1.22	4	0	1.94	1.24	4	0	1.19	1.14	4	0
C01			4	0.15	0.36	1	0	0.32	0.73	3	0	0.19	0.54	2	0
C02	SLS	0.3	48 h	0.85	0.91	3	0	1.74	1.33	4	0	1.11	1.13	3	0
C02			24	0.60	0.88	3	0	1.28	1.23	4	0	0.74	1.01	3	0
C02			4	0.13	0.40	2	0	0.21	0.55	2	0	0.11	0.31	1	0
C03	SLS	0.1	48 h	0.23	0.52	2	0	0.55	0.83	3	0	0.30	0.59	3	0
C03			24	0.32	0.66	3	0	0.60	0.88	3	0	0.32	0.59	2	0
C03			4	0.23	0.52	2	0	0.00	0.00	0	0	0.06	0.32	2	0
C04	PG	50	48 h	1.00	0.91	3	0	0.64	0.82	3	0	0.40	0.65	2	0
C04			24	0.66	1.03	4	0	0.28	0.50	2	0	0.11	0.31	1	0
C04			4	0.04	0.20	1	0	0.06	0.25	1	0	0.11	0.31	1	0
C05	PG	30	48 h	0.85	0.96	4	0	0.68	0.78	2	0	0.45	0.75	3	0
C05			24	0.38	0.68	2	0	0.26	0.53	2	0	0.21	0.46	2	0
C05			4	0.02	0.15	1	0	0.00	0.00	0	0	0.09	0.28	1	0
C06	SL	2	48 h	1.30	1.08	4	0	1.13	1.03	3	0	0.70	0.91	3	0
C06			24	1.40	1.08	3	0	1.38	1.07	3	0	1.11	1.03	3	0
C06			4	1.00	1.23	4	0	0.49	0.75	2	0	0.23	0.63	3	0
C07	SL	1	48 h	0.55	0.77	3	0	0.34	0.56	2	0	0.19	0.50	2	0
C07			24	0.66	0.76	3	0	0.64	0.70	2	0	0.38	0.61	2	0
C07			4	0.53	0.93	3	0	0.19	0.45	2	0	0.15	0.47	2	0
C08	IPM	100	48 h	0.23	0.52	2	0	0.11	0.31	1	0	0.21	0.46	2	0
C08			24	0.04	0.20	1	0	0.04	0.20	1	0	0.15	0.55	3	0
C08			4	0.04	0.20	1	0	0.00	0.00	0	0	0.13	0.45	2	0
C09	BC	0.1	48 h	1.09	1.08	3	0	0.79	0.88	3	0	0.72	0.99	3	0
C09			24	1.11	1.34	4	0	0.87	1.10	4	0	0.55	0.83	3	0
C09			4	0.32	0.59	2	0	0.02	0.15	1	0	0.04	0.20	1	0
C10	BC	0.05	48 h	0.77	0.94	3	0	0.55	0.77	3	0	0.45	0.69	3	0
C10			24	0.40	0.65	2	0	0.30	0.69	4	0	0.21	0.59	3	0
C10			4	0.13	0.45	2	0	0.09	0.28	1	0	0.04	0.20	1	0
C11	Oleth-10	10	48 h	0.49	0.78	2	0	0.43	0.62	2	0	0.17	0.48	2	0
C11			24	0.38	0.80	3	0	0.17	0.52	2	0	0.26	0.49	2	0
C11			4	0.13	0.40	2	0	0.04	0.20	1	0	0.09	0.35	2	0
C12	Oleth-10	5	48 h	0.81	1.06	4	0	0.47	0.75	3	0	0.34	0.60	2	0
C12			24	0.49	0.83	3	0	0.30	0.59	2	0	0.30	0.51	2	0
C12			4	0.09	0.35	2	0	0.06	0.25	1	0	0.04	0.20	1	0
C13	WP		48 h	0.17	0.43	2	0	0.04	0.20	1	0	0.11	0.37	2	0
C13			24	0.04	0.20	1	0	0.00	0.00	0	0	0.04	0.20	1	0
C13			4	0.04	0.20	1	0	0.02	0.15	1	0	0.09	0.35	2	0
C14	DW		48 h	0.45	0.65	2	0	0.32	0.63	3	0	0.21	0.46	2	0
C14			24	0.21	0.51	2	0	0.15	0.42	2	0	0.36	0.67	3	0
C14			4	0.06	0.32	2	0	0.13	0.45	2	0	0.04	0.29	2	0
C15	Saline		48 h	0.26	0.49	2	0	0.06	0.25	1	0	0.06	0.25	1	0
C15			24	0.11	0.31	1	0	0.06	0.25	1	0	0.11	0.31	1	0
C15			4	0.09	0.35	2	0	0.02	0.15	1	0	0.06	0.32	2	0

score,[17] which is based on the Japanese criteria, were much the same as those calculated using the new criteria.

DISCUSSION

Using both new and standard criteria, we were able to detect irritation after 24 and 48 h of patch application. However, it was difficult to detect irritation after 4 h of application because the incidence of irritation was low. There was a significant difference in the incidence of irritation after 24 and 48 h of application for PG (30%) solution, SL (1%) solution and oleth-10 (10%), substances for which irritation levels were low. However, no such difference was found for substances that cause greater irritation, that is, there was no marked difference in the incidence of irritation by application time. Plasters attached for 48 h caused a rash in some volunteers (Fig. 4), which indicates that leaving patches in place for this length of time places a greater burden on people undergoing the test.

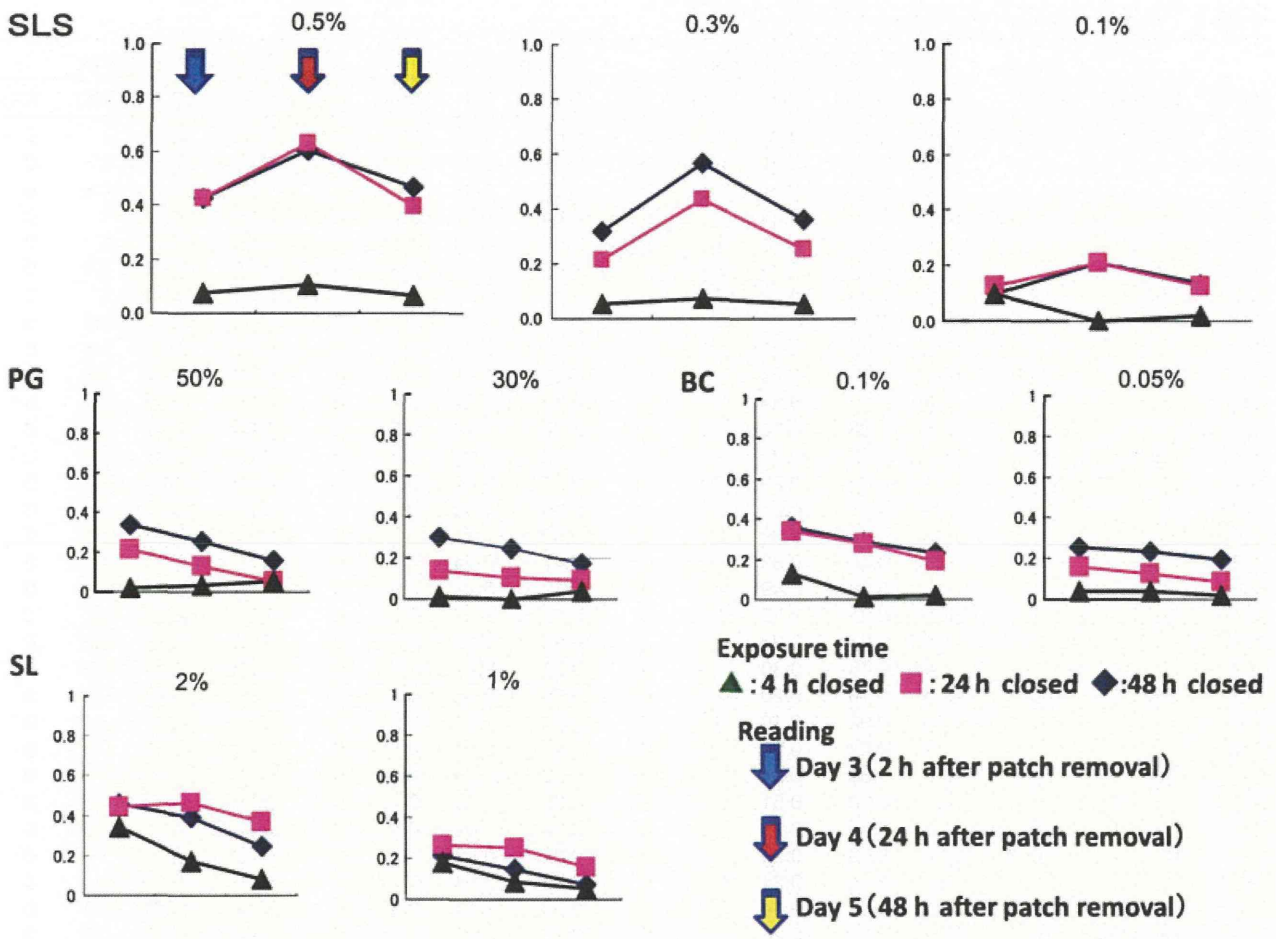

Figure 3. Japanese standard. BC, benzalkonium chloride; PG, Propylene glycol; SL, sodium laurate; SLS, sodium lauryl sulfate.

For patch application times of 24 and 48 h, the incidence of skin irritation reaction at 24 h after was relatively higher or almost the same, as compared to 48 h after patch removal. This indicates that skin irritation reactions can be examined at 24 h after patch removal.

The samples used in this study are all commonly available. In particular, SLS is used as a positive control in research on the evaluation of primary skin irritation.[17–19] These reports describe the concentration of SLS, as well as the application time, observation period and part of the body to which SLS is applied. Many reports suggest a concentration of between 0.1% and 2% for use in studies; SLS is usually applied to the skin using a patch for 4, 23, 24 or 48 h.

Testing with an application time of 4 h was developed to predict acute skin irritation to chemical substances and to rank substances according to the resulting irritation. Because the main objective of this method is to detect skin irritation and the toxicity of chemical substances, samples are usually used at high concentrations and short exposure periods. The concentration of SLS – used as a positive control in such tests – is 20%, and a positive rate of at least 30% is required for the test to be valid.[10] In clinical practice, the 48-h patch test is usually used, although 23- or 24-h patch tests can also be used for evaluation of skin irritation.

In many reports, an observation period of 1, 24 or 48 h after patch removal is selected, irrespective of the application time, with patches usually attached to the upper back, forearmor upper arm. Löffler *et al.* performed a 48-h patch test with various SLS concentrations and reported that with a 0.5% SLS aqueous solution there was a strong correlation between reactions on the upper back and on the forearm, and that the reactions on the forearm were more noticeable than those on the upper back. They also reported that a 0.5% SLS aqueous solution, applied to the forearm for 48 h, was useful for evaluation of skin irritation and that mild irritation values were observed at 24 h after patch removal.[5]

In the present study using SLS 0.5% aqueous solution, 42.6% (20/47) of volunteers had a clear positive response ($\geq$3 according to the new criteria for skin irritation and + or higher according to the standard Japanese criteria) at 24 h after patch removal on the 24-h patch test; 40.4% (19/47) had such a response on the 48-h patch test. Although there are

Table 4. The summary of results in Japanese standard

Chem	Sample	Conc. (%)	hour	Evaluation 2 h after				Evaluation 24 h after				Evaluation 48 h after			
				Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min
C01	SLS	0.5	48 h	0.43	0.35	1	0	0.67	0.50	2	0	0.47	0.34	1	0
C01			24	0.49	0.51	2	0	0.69	0.50	2	0	0.44	0.45	2	0
C01			4	0.07	0.18	0.5	0	0.11	0.23	1	0	0.06	0.17	0.5	0
C02	SLS	0.3	48 h	0.32	0.30	1	0	0.63	0.53	2	0	0.36	0.36	1	0
C02			24	0.21	0.29	1	0	0.46	0.44	2	0	0.26	0.33	1	0
C02			4	0.05	0.16	0.5	0	0.07	0.18	0.5	0	0.05	0.16	0.5	0
C03	SLS	0.1	48 h	0.10	0.20	0.5	0	0.21	0.29	1	0	0.14	0.25	1	0
C03			24	0.13	0.24	1	0	0.21	0.29	1	0	0.13	0.22	0.5	0
C03			4	0.10	0.20	0.5	0	0.00	0.00	0	0	0.02	0.10	0.5	0
C04	PG	50	48 h	0.34	0.28	1	0	0.26	0.29	1	0	0.16	0.24	0.5	0
C04			24	0.23	0.39	2	0	0.13	0.22	0.5	0	0.05	0.16	0.5	0
C04			4	0.02	0.10	0.5	0	0.03	0.12	0.5	0	0.05	0.16	0.5	0
C05	PG	30	48 h	0.32	0.37	2	0	0.24	0.25	0.5	0	0.17	0.26	1	0
C05			24	0.14	0.23	0.5	0	0.11	0.21	0.5	0	0.10	0.20	0.5	0
C05			4	0.01	0.07	0.5	0	0.00	0.00	0	0	0.04	0.14	0.5	0
C06	SL	2	48 h	0.48	0.39	2	0	0.39	0.33	1	0	0.24	0.29	1	0
C06			24	0.45	0.33	1	0	0.47	0.34	1	0	0.37	0.32	1	0
C06			4	0.38	0.49	2	0	0.17	0.24	0.5	0	0.09	0.22	1	0
C07	SL	1	48 h	0.21	0.27	1	0	0.15	0.23	0.5	0	0.07	0.18	0.5	0
C07			24	0.27	0.27	1	0	0.26	0.25	0.5	0	0.16	0.24	0.5	0
C07			4	0.18	0.30	1	0	0.09	0.19	0.5	0	0.05	0.16	0.5	0
C08	IPM	100	48 h	0.10	0.20	0.5	0	0.05	0.16	0.5	0	0.10	0.20	0.5	0
C08			24	0.02	0.10	0.5	0	0.02	0.10	0.5	0	0.05	0.19	1	0
C08			4	0.02	0.10	0.5	0	0.00	0.00	0	0	0.04	0.14	0.5	0
C09	BC	0.1	48 h	0.36	0.34	1	0	0.29	0.29	1	0	0.23	0.31	1	0
C09			24	0.38	0.51	2	0	0.30	0.40	2	0	0.19	0.27	1	0
C09			4	0.13	0.22	0.5	0	0.01	0.07	0.5	0	0.02	0.10	0.5	0
C10	BC	0.05	48 h	0.26	0.29	1	0	0.23	0.29	1	0	0.19	0.27	1	0
C10			24	0.16	0.24	0.5	0	0.15	0.34	2	0	0.09	0.22	1	0
C10			4	0.04	0.14	0.5	0	0.04	0.14	0.5	0	0.02	0.10	0.5	0
C11	Oleth-10	10	48 h	0.16	0.24	0.5	0	0.18	0.24	0.5	0	0.06	0.17	0.5	0
C11			24	0.14	0.27	1	0	0.05	0.16	0.5	0	0.12	0.21	0.5	0
C11			4	0.05	0.16	0.5	0	0.02	0.10	0.5	0	0.03	0.12	0.5	0
C12	Oleth-10	5	48 h	0.30	0.40	2	0	0.18	0.26	1	0	0.14	0.23	0.5	0
C12			24	0.18	0.28	1	0	0.12	0.21	0.5	0	0.14	0.23	0.5	0
C12			4	0.03	0.12	0.5	0	0.03	0.12	0.5	0	0.02	0.10	0.5	0
C13	WP		48 h	0.07	0.18	0.5	0	0.02	0.10	0.5	0	0.04	0.14	0.5	0
C13			24	0.02	0.10	0.5	0	0.00	0.00	0	0	0.02	0.10	0.5	0
C13			4	0.02	0.10	0.5	0	0.01	0.07	0.5	0	0.03	0.12	0.5	0
C14	DW		48 h	0.18	0.24	0.5	0	0.14	0.25	1	0	0.10	0.20	0.5	0
C14			24	0.09	0.19	0.5	0	0.06	0.17	0.5	0	0.15	0.25	1	0
C14			4	0.02	0.10	0.5	0	0.04	0.14	0.5	0	0.01	0.07	0.5	0
C15	Saline		48 h	0.12	0.21	0.5		0.03	0.12	0.5	0	0.03	0.12	0.5	0
C15			24	0.05	0.16	0.5	0	0.03	0.12	0.5	0	0.05	0.16	0.5	0
C15			4	0.03	0.12	0.5	0	0.01	0.07	0.5	0	0.02	0.10	0.5	0

differences between the upper back and forearm, the results obtained in the present study agree with those of earlier studies, suggesting that the conditions in the present study allow mild skin irritations to be accurately detected. There were no important differences in the results on irritation reaction obtained according to the standard Japanese criteria for determining skin irritation and the new criteria.

In conclusion, a patch application time of 24 h is sufficient to detect primary skin irritation from irritants in cosmetics and other common household products. In addition, we found that skin irritation reactions were strongest at 24 h after patch removal and that the reaction tended to be weaker at 48 h after patch removal. These results suggest that, for optimum results, patch testing to evaluate irritants can be performed by means of a 24-h patch test with a follow-up reading at 24 h after patch removal. In addition, an application time of 24 h places less of a burden on patients than a 48-h patch test.

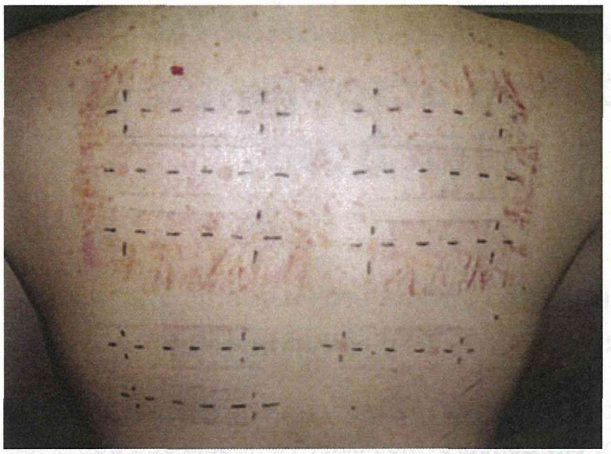

Figure 4. The left upper back was used for 48-h patch application, and the right rash is clearly more severe on the left upper back.

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Whole-exome sequencing identifies *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 *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 *ADAM10* mutations. We identified a total of three truncating mutations, a splice site mutation and a missense mutation in *ADAM10*. We searched for mutations in the *KRT5* gene, a causative gene for the similar pigmentation disorder Dowling-Degos disease (DDD), in all the patients and found no *KRT5* mutation. These results reveal that mutations in *ADAM10* are a cause of RAK and that RAK is an independent clinical entity distinct from DDD.

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 *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

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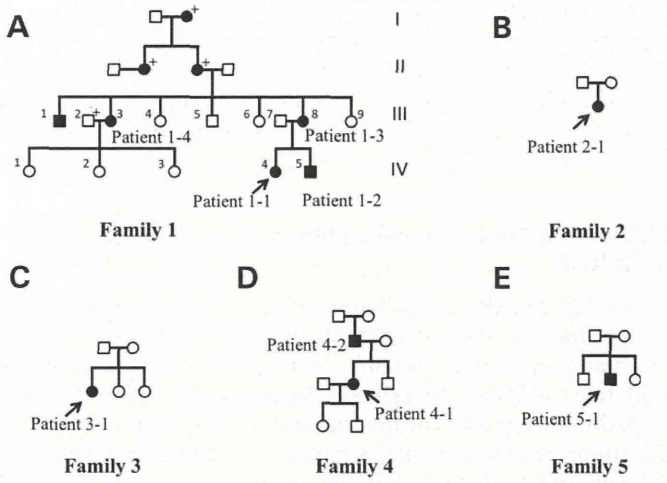

Figure 1. Pedigrees of the five families with reticulate acropigmentation of Kitamura (RAK) included in the present study. Arrows indicate the probands. Whole-exome sequencing was performed on Family 1.

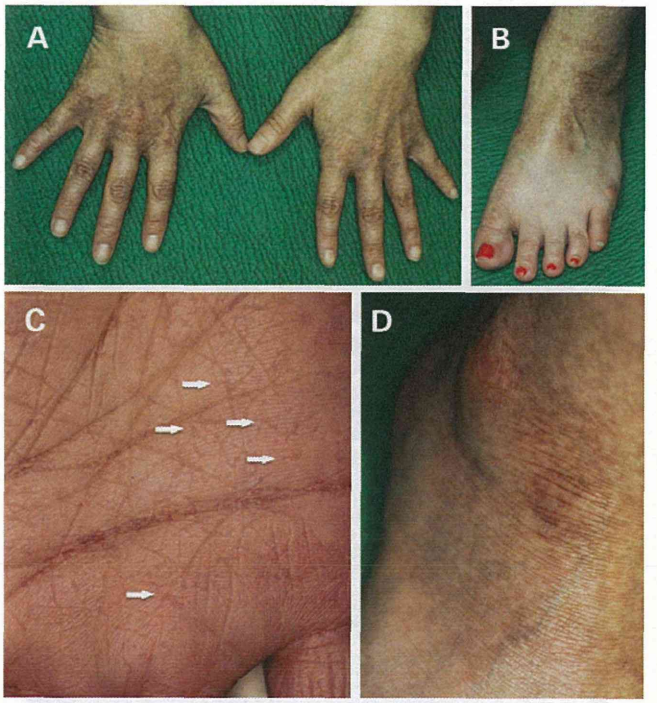

Figure 2. Clinical photographs of a typical RAK patient. Patient 1-1 (IV-4 in Family 1 in Fig. 1) had reticular pigmented macules on the dorsal hands and the left foot (**A** and **B**). Arrows indicate pits on the palm (**C**). Brown macules on the dorsa of the foot are slightly depressed (**D**).

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

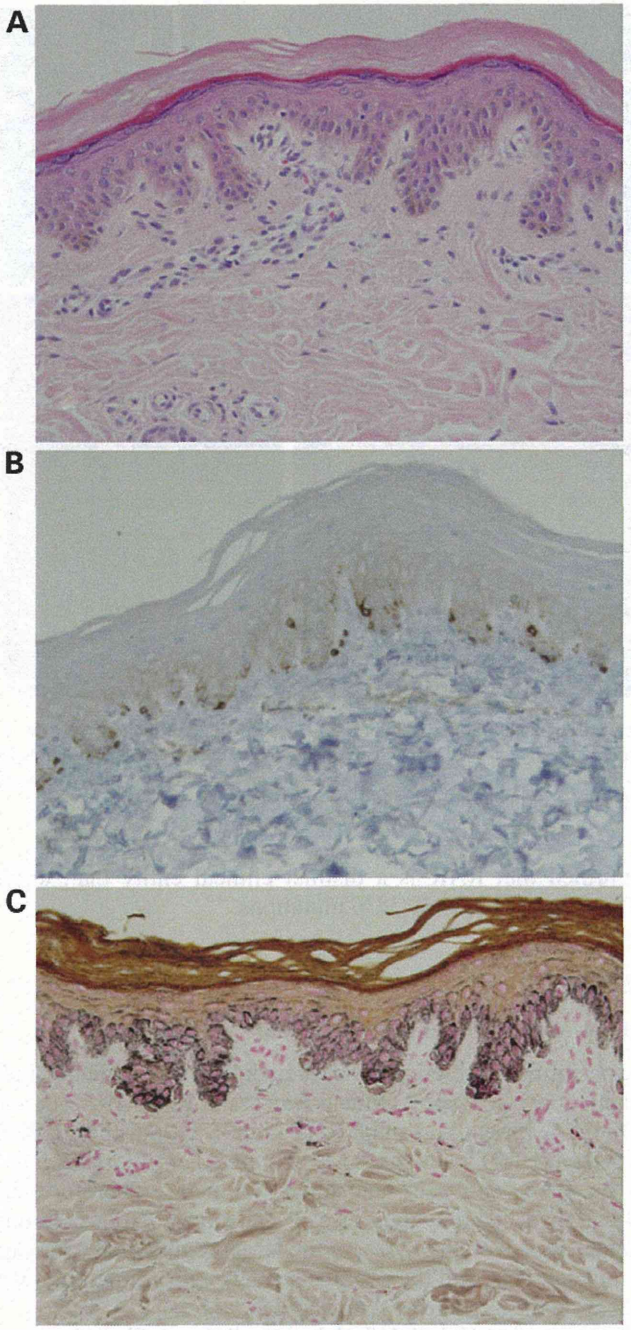

Figure 3. Histopathology of skin lesions of RAK. A brown macule on the back of the hand from Patient 3-1 shows mild and compact hyperkeratosis and elongation of rete ridges (hematoxylin and eosin staining) (**A**). MART1 (**B**) and Fontana-Masson (**C**) stains show a slightly increased number of melanocytes and hypermelanosis, respectively.

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