ceive non-invasive treatments for the purpose of anti-skin aging or slimming.

Among these procedures, a hand massage can be a good alternative not only to a facial but also to a full body treatment. It can be a relaxing, rejuvenating and sometimes becomes a therapeutic method for flabby skin. However, there have been only a few studies that have evaluated the effectiveness of a hand massage based on medical approaches [1]-[4].

The application of a treatment by hand or a hand massage is one of the commonly used non-invasive cosmetic procedures, and it not only provides the skin with nourishment and improves the circulation, resulting in rejuvenated skin, but also is known be a good way to relieve stress. On the other hand, therapists have to continue to work for long hours and utilize their skin and muscles in every possible type of motion and injuries of the hands and back are common among manual therapists. In addition, therapists sometimes become exhausted after performing the hard work for several days in succession, although certain breathing and stretching exercises may help.

In such situation, the similar massage can be performed with appropriate products, such as stones or sticks. The quality or the effect of the treatment depends on how well therapists have been trained, how skilled they are, and how they feel when they are performing the treatment. Therefore, a treatment with these products may be helpful for practitioners, because it enables them to uniformly and stably add power and to control the intensity of the power applied to the subjects' skin, making them more comfortable. For example, a hot stone massage is a special kind of massage where therapists use and handle smooth, heated stones to massage the subject. By using the stones, the therapists can work more accurately, more quickly and more easily compared with the treatment using just their hands without the stones.

We recently developed a new device for body treatment using a tree instead of a stone. The device is made from Hinoki cypress and has a peculiar shape, which is perfectly configured for full body of humans. In this study, we examined the effectiveness and usefulness of this novel tool (Power Tree®) for body massage by evaluating several dermatological and psychological parameters.

2. Materials and Methods

We enrolled 10 healthy female volunteers (age range, 24 - 55 years; mean age, 40.5 years) in the present study. We applied the newly developed novel-shaped tool, the Power Tree®, made from Hanoi cypress, for a full massage body massage (Figure 1).

A typical full body massage consists of effleurage, friction, pressure, smoothing and kneading, and these treatments are performed in order on appropriate sites of the body with a mixture of massage oil (Table 1), typically for 60 minutes. In all of these steps, the Power Tree® was used instead of the hands and the treatments were performed by three female therapists in the present study.

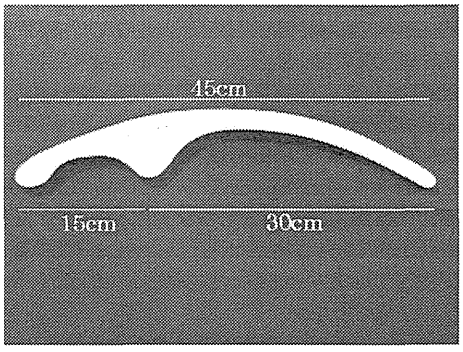


Figure 1. The newly-developed device, the Power Tree®, made of Hinoki cypress.

Table 1. The major chemical constitutions of the essential oils used in the study.

Hydrogenated poly (C6-12 olefin)	35%
Oryza sativa (rice) bran oil	35%
Cetylethylhexanoate	30%

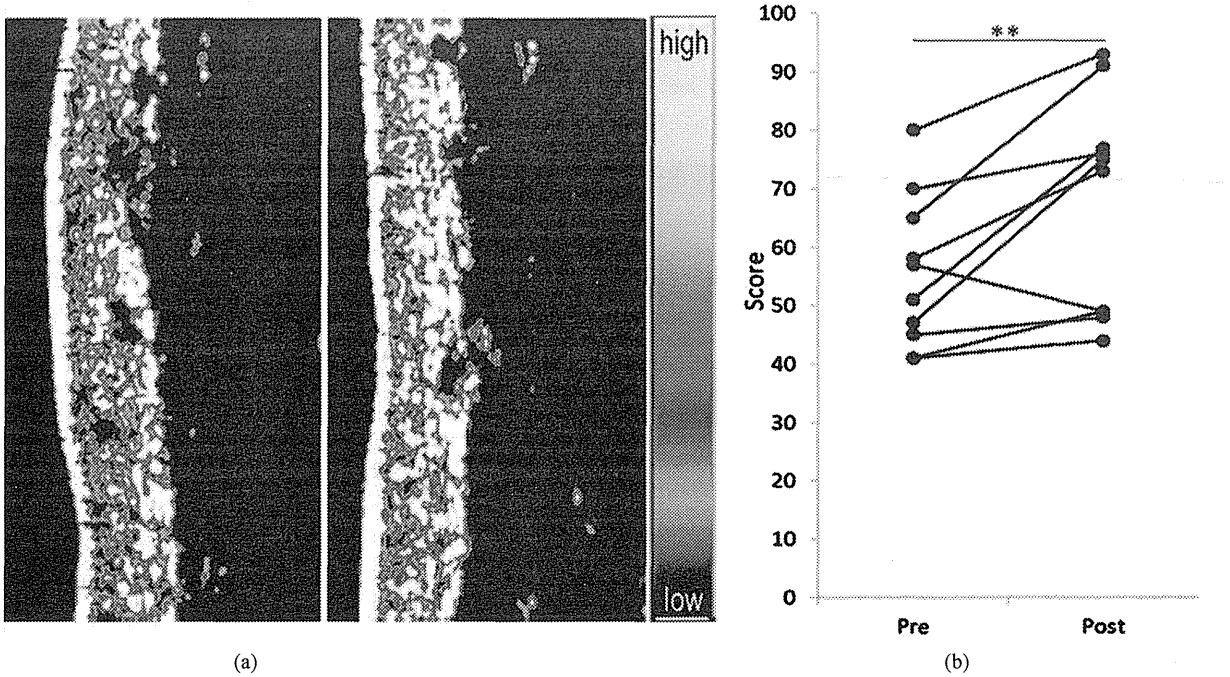
We analyzed the changes in the dermal collagen score, skin temperature and release of the salivary enzyme alpha-amylase, before and immediately after the body massage treatment by using a SkinLab Ultrasound system (DermaLab®, Cortex Technology, Denmark), a Thermo Shot F20 (NEC Avio Infrared Technologies Co., Ltd, Japan) and a Cocoro Meter (Nipro Co., Japan), respectively according to the manufacturers' instructions. The cross-sectional skin image obtained by the SkinLab device represents the intensity of collagen signals, where high density (yellow or red) areas contain abundant collagen. Before and after the procedure, ultrasound images were compared with an average density in the range 0 - 100. The higher the density in the dermis, the higher the collagen score is. This dermal collagen score was measured at the lateral site of the left thigh. The skin temperature was measured at three points; the center of the bilateral scapulae and the bilateral posterior site of the thigh before and after the procedure by using infrared thermal imaging camera. The Cocoro Meter is a stress detector and the level of the comprehensive stress can be evaluated by measuring the amount of amylase in the saliva with this meter after putting its spatula on the subject's tongue for 60 seconds. In addition, the Spielberger State-Trait Anxiety Index (STAI) (Chiba Test Center Co., Ltd, Tokyo) was measured as described previously [5] [6] to assess the subject' levels of anxiety. The STAI inventory is used to assess both state and trait anxiety (SA and TA) separately. Each type of anxiety has its own scale of 20 different questions that are scored. Scores range from 20 to 80, with higher scores correlating with greater anxiety. All of the measurements were carried out immediately after the therapy and there was no skin abnormality including edema and erythema.

All of the analyses were performed after obtaining institutional approval and written informed consent from the subject and the study was conducted according to the principles of the Declaration of Helsinki. The results are expressed as the means with SD. The statistical analyses were performed using the two-tailed student's t test for independent samples. Significant differences were recognized at p values ≤ 0.05.

3. Results

The sixty-minute treatment with this device was smoothly performed without any burden on the therapists in the present study.

The dermal collagen score assessed by the SkinLab system increased from 55.5 ± 13 to 67.5 ± 18.5 by the treatment (Figure 2(a), Figure 2(b)) and this change was statistically significant ($p = 0.005$). In addition, the skin temperature of the back, right thigh and left thigh (mean ± SD pre-treatment: 34.5 ± 0.9, 32 ± 0.7 and 32.4



(a) (b)

Figure 2. An image showing the changes in dermal collagen score in a representative subject (a) and all of the data regarding the changes in the dermal collagen score (b). $^{**}p < 0.01$.

± 0.7, respectively) was increased significantly by the treatment (post-treatment 36.1 ± 1, 35.1 ± 1 and 35 ± 0.9, respectively, $p < 0.001$) (**Figure 3(a)**, **Figure 3(b)**).

Regarding the level of general stress, the concentration (kU/l) of amylase in the saliva was not significantly different pre- and post-procedure (mean + SD: 58 + 40, 64 ± 28, respectively) ($p = 0.3$) (data not shown). However, the mean ± SD of SA and TA scores before the procedure were 43 ± 8 and 46 ± 10, respectively, and changed to 27 ± 5 and 42 ± 9, respectively, after the treatment. Both of these changes were statistically significant ($p = 0.0002$ for the SA, $p = 0.009$ for the TA) (**Figure 4**).

4. Discussion

There have been several reports of the psychological and physiological efficacy of hand massage therapy;

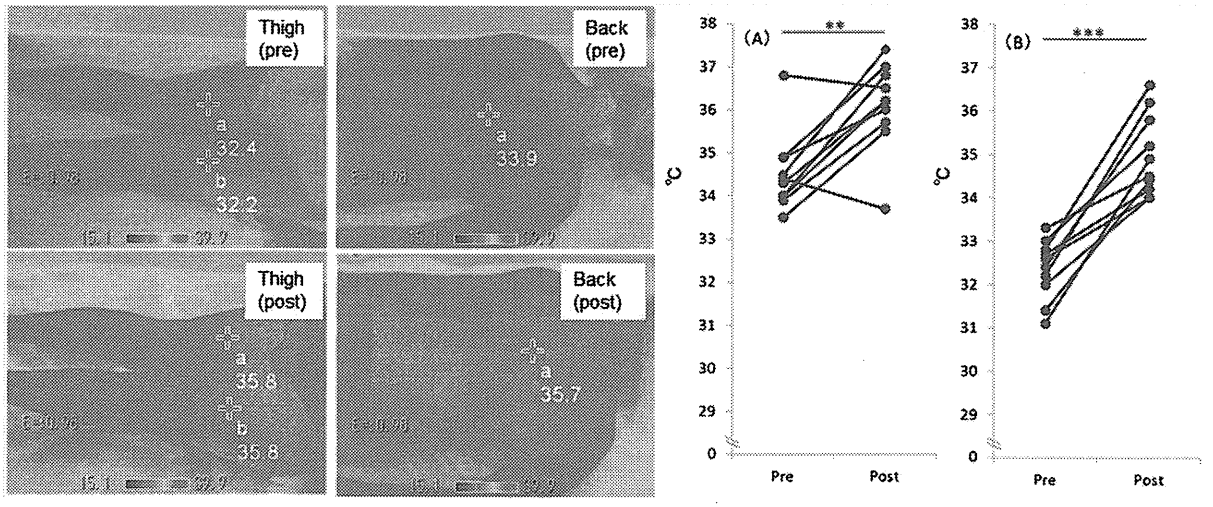


Figure 3. A representative image showing the changes in the skin temperature in a subject (a) and all of the data for the changes in skin temperature ((A) back; (B) left thigh). $^{**}p < 0.01$, $^{***}p < 0.001$.

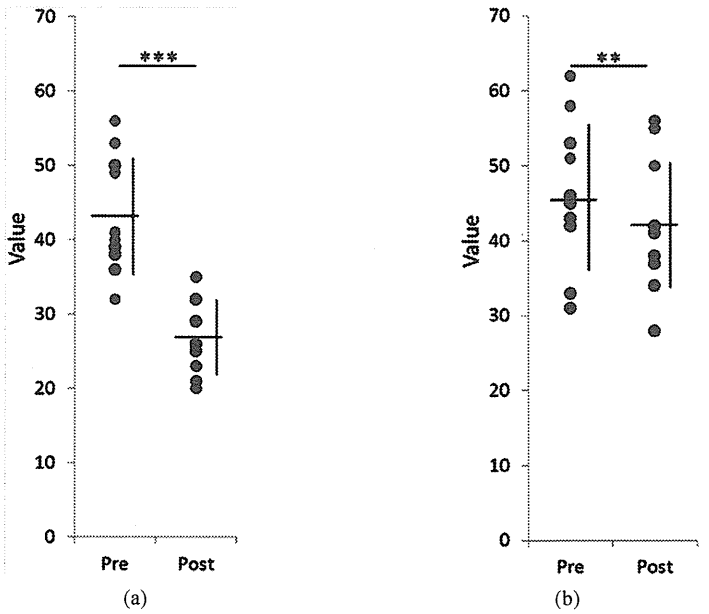


Figure 4. The changes in the STAI score. (a) State anxiety; (b) Trait anxiety. Vertical bar = SD, horizontal bar = mean value. $^{**}p < 0.01$, $^{***}p < 0.001$.

relaxation, improvement of the score estimated by the STAI and a decrease in the levels of cortisol and nor epinephrine [1]-[5].

In the present study, we evaluated the usefulness of a special tool; the Power Tree®-mediated massage therapy by assessing various parameters including the dermal collagen score, skin temperature, the level of stress based on the concentration of salivary amylase as well as the STAI score. The collagen stimulating effect of the Power Tree®-mediated treatment in the dermis visualized by the DermaLab® system was confirmed.

We also observed that the Power Tree® increased the skin temperature after the massage. However, there was little or no change in the level of salivary amylase after the procedure. The level of salivary amylase is one of the valuable biological indicators of physiological and psychological stress reactions. The lack of any differences between the salivary amylase levels before and after treatment may have been due to a lack of stress pre-procedure or the discomfort associated with the use of the Power Tree®, even though they were receiving a treatment considered to have a relaxing effect.

In the present study, we examined the psychological effects of the massage by evaluating the STAI scores, and we observed that the post-procedure scores were significantly lower for both the SA and TA compared to those pre-procedure. These results suggest that beneficial effects in terms of anxiety reduction were obtained by the full body massage using the Power Tree®. The improvement of the TA following the procedure implies the possibility that there is a healing effect induced by Power Tree®-mediated message therapy.

5. Conclusion

In conclusion, a newly-developed device, the Power Tree®, is a powerful and useful tool for reflexology during full body massage therapy, as evaluated by several medical parameters. By using this device, massage therapy may produce beneficial physiological effects as well as psychosocial improvement.

Because we performed this study in only a small number of subjects, and examined the data over a short period before and immediately after the treatment, it is necessary to validate the effects a longer period of time after the Power Tree®-mediated massage in a future study, and to examine the impact of the treatment in patients experiencing high levels of stress prior to the treatment.

Conflict of Interests

One of the authors (S.M.) is a research adviser for the Bloom Classic Co.

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Generalized milia in an infant with full trisomy 13

Dear Editor,

Milia are classified as primary or secondary depending on the underlying mechanism.[1] Primary milia commonly appear on the face in young people, while multiple eruptive milia rarely occur.[2] Primary milia are also common in newborns, with rashes appearing on the face several days after birth, followed

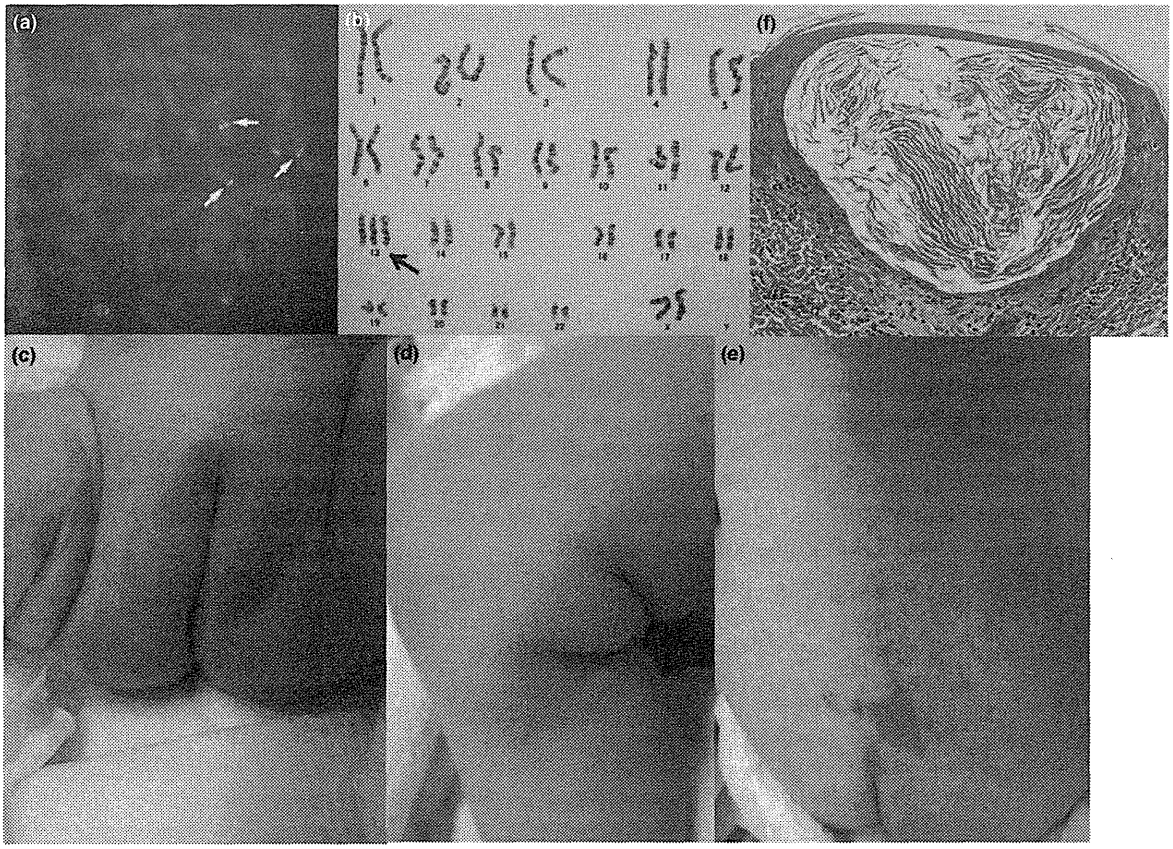

Figure 1. On genetic testing, fluorescence *in situ* hybridization (a) detected three signals (ish + 13 [RB1 × 3], shown by arrows) on chromosome 13 (green) in all 100 cells examined, and a G-band analysis (b) detected three copies of chromosome 13 (47, XX, +13, shown by an arrow). These genetic examinations led to a definitive diagnosis of full trisomy 13. At the first visit at 3 months of age, multiple white papules measuring 1–2 mm in size were found to be scattered on the perineum (c), inner right lower extremity (d), buttocks and lower back (e). A histopathological examination of the white papules showed a small cyst-like structure extending from the epidermis to the upper dermis. Layers of keratinous material were found in the lumen (hematoxylin–eosin, original magnification ×200) (f).

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by spontaneous resolution within a few weeks. Secondary milia develop following the formation of retention cysts via epidermal implantation after injury due to burns or bullous diseases.[1]

Trisomy 13 (or Patau syndrome) is a rare congenital malformation caused by an extra copy of chromosome 13.[3] Full and translocation trisomy 13 account for 80% and 15% of all cases, respectively, with other forms exhibiting a mosaic pattern. Trisomy 13, particularly full and translocation 13 trisomy, is known to have an extremely poor prognosis, as 90% of affected infants die before reaching 1 year of age.[3,4] Trisomy 13 is associated with various external and organ malformations; however, skin changes other than capillary hemangioma and nuchal thickening are rare. To date, there has been only one report of translocation trisomy 13 with generalized milia.[5] We herein report an atypical case of full trisomy 13 in an infant who developed generalized milia without resolution.

A 3-month-old female without a family history was referred to our department for an assessment of generalized papules. Following cesarean section at 37 weeks of gestation, dextrocardia, bilateral cystic kidneys, hydroureter, ventricular septal defect, hypertrophic pyloric stenosis and bilateral hydronephrosis were noted. In addition, various findings related to abnormal ectodermal development were observed, including a cleft lip and palate, capillary hemangioma, extra nuchal skin and overlapping fingers. A diagnosis of full trisomy 13 was made based on genetic testing (Fig. 1a,b).

Three months after birth, the patient developed multiple white military papules on the trunk and limbs. The clinical findings at the first visit revealed multiple white miliary papules scattered over the patient's body (Fig. 1c–e). A cyst-like structure was observed histologically (Fig. 1f), and a definitive diagnosis of primary milia was made. Unfortunately, the patient died of heart failure at 10 months of age.

Primary milia affect 50% of normal neonates, usually exhibiting spontaneous remission within several weeks.[1] In the present case, however, the milia developed outside the neonatal period (3 months after birth) and were generalized. Because no prior skin diseases were detected, this case represents primary milia, although it is atypical. The patient had trisomy 13; however, no genetic disorders (e.g. Bazex–Dupre–Christol syndrome) that could have caused the milia[1] were noted. Capillary hemangioma and extra skin at the nape were observed, typical skin conditions observed in trisomy 13. Only one previous case of generalized milia associated with trisomy 13 has been reported, in which the patient had translocation trisomy 13 and presented with milia at birth.[5] Although the association between milia and chromosomal abnormalities is unclear, trisomy 13 is known to be associated with various ectodermal malformations; therefore, the mechanism may involve dysplasia of ectodermal tissue associated with the chromosomal abnormality.

CONFLICT OF INTEREST: The authors have no conflicts of interest to disclose.

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Immunohistochemical analysis of O6-methylguanine-DNA methyltransferase in human melanoma in comparison with skin squamous cell carcinoma

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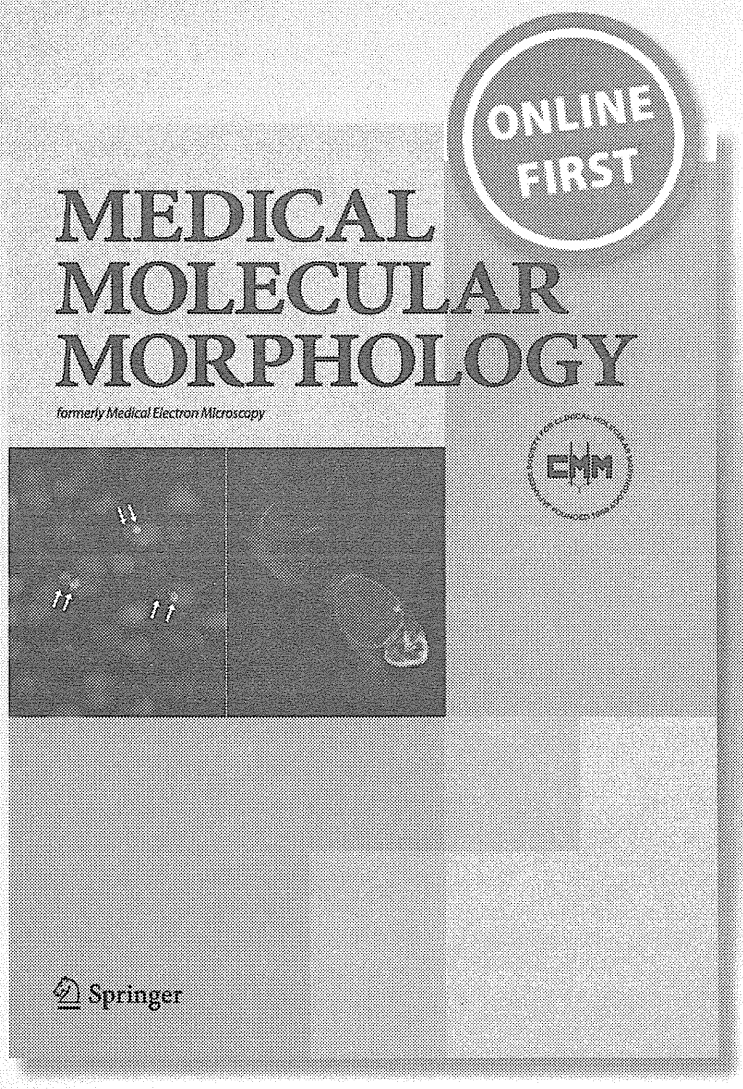

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ORIGINAL PAPER

Immunohistochemical analysis of O^6-methylguanine-DNA methyltransferase in human melanoma in comparison with skin squamous cell carcinoma

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Abstract Alkylating agents, often used for chemotherapy in patients with melanoma, can produce O^6-alkylguanine (O^6AG) which is related to tumor cell killing after treatment with alkylating agents. O^6AG is effectively eliminated by O^6-methylguanine-DNA methyltransferase (O^6MGMT) and its level is correlative to the resistance to alkylating agents. However, little is known about the relationship of O^6MGMT to the characteristics of melanoma. This study investigated the expression of O^6MGMT in 12 melanomas and compared it with that in 11 skin squamous cell cancers (SCCs) immunohistochemically to evaluate the O^6MGMT activity in melanoma and its clinical significance. All of the SCC samples had high O^6MGMT expression, while the expression of O^6MGMT in melanoma was diverse and 4 out of 12 samples had no or extremely low O^6MGMT activity. Out of 6 lesions obtained from metastasis, 4 had a high O^6MGMT activity. Two out of 3 cases with a low O^6MGMT activity in each primary lesion did not show any evidence of metastasis or local recurrence. The evaluation of O^6MGMT activity in melanoma may, therefore, be useful to determine the characteristics of tumor in each melanoma case. In addition, the present study implies the possibility of selective cancer chemotherapy for melanoma in the near future.

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Keywords Alkylating agent · DNA repair · Melanoma · O^6-alkylguanine · O^6-methylguanine-DNA methyltransferase · Skin squamous cell carcinoma

Introduction

Alkylating agents such as 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC) and 1-(4-amino-2-methyl-5-pyridimil)methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU) often used for cancer chemotherapy in melanoma and brain tumors, can produce various kinds of adducts to the bases in DNA [1–3]. O^6-alkylguanine (O^6AG) is thought to be the major lesion related to tumor cell killing after treatment with alkylating agents, because the presence of O^6AG in DNA may induce interstrand cross-linking or mispair with thymine during semiconservative DNA synthesis, thus resulting in the occurrence of G:C-A:T transversion type of mutations [4, 5]. O^6AG is effectively eliminated by a DNA repair enzyme, O^6-methylguanine-DNA methyltransferase (O^6MGMT), which can transfer the alkyl group from the O^6 position in the guanine base. The level of O^6MGMT in tumors is variable; however, brain tumors and melanomas have lower O^6MGMT activity in comparison to those in other epithelial malignant neoplasms and its level is correlative to the resistance to alkylating agents based on several studies using cultured tumor or transformed cell lines [6–9]. In contrast, the level of O^6MGMT in cultured cells from squamous cell cancers (SCCs) is usually high, thus suggesting that there is no clinical indication to use alkylating agents for the treatment of patients with SCCs [10]. Some studies noted that the inactivation of O^6MGMT by the methylation in the promoter region in *O^6MGMT* gene is related to both the effectiveness of temozolomide, an alkylating agent, and the

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survival of patients with a brain tumor [11–16]. O^6MGMT knockout mice have been shown to be sensitive to killing by the chemotherapeutic alkylating agents [17]. However, other clinical studies about the relationship between O^6MGMT level and the response to alkylating agents have yielded different results in patients with brain tumors and melanoma [17–21]. Furthermore, little is known about the usefulness of measuring the level of *O^6MGMT* in melanoma specimens to predict the phenotype of the melanoma and the effectiveness of alkylating agents against melanoma and the clinical outcome in melanoma-prone patients. This study investigated the expression of *O^6MGMT* in specimens from Japanese patients to explore the clinical significance and to evaluate the *O^6MGMT* activity in melanoma.

Materials and methods

Patients

Fourteen paraffin-embedded tumor samples obtained from 12 melanoma patients that were treated by use of alkylating agents between January 1999 and July 2011 were included in the present study. In addition, 11 skin SCC samples obtained from the same period were also used. SCC is not commonly treated by alkylating agent. The patients' age ranged between 55 and 101 years (mean 78.6) and 35 and 94 years (mean 72.5) in the melanoma and SCC patients, respectively.

The clinical characteristics of the patients with melanoma and SCCs including the clinical stage, chemotherapy, and survival status/prognosis were summarized in Tables 1 and 2 respectively.

Two independent samples of metastatic tumor lesions were obtained in melanoma cases 1 and 8. In 7 of the 14 specimens obtained from primary melanoma lesions, 6 were from metastatic lesions (4 of skin metastasis and 2 of lymph node metastasis) and one was from a skin recurrence.

Six of the 14 specimens of melanoma from non-primary lesions were obtained after chemotherapy using DTIC (Dacarvazine™) and ACNU (Nidran™), which are both alkylating agents and commonly used for melanoma patients in Japan. The clinical stage listed in Table 1 was classified according to the Union Internacional Contra la Cancrum (UICC)/American Joint Committee on Cancer (AJCC), 2009 [22].

All of the SCC samples (4 male and 7 female patients) were from primary lesions without any chemotherapy. The clinical stage listed in Table 2 was classified by UICC/AJCC, 2009 [22].

Clinical data were collected from the medical files, including the patient age, sex, disease duration, associated medical condition/information, treatment and response to treatment and survival status.

The protocol of the present study was approved by the institutional review board at Osaka Medical College and the study was conducted according to the Declaration of

Table 1 Clinical characteristics of the melanoma patients evaluated in the present study

Case no.	Age/sex	Location of melanoma	Clark level	Clinical stage	Therapy[a]	Survival period and status[b]
1	35/M	Abdomen	IV	IIa	Surgical resection, chemotherapy	5 y, unknown
2	54/F	Upper extremity	Unknown	IIIb∼IV	Surgical resection, chemotherapy	6 y, dead
3	74/M	Upper extremity	II	Ia∼IIc	Surgical resection, chemotherapy	1 y 5 m, dead
4	75/F	Lower arm	II	IIb	Surgical resection, chemotherapy	1 y 6 m, alive
5	60/F	Sole	II	IIb	Surgical resection, chemotherapy	2 y 2 m, alive
6	59/F	Sole	Unknown	IIIb	Surgical resection, chemotherapy	7 y 10 m, alive
7	81/F	Vagina	II	IIc	Surgical resection, chemotherapy	1 y 6 m, dead
8	78/M	Nose	Unknown	Unknown	Surgical resection, chemotherapy	7 y 6 m, dead
9	79/M	Sole	II	IIa	Surgical resection, chemotherapy	4 y 2 m, alive
10	69/F	Lower leg	I	IIIb	Surgical resection, chemotherapy	8 m, dead
11	49/M	Head	I	IIIa	Surgical resection, chemotherapy	1 y 2 m, dead
12	25/F	Lower leg	I	IIIc	Surgical resection, chemotherapy	1 y 1 m, alive

M male, *F* female

[a] All of the patients had repeated chemotherapy using DTIC and ACNU

[b] *y* year, *m* month, survival after the surgical resection

Table 2 Clinical characteristics of the SCC patients evaluated in the present study

Case no.	Age/sex	Location of SCCs	Clinical stage	Therapy[a]
1	35/F	Vagina	IV	Surgical resection
2	74/M	Nose	I	Surgical resection
3	62/F	Lower leg	III	Surgical resection
4	94/F	Lower arm	Unknown	Surgical resection
5	75/F	Lower leg	I	Surgical resection
6	74/M	Hand	I	Surgical resection
7	72/M	Finger	I	Surgical resection
8	86/F	Finger	II	Surgical resection
9	62/F	Hand	I	Surgical resection
10	85/M	Ear	I	Surgical resection
11	78/F	Hand	I	Surgical resection

M male, *F* female

[a] All of the patients did not have any chemotherapy

Helsinki principles. All of the analyses were performed after obtaining informed consent from each surviving patient.

Immunohistochemistry

Immunohistochemistry was performed with the EnVision system (Dako, Japan, Tokyo) according to the manufacture's instruction. First, 4 μm sections of formalin-fixed paraffin-embedded tissue were prepared, mounted on slides, dewaxed and rehydrated. They were placed in pre-warmed citrate buffer (pH 6.0) and boiled in a water bass at 98 °C for 40 min. Still in the citrate buffer, the slides were cooled at room temperature for 20 min. Subsequently, after incubation in blocking solution of 3 % H_2O_2, 1:50 dilution of anti-MGMT antibody (MT1.3) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was added and the slides were incubated for 2 h at room temperature. Slides were washed in phosphate buffered saline (PBS) and antibody binding was visualized with the DMB (3,3′-diaminobenzidine chromogen solution). The slides were then washed in water, counter-stained with hematoxylin, dehydrated and mounted with cover slides. As negative control, we used sections stained without the first antibody.

For each specimens, 500–1,000 tumor cells were observed and the frequency of positively stained cells were calculated by the ratio of number of positive cells to total cells counted [14, 23].

Results

All of the SCC samples were strongly and positively immunohistochemically stained by the O^6MGMT antibody and its expression was not related to the clinical stage of SCCs, patient's age at the time of resection, anatomical location of the tumor, and sex of the patients, as shown in Table 3. More than 90 % of the tumor cells in each specimen were found to express O^6MGMT in 9 out of the 11 cases and the remaining 2 cases had the immunohistochemical signal of this enzyme in about 70 % of the tumor cells in each specimen. The representative data of the expression of O^6MGMT in SCCs are shown in Fig. 1.

Table 3 Immunohistochemical expression of O^6MGMT in patients with SCCs and melanoma

SCCs		
SCC case no.	Tumor lesion obtained for the study	% of O^6MGMT positive cells
1	Primary lesion	90
2	Primary lesion	70
3	Primary lesion	90
4	Primary lesion	90
5	Primary lesion	90
6	Primary lesion	90
7	Primary lesion	90
8	Primary lesion	90
9	Primary lesion	90
10	Primary lesion	90
11	Primary lesion	70
Melanoma		
Primary lesion group		
Melanoma case no.		% of O^6MGMT positive cells
4		0
6		90
7		90
9		40
10		90
11		60
12		30
Metastatic or recurrent lesion group		
Melanoma case no.	Tumor lesions obtained for the study	% of O^6MGMT positive cells
1	LN metastasis	50
	Skin metastasis	90
2	LN metastasis	90
3	Skin metastasis	90
5	Recurrent lesion	0
8	Skin metastasis	90
	Skin metastasis	10

The expression of O^6MGMT in total melanomas was diverse and 4 out of 12 samples had no or extremely low (10 and 30 %) O^6MGMT activity (Table 3; Fig. 2). Limited to the 7 primary lesions, one (case 4) had no and two (cases 9 and 12) had low (expression in less than 40 % of tumor cells) O^6MGMT activity. The remaining 4 cases (cases 6, 7, 10 and 11) with primary lesions demonstrated a high O^6MGMT activity (60 % and more than 90 % of tumor cells were positively stained by the antibody). On the other hand, more than 90 % of the tumor cells were positively stained by anti-O^6MGMT antibody in 4 out of 6 specimens obtained from distant or lymph node metastasis. On the prognosis, one patient (case 6) with a high activity had a good prognosis, while among the 2 cases with a low O^6MGMT activity (less than 40 % of tumor cells expressing this enzyme), one (case 9) survived for 4 years and the other (case 4) for 1½ years without any recurrence or metastasis.

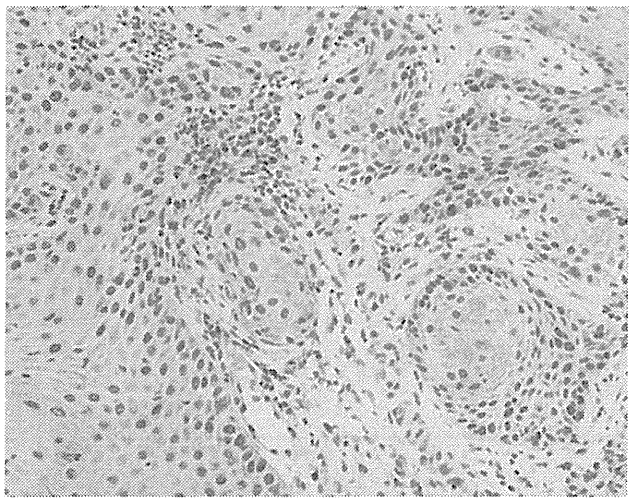

Fig. 1 Representative immunohistochemical staining of the resected SCC specimen (case 9). More than 90 % of the tumor cells expressed strong reactivity with antibody to O^6MGMT (objective magnification; ×20)

Samples were obtained twice independently from metastatic lesions from cases 1 and 8. The level of expression of O^6MGMT was different in both cases between two samples of the same patient. In case 1, after repeated chemotherapy using DTIC and ACNU, the level increased from 50 to 90 %. On the other hand, in case 8, the level of expression of O^6MGMT in two samples was 90 and 10 %, in spite of two independent skin metastases after the chemotherapy.

Discussion

The present study analyzed the immunohistochemical expression of O^6MGMT, which is a DNA repair enzyme, presumably related to the cytotoxic effect of cancer cells to alkylating agents [6–9]. Several studies have measured the expression of O^6MGMT in melanoma samples from

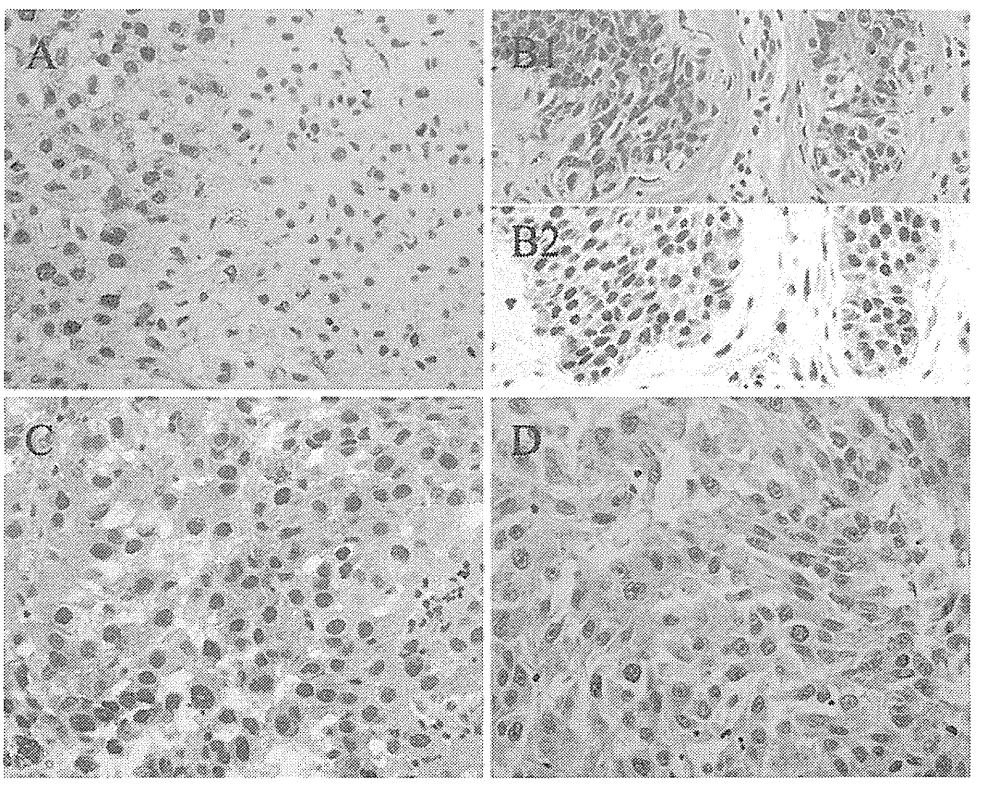

Fig. 2 Representative immunohistochemical staining for O^6MGMT of the resected melanoma specimens (objective magnification; ×20). **a** Lymph node metastasis in case 1. About half of melanoma cells are positive (*left*), and the half of cells are negative (*right*). **b** Skin metastasis in case 1. (*B1* Hematoxylin and eosin staining. Melanoma cell nests are seen in the basal region of epidermis. *B2* Immunohistochemical staining. Melanoma cells, as normal keratinocytes of epidermis, show positive reactivity.) **c** First skin metastasis in case 8. More than 90 % of melanoma cells are positive for O^6MGMT. **d** Second skin metastasis in case 8. Melanoma cells are negative for O^6MGMT

American and European patients [17–21] and there is only one study that measured the enzyme levels of melanoma tissues from Japanese [24]. Moreover, there is only one report to estimate the level of O^6MGMT in pulmonary SCC samples [10]. The present study examined the expression of O^6MGMT immunohistochemically in Japanese melanoma cases. In addition, the study estimated the level of O^6MGMT in skin SCC samples, and compared with the expression to that in melanoma samples.

The level of O^6MGMT expression in all specimens of skin SCC was as high as reported previously [10], in contrast, that in melanoma samples varied widely and a ratio of positively stained cells in each specimen was from 0 % to more than 90 %.

There are several in vitro studies, in which SCC cells are proficient of O^6MGMT and melanoma cells are generally O^6MGMT deficient. Approximately 20 % of human tumor cell strains [6], 7 of 11 human fibroblast cell strains transformed by simian virus 40 [9], or about one-third of human lymphoblastoid cell strains immortalized by Epstein-Barr virus [7, 8] are deficient in the ability to repair O^6AG in DNA. Such a phenotype is termed Mer^- or Mex^- [8]. The present study showed that skin SCC cells strongly expressed O^6MGMT, while the expression in melanoma cells was relatively low in clinical samples from patients. This observation clearly suggests that SCC cells are resistant and melanoma cells are somewhat sensitive to killing by alkylating agents in cancer-prone subjects. Augustine et al. [25] reported that low O^6MGMT expression was associated with sensitivity to temozolomide (TMZ), an alkylating agent commonly used to treat neurological and melanocytic malignancies, in melanoma cells in vitro. Ma et al. [19, 20] evaluated O^6MGMT expression in metastatic lesion of melanoma patients and showed a tendency of lower O^6MGMT expression in responders to DTIC-based chemotherapy in comparison to non-responders. The current study found that primary lesions from cases 6, 7, 10 and 11 had a high O^6MGMT activity and among them only case 6 survived with a good prognosis. However, cases 9 and 4 with a low O^6MGMT activity survived for more than 4 and 1½ years, respectively, without any recurrence or metastasis. The loss of O^6MGMT activity was observed in 2/12 (17 %) specimens (primary lesions of case 4 and the recurrent lesion of case 5). Case 4 is still alive for 1½ years after the resection of the primary lesion. In case 5, we examined the recurrent lesion which had appeared 8 years after the first surgical resection of the primary lesion. In addition, case 5 is still alive more than 2 years after the appearance and the resection of the recurrent lesion. There may be the tendency of the loss of O^6MGMT in responders to DTIC-based chemotherapy.

The present study examined the immunohistochemical expression of O^6MGMT in two independently obtained specimens in cases 1 and 8. Interestingly, the level of O^6MGMT expression tended to increase in case 1 after repeated chemotherapy using DTIC and ACNU. Ma et al. [19, 20] described a higher O^6MGMT expression level in metastatic melanoma lesions following DTIC-based chemotherapy in comparison with that before therapy. They suspected that the appearance of metastatic lesions after chemotherapy may be caused by the increased O^6MGMT activity, thus resulting in increased alkylating agents resistant tumor cells selected by the treatment. Their observation is consistent with the data by Moriwaki et al. [24] describing a higher O^6MGMT activity in melanoma tissues after chemotherapy with DTIC plus ACNU than that before treatment. The current findings of a change in the O^6MGMT level after the chemotherapy imply the adoptive response of the tumor cells treated by the alkylating agents, resulting in the resistance to chemotherapeutic agents [26, 27]. On the other hand, the levels of the expression of O^6MGMT in two independent samples in case 8 were different (90 and 10 %) after the chemotherapy using DTIC and ACNU. This result implies the heterogeneity of advanced melanoma [28].

The molecular mechanism of shifting from the Mer^+/Mex^+ to Mer^-/Mex^- phenotype is related to the methylation in the promoter region of the *O^6MGMT* gene [11–14], not due to a mutation or polymorphism of the gene [15]. Recent studies reported that the decreased O^6MGMT activity due to methylation in promoter region of the gene enhanced the therapeutic response to temozolomide (TMZ) [14, 15]. On the other hand, there are several studies demonstrating that there is lack of association between the methylation status of the promoter region in the *O^6MGMT* gene and the chemotherapeutic response to alkylating agents [11, 18, 21], although all of these data were obtained from patients with an advanced stage of melanoma. In the present study, the shift from Mer^-/Mex^- to Mer^+/Mex^+ phenotype may be related to an epigenetic change of the *O^6MGMT* genome, such as DNA methylation, although the methylation status in the promoter region of *O^6MGMT* gene was not determined.

In conclusion, immunohistochemistry with a monoclonal anti-MGMT antibody was used to demonstrate the expression of O^6MGMT in primary and metastatic lesions from melanoma patients in comparison with that in primary lesions from skin SCC patients. These data in the present study are consistent with the hypothesis that the level of *O^6MGMT* may contribute to the resistance to cancer treatment using DTIC and ACNU. Taken together, the evaluation of O^6MGMT activity in melanoma may, therefore, be useful to determine the tumor phenotype and predict the efficacy of alkylating agents in each melanoma case.

Although additional studies with larger groups of patients that have received chemotherapy are required to

develop a useful marker to predict the response of chemotherapy and patients' prognosis in melanoma, there seems to be the possibility of selective cancer chemotherapy in melanoma in the near future.

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Conflict of interest The authors state no conflict of interest.

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ORIGINAL ARTICLE

Trichothiodystrophy group A: A first Japanese patient with a novel homozygous nonsense mutation in the *GTF2H5* gene

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ABSTRACT

Trichothiodystrophy group A (TTD-A) is one of the three types of photosensitive TTD and is a very rare genodermatosis with deficient post-ultraviolet (UV) DNA repair. We herein describe the first Japanese case with a novel mutation in the *GTF2H5* gene responsible for TTD-A. A 5-year-old male, born as a collodion baby from healthy non-consanguineous parents, exhibited sun sensitivity, brittle hair, ichthyosis, cataracts and mental/physical retardation. He demonstrated neither neurological abnormalities nor pigmentary changes following sun exposure. The patient's primary fibroblasts were hypersensitive to killing by UV (D_0 = 1.5 J/m^2), and the post-UV unscheduled DNA synthesis was 13% of normal. A host cell reactivation complementation analysis showed a decreased DNA capacity without recovery after transfecting any xeroderma pigmentosum genes. We identified a novel homozygous mutation (c.166G>T) in the coding region of the *GTF2H5* gene that resulted in a predicted amino acid change: p.E55X. Thus far, only one Japanese case of TTD with a mutation of the *XPD* gene had been reported. The present case is the first of TTD-A and the second case of TTD in Japan, suggesting that it is necessary to differentiate TTD from other photosensitive disorders, although the incidence of TTD is very low in Japan compared to that observed in Western countries.

Key words: brittle hair, DNA repair, Japanese, photosensitivity, trichothiodystrophy group A.

INTRODUCTION

Trichothiodystrophy (TTD, Mendelian Inheritance in Man 601675) is a very rare autosomal recessive disorder characterized by the presence of sulfur in cystein-deficient brittle hair, ichthyosis, intellectual impairment, decreased fertility and a short statue. Approximately half of reported patients with TTD have photosensitivity, associated with cellular deficiency in nucleotide excision repair (NER) of ultraviolet (UV)-induced DNA damage.[1,2] Among patients with photosensitive TTD, there are three different genetic groups based on inherited mutations in three of the 10 subunits of the basal transcription factor TFIIH: xeroderma pigmentosum group B (XPB), XP group D (XPD) and TTD group A (TTDA, GTF2H5).[3,4] Mutations in at least one gene, *MPLKIP*, have been reported to cause a non-photosensitive form of TTD.[5]

Photosensitive TTD was reported to be very rare, with an overall incidence of TTD of 1.2 per million in Western countries.[6] In Asian countries, the incidence is too low to obtain an accurate assessment. Several dozen photosensitive TTD patients have been reported from Europe and the USA, most of whom were found to have mutation in the *XPD* gene. Only two cases of TTD involved mutations in the *XPB* gene, while four patients from three families were determined to have TTD-A, with only three mutations reported from these TTD-A families.[3] In Asia, only two patients with TTD have been reported from Japan and India; the Japanese case was found to have a mutation in the *XPD* gene.[7]

In this article, we describe the first Japanese case of TTD-A caused by a novel homozygous mutation in the responsible gene (*GFT2H5*) for this rare disease. This case represents the third Asian and second Japanese case of TTD.

METHODS

Report of the patient

A Japanese male neonate was born at term as a collodion baby (Fig. 1a) from non-consanguineous parents. His mother did not experience any problems during pregnancy. A physical examination revealed no evident skeletal or visceral lesions.

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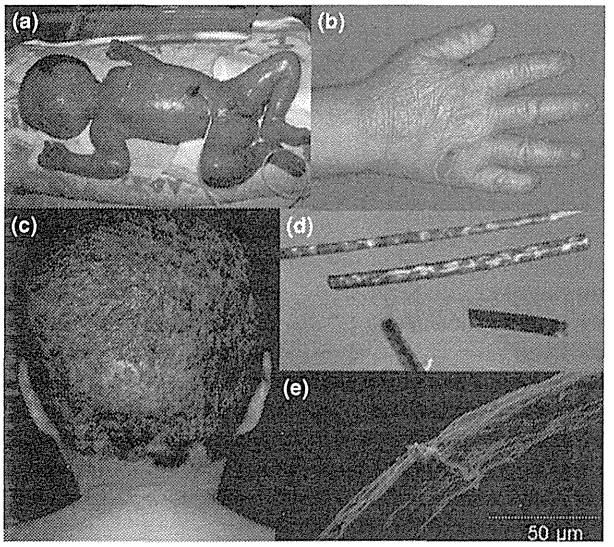

Figure 1. Clinical features of the present patient. (a) The patient was born as a collodion baby. (b) His skin was dry and partially ichthyotic. (c) He exhibited a wide variety of phenotypes including brittle hair. (d) Polarizing microscopy showed characteristic bright and dark regions, a so-called "tiger-tail" appearance. (e) Scanning electron microscopy revealed numerous surface irregularities and findings of trichorrhexis nodosa.

However, mental and physical retardation was subsequently noted. At 1 year of age, the patient was noticed to sunburn easily following sun exposure. At 5 years of age, he presented to our dermatology department for an evaluation of photosensitivity. His skin was dry and partially ichthyotic. His hair was brittle (Fig. 1b) and formed brush-like nodes. He also had cataracts. His hair displayed bright and dark bands, characteristic of "tiger-tail" banding under polarized light (Fig. 1c), and electron microscopy showed a longitudinal split into small fibers, a typical finding of trichorrhexis nodosa (Fig. 1e). There were no other symptoms, including repeated infection and no family history of fragile hair. A biochemical analysis of the patient's scalp hair demonstrated the brittleness to be associated with decreased levels of cystein/cystine in hair proteins to 30% of those observed in normal individuals.

DNA repair and genetic analyses

As a diagnosis of photosensitive TTD was clinically suspected, post-UV DNA repair and molecular studies using primary dermal fibroblasts (cell strain: STUC) obtained from a skin biopsy specimen were performed. A primary fibroblast culture (XP2OS [XPA cells] and N-25 [normal cells]) was used as a control. DNA repair tests, such as the measurement of post-UV unscheduled DNA synthesis (UDS) and post-UV cell survival, were performed using a germicidal lamp emitting predominantly 254-nm light (GL10; Toshiba Electric, Tokyo, Japan) as previously described.[8] The fluence rates were measured using a UV radiometer, UVR-1 (Topcon, Tokyo, Japan). We utilized plasmid host cell reactivation (HCR) to evaluate the cellular DNA repair capacity and perform complementation assignment. Briefly, the plasmid pLuc harboring the *luciferase* gene, was diluted to 40 μg/mL with sterile water and irradiated with UV in a plastic tissue culture dish on ice. Irradiation was performed with germicidal lamps emitting predominantly 254-nm light to a dose of 1500 J/m^2. Fibroblasts were transfected with either UV-irradiated or non-irradiated pLuc (0.1 μg of DNA per well) with expression vectors containing wild-type *XP* cDNA (*XPA, XPB, XPC, XPD, XPF* and *XPG*) in pcDNA3.1 using Effectene transfection reagent (Qiagen, Hilden, Germany). Forty-eight hours after incubation, the cells were lysed with lysis solution (LCβPGC-51; Toyo Ink, Tokyo, Japan) and the luciferase activity was measured using a luciferase assay system (PGL1500; Toyo Ink). The DNA repair capacity was expressed as the percentage of residual luciferase activity following repair of UV-irradiated pLuc compared with non-irradiated pLuc.[8] For the DNA analysis, we cloned the cDNA encoding TTDA protein (an 8-kDa protein: p8) from primary fibroblasts derived from N-25 and the patient. We expressed these p8 in pcDNA3.1. According to the bioinformative analysis of genomic DNA of TTDA, there are three exons in the gene. All three exons and flanking introns of the TTD-A responsible gene (*GTF2H5*; Fig. 2a) in genomic DNA extracted from a normal subject, STUC and the parents' peripheral blood cells were amplified via polymerase chain reaction (PCR) with EX Taq DNA Polymerase (Takara Bio, Shiga, Japan) and the following appropriate primers: TTDA-1F, GGC TGG CTC CAC CCA CAA TA; TTDA-1R, CAG TGT TCC CAC ACC CCA CT (exon 1); TTDA-2F, CAG AGG CAG ATC CCC AAA GT; TTDA-2R, CTG TGC CAC TTG TTA AAA GCG (exon 2); TTDA-3F, GCT CAA GTC TCT GTG ATG TG; and TTDA-3R, AAA GCC AAA TTA CAG CCA ACT G (exon 3).

The PCR products were sequenced using an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions with the previously described primers.

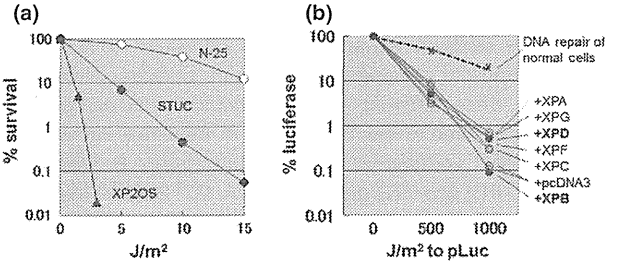

Figure 2. (a) Post-ultraviolet (UV) survival and host cell reactivation assay for the assignment of xeroderma pigmentosum (XP) complementation group in STUC cells. The sensitivity to killing by UV in the STUC cells was much higher than that of normal N-25 cells. (b) The luciferase activity was very low in the STUC cells compared to that in normal N-25 cells and no increased luciferase activity was observed when any of the wild-type *XP* cDNA including *XPB*, *XPD* or the empty vector (*pcDNA3.1*) was transfected into the cells from the patient.

All genetic and cellular analyses using the patient's cells were performed after obtaining institutional approval and written informed consent from the patient's parents and the study was conducted according to the principles of the Declaration of Helsinki.

RESULTS

The post-UV survival and post-UV-induced UDS of primary fibroblasts obtained from the patient were compared to those of cells from a normal subject (N-25) and typical XPA patient (XP2OS). The sensitivity to killing by UV in the STUC cells was much higher than that observed in the normal N-25 cells. The D_0 value (a UV dose that results in 37% cell survival) of the cells obtained from the patient was 1.5 J/m^2, which was much lower than that of the normal N-25 cells (D_0 value: 6.0 J/m^2; Fig. 2a). However, these cells were less sensitive to UV than those of XP2OS (D_0 value: 0.3 J/m^2). The level of UV-induced UDS in the STUC cells was 13% of that of the normal cells. An XP complementation group analysis was performed using a HCR assay in which the cells were co-transfected with a UV-damaged luciferase gene expression vector in addition to expression vectors harboring cloned wild-type *XP*. No increased luciferase activity was observed following transfection of the wild-type *XP* cDNA into the cells obtained from the patient, and the luciferase activity was very low following co-transfection of the cells with expression vectors harboring *XP* genes, including the *XPB* and *XPD* genes, two of the genes responsible for TTD, or the empty vector (Fig. 2b). These clinical and cell biological findings suggested that the patient had TTD group A. Therefore, we sought to identify molecular abnormalities in the *TTDA* gene (Fig. 3a) using appropriate primers and ultimately found that this individual carried a homozygous transversion (c.166G>T) at codon 55, converting GAG (glutamine) to a TGA stop codon (p.E55X; Fig. 3b), with truncated highly conserved proteins (presumably 76% of the normal length). The patient's parents had the same heterozygous mutation (Fig. 3c).

DISCUSSION

Trichothiodystrophy falls within the category of UV-sensitive genetic disorders, such as XP, Cockayne syndrome (CS), XP/CS complex and UV-sensitive syndrome, all of which are related to the deficiencies in the DNA repair pathway of post-UV DNA damage. TTD is a rare inherited condition that involves hair (abnormal brittle hair) and skin (ichthyosis) in addition to physical/developmental delays. The photosensitive type of TTD is mutated in *XPB*, *XPD* and *GTF2H5* (a gene for TTDA/p8), three of the units of TFIIH, which functions as a transcription factor and recruits to the site of UV-induced DNA damage, resulting in subsequent steps of NER: opening, incision and excision. An 8-kDa protein is important for maintaining UV irradiation resistance[9] and is essential for NER and transcription.[10,11]

Thus far, only one Japanese TTD patient, who exhibited compound heterozygous *XPD* mutations (c.2164C>T/del67-69),

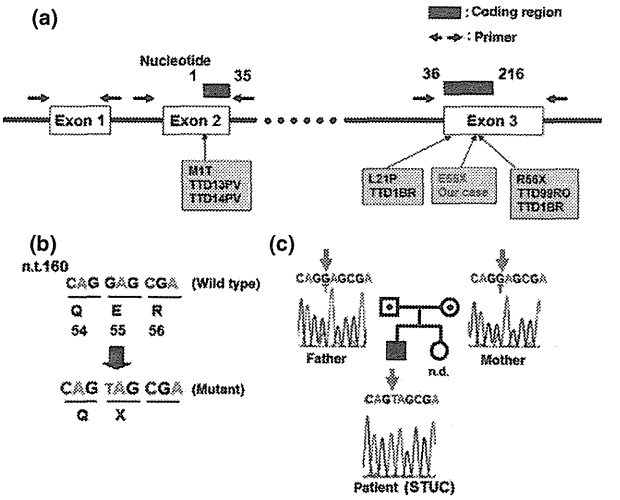

Figure 3. (a) Structure of the *GTF2H5* gene, constructed primers for DNA sequencing and sites and types of amino acid changes in previously reported trichothiodystrophy group A (TTD-A) cases. (b) A nucleotide sequence analysis of the *GFT2H5* gene in the present patient. We identified a homozygous T-to-C change in exon 3 of the *GTF2H5* cDNA (c.166G>C) with a predicted amino acid change (pE55X). (c) A nucleotide sequence analysis of the *TTDA* gene in the patient's parents. Both parents were determined to have the same heterozygous mutation (c.166G>C).

resulting in an amino acid change (R722W/S23del), has been reported.[7] We herein described the second TTD and first Japanese TTD-A patient with a novel homozygous *GTF2H5* mutation. The responsible gene for TTD-A is *GFT2H5*, a component of the TFIIH basal transcription factor, p8, which is involved in NER and RNA transcription induced by RNA polymerase II and is necessary for the stability of the TFIIH complex. The *GFT2H5* gene contains three exons for an 8-kDa protein (71 amino acids). The homozygous c.166G>C change in exon 3 of the *GTF2H5* gene observed in the present case is predicted to result in an amino acid change: p.E55X.

Until recently, there have been only four cases of TTD-A among three families reported worldwide. Each of these families had a different inactivating mutation resulting in a truncated protein: M1T (no start) in TTD13PV and TTD14PV cells, L21P in TTD1BR cells, and R56X in TTD99RO and TTD1BR cells. The mutation detected in the present case is located very close to the sites in previously reported patients; TTD99RO and TTD1BR with homozygous and heterozygous R56X changes, respectively. Therefore, this region in exon 3 may play an important role in the NER as well as transcription activity. As we have not performed TTDA protein analysis, we cannot verify the formation of TTDA proteins truncated by 24% and there is another possibility of no TTDA expression by nonsense-mediated TTDA mRNA decay. However, each of the abnormalities may be related to the various symptoms of TTD noted in the present patient because mutant p8 proteins (L21P and R56X) have been reported to not interact with p52, resulting in a reduced quantity

of TFIIH[10] and a mutant p8 protein (E55X) similar to R56X bearing p8 protein is thought to not function normally.

Thus far, only one Japanese case of TTD with a mutation of the *XPD* gene has been reported. STUC is the second TTD and first TTD-A reported patient in Japan, suggesting that TTD is rarer in Japan than in Western countries (1.2 per million). However, physicians must always differentiate TTD from other photosensitive disorders in dermatology patients.

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CONFLICT OF INTEREST: The authors declare that they have no conflict of interest associated with this study.

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SHORT COMMUNICATION

Detection of Apoptotic Keratinocytes in a Case of Bullous Pemphigoid Developed after Graft-versus-host Disease

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Graft-versus-host disease (GVHD) is a frequent complication following haematopoietic stem cell transplantation. GVHD reveals various skin lesions, such as maculo-papular rash, brownish pigmentation, and scleroderma (1). Although GVHD may present subepidermal blisters caused by vacuolar degeneration of basal cells, the association of bullous pemphigoid (BP) and GVHD is extremely rare (1).

We report here a case of BP that developed 4 months after allogeneic bone marrow transplantation (BMT). Interestingly, histopathology revealed necrotic keratinocytes scattered in the spongiotic epidermis, composed of subepidermal blister roof. We further investigated the necrotic keratinocytes for the presence of apoptotic cells and cleaved caspase 3.

CASE REPORT

A 57-year-old Japanese woman was diagnosed with acute myelogenous leukaemia in November 2002. She achieved complete remission after chemotherapy with cytarabine, daunorubicin and gemcitabine. In June 2004, the patient underwent allogeneic BMT from her human leukocyte antigen (HLA)-identical older sister. GVHD prophylaxis was attempted with cyclosporine and short-term treatment with methotrexate. Complete remission of the acute myelogenous leukaemia and 100% donor chimerism were confirmed. In October 2004 (4 months after BMT), extensive exudative erythemas with blisters appeared over the patient's entire body surface (Fig. 1a). A biopsy from a fresh vesicle showed subepidermal blistering and moderate infiltration of both eosinophils and lymphocytes in both the dermis and the bulla (Fig. 1b). Dyskeratotic keratinocytes were observed in the epidermis at the roof of the bulla with spongiosis (Fig. 1b, c). Shrunken keratinocytes were also demonstrated in the blister roof of different blisters (Fig. 1d). Direct immunofluorescence of skin biopsy showed linear immunoglobulin G (IgG) deposition in the epidermal basement membrane zone (BMZ). Indirect immunofluorescence of normal human skin revealed circulating IgG anti-BMZ antibodies. Indirect immunofluorescence of 1 M sodium chloride (NaCl)-split skin sections showed IgG reactivity with the epidermal side of the split (Fig. 1e). No circulating anti-BMZ antibodies were detected in the older sister. BP180 and BP230 enzyme-linked immunosorbent assays (ELISAs) of the sera from the patient and her sister were negative. Immunoblot analysis of normal human epidermal extracts did not detect IgG antibodies to either BP180 or BP230. Immunoblot analysis of recombinant proteins of NC16a and C-terminal domains of BP180 showed negative results. Oral prednisolone, 1 mg/kg/day, was started, leading to complete disappearance of the skin lesions within one month. Oral prednisolone was discontinued 6 months later. One year later, the patient died from brain metastasis of leukaemia and progressive renal failure.

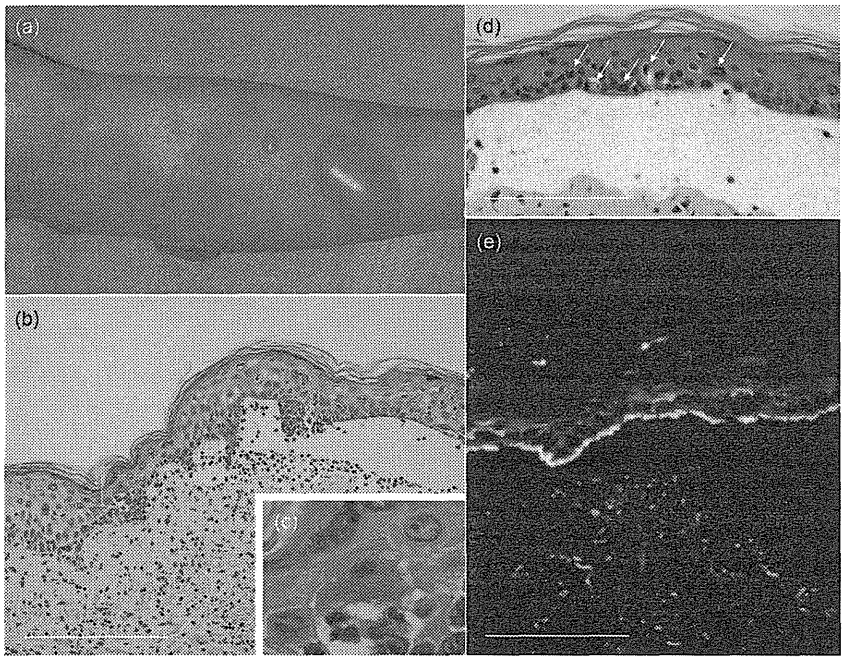

Fig. 1. (a) Clinical feature on the right upper limb. (b–d) Histopathological features. (b) Lower and (c) higher magnifications for a blister. (d) Shrunken keratinocytes in the epidermis of another blister. (e) Indirect immunofluorescence of 1 M sodium chloride (NaCl)-split skin for IgG antibodies. *Scale bars*: (b, d, e) 360 μm.

In order to characterize the necrotic keratinocytes in the blister roof, we conducted transferase dUTP nick end labelling (TUNEL) staining with the ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Merck Millipore, Darmstadt, Germany) (2–4). In the lower epidermis, some keratinocytes facing the vesicle were TUNEL-positive (Fig. 2 a–c). Next, immunofluorescence using anti-cleaved caspase 3 (Asp175) rabbit polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA) was performed on deparaffinized lesional skin sections (5, 6). Keratinocytes with shrunken nuclei showed cleaved caspase 3 in both the cytoplasm and the nucleus (Fig. 2 d–f). There were no keratinocytes that were positive for TUNEL and/or cleaved caspase 3 in BP without BMT (data not shown).

DISCUSSION

Bullous lesions are unusual as skin manifestations of GVHD after BMT (1).

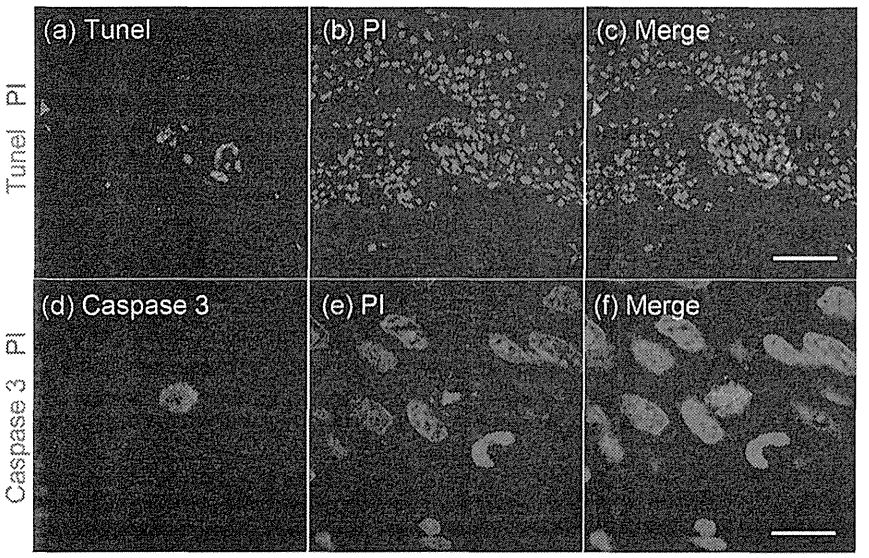

Fig. 2. (a–c) Double staining figures of transferase dUTP nick end labelling (*TUNEL*) and propidium iodide (PI). *Scale bars*: 150 μm. (d–f) Double staining figures of cleaved caspase 3 and PI. *Scale bars*: 30 μm.

In our case, bullous-type chronic cutaneous GVHD with subepidermal blister formation should be considered as a differential diagnosis. However, our case showed linear IgG deposits in the BMZ and circulating anti-BMZ antibodies on immunofluorescence studies, although no reactivity with either BP230 or BP180 was detected on ELISAs and immunoblot analyses. Therefore, we diagnosed our case as BP developed after GVHD.

In our previous study of chronic GVHD, apoptosis occurred and caspases 3 and 8 were cleaved in skin lesions with lichenoid tissue reaction (7). In this study, some keratinocytes with shrunken nuclei were positive for both TUNEL and cleaved caspase 3. Hence, we speculate that apoptosis was also a causative factor in our case for blister formation.

There is one case report of BP that had subepidermal blisters with necrosis of keratinocytes in the blister roof. The eruptions were multiple with centrifugal erythema and blisters. Blisters were annular and narrow and along the margins of the erythema, which showed polycyclic configurations (8). We conclude that caspase activation may have occurred and be responsible for blister formation in this case also.

There are several reports of BP after allogeneic BMT (See (9) and references therein). A recent study has also shown a statistically significant association between BMZ antibodies and GVHD after BMT (10). Izumi et al. (9) speculated that the exposure of BMZ proteins due to the attack to basal cells in GVHD induced antigen exposure. They also stated that, in addition to GVHD-induced predisposition to autoimmunity, treatments for GVHD, including prednisolone and cyclosporine, could disturb the immune system and the Th1/Th2 balance. Thus, in GVHD, the liberation of antigens due to keratinocyte damage, and the disturbance of the immune system by prednisolone and/or cyclosporine might induce the production of anti-BMZ antibodies, as described previously (10).

This is the first study to demonstrate apoptotic keratinocytes in the roof of blisters in BP developed after GVHD. We conclude that, in our case, subclinical GVHD might continue and induce BP after remission of GVHD.

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The authors declare no conflicts of interest.

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