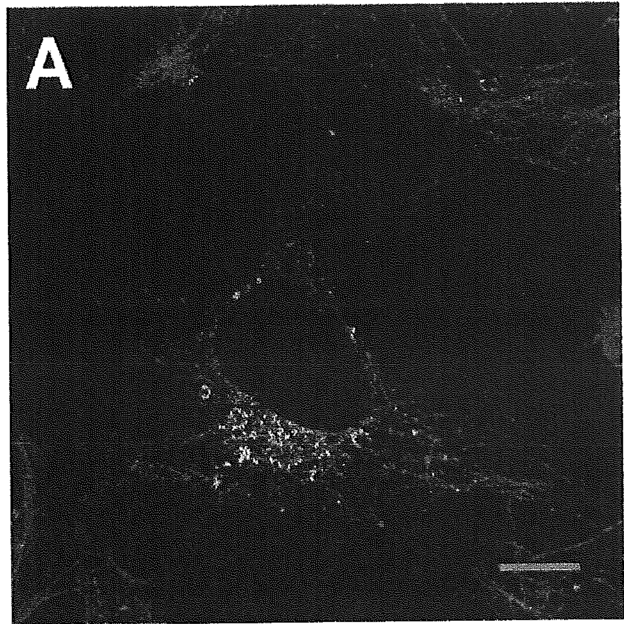
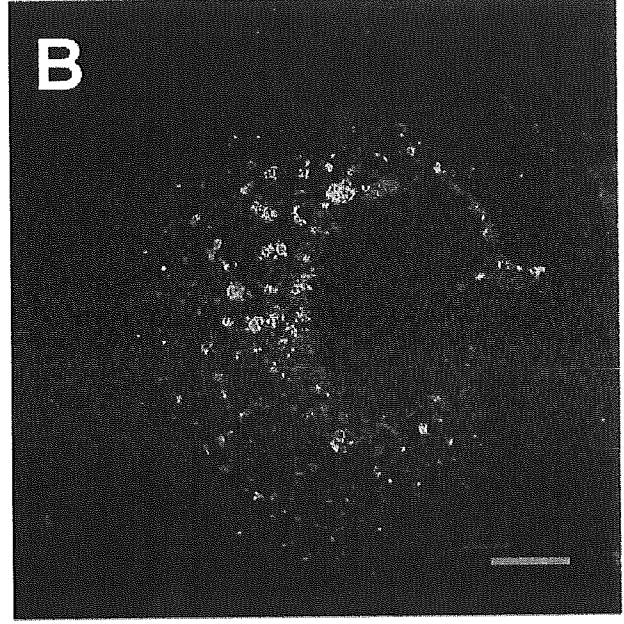
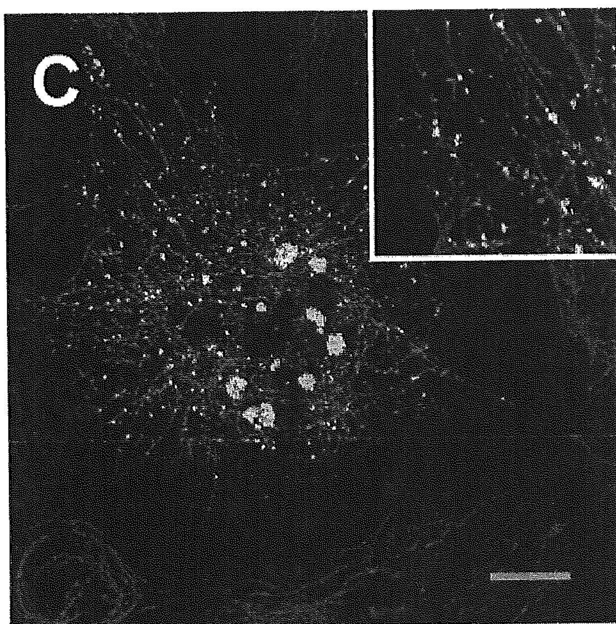
Noc (-) Noc (+)

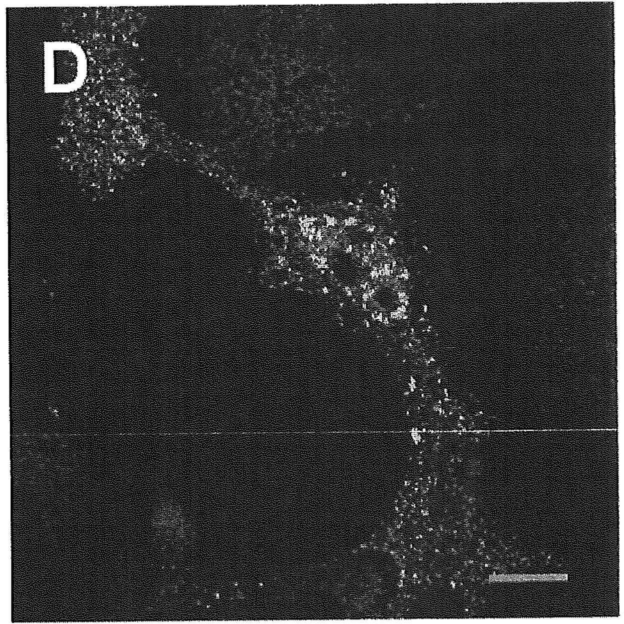
Rab7wt





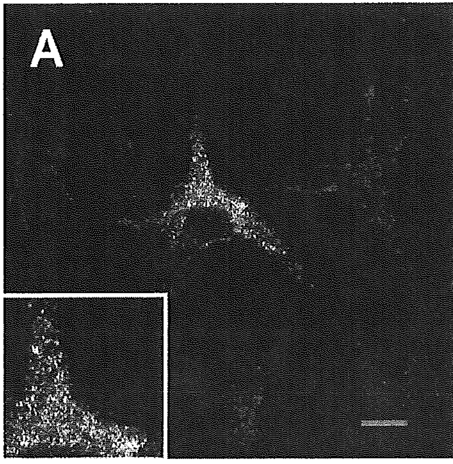
Rab7N125I

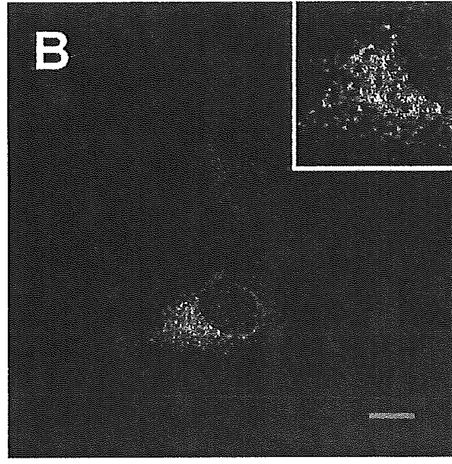


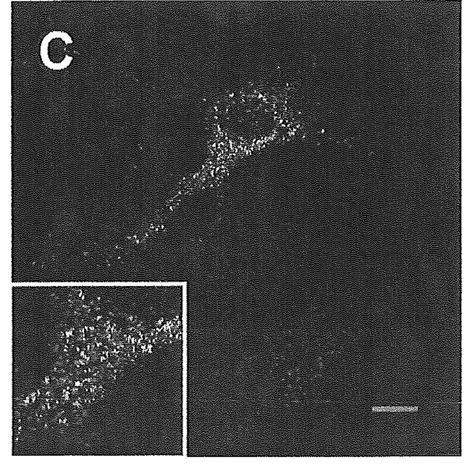


TYRP1 TYR gp100

Rab7wt







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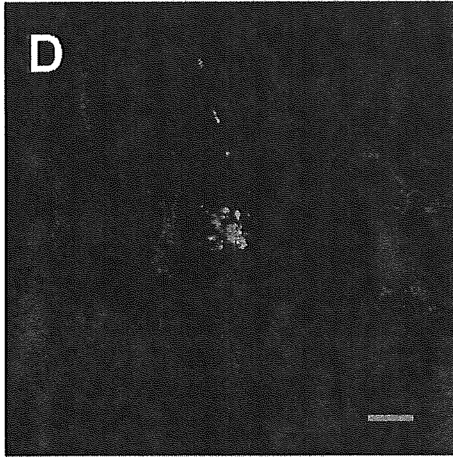

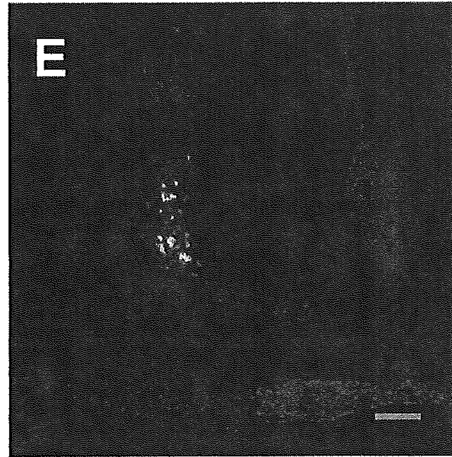

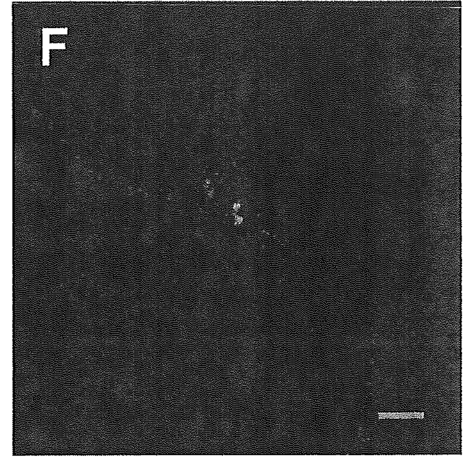

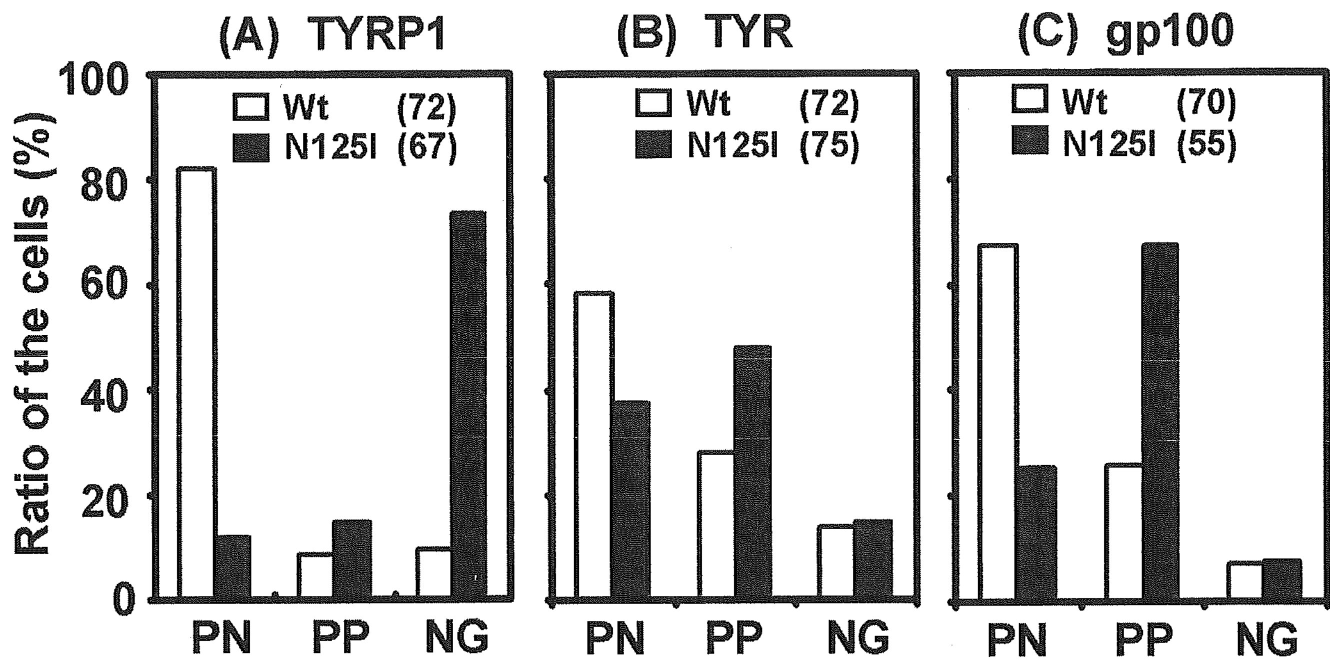
(A) TYRP1
(B) TYR
(C) gp100
Ratio of the cells (%)
100
80
60
40
20
0
Wt (72)
N125I (67)
Wt (72)
N125I (75)
Wt (70)
N125I (55)
PN
PP
NG

Unique Sequence of *Melanocortin 1 Receptor* Gene in Japanese Melanoma; Only Val92Met Is Associated with Higher Susceptibility

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Short title: *MC1R* sequence of Japanese melanoma

Abbreviations used:

ALM: acral lentiginous melanoma

α-MSH: alpha melanocyte-stimulating hormone

BCC: basal cell carcinoma

JST: Japanese skin type

LMM: lentigo maligna melanoma

MC1R: melanocortin 1 receptor

NM: nodular melanoma

PCR: polymerase chain reaction

SCC: squamous cell carcinoma

SSM: superficial spreading melanoma

SUMMARY

Binding of α-MSH to melanocortin 1 receptor (MC1R) initiates melanogenesis through upregulation of cAMP in melanocytes. Specific amino acid changes of MC1R such as Arg151Cys, Arg160Trp and Asp294His have been reported to be associated with a potent predisposition to fair skin, red hair and also to the development of cutaneous melanoma in Caucasians. To elucidate whether specific variants of MC1R are associated with higher susceptibility to melanoma development in Japanese, we analyzed nucleotide sequences of MC1R-encoding gene of blood leukocytes from 95 melanoma patients and 92 non-melanoma controls. A total of seven variants were found to be different from MC1R prototype. Among them, Val92Met was detected in 22 (23.2%) cases and 11 (12.0%) controls, revealing statistical significance in individual ($p<0.05$, Odds ratio=2.2), and showed specific association with non-acral lentiginous melanoma subtypes ($p<0.05$). Furthermore, this variant was observed to appear or disappear in some melanoma tissues compared with that of leukocytes from the same patients. Arg163Gln was found in 94 melanoma patients (98.9%) and 89 controls (96.7%), thus this variant was considered a wild-type allele of Japanese. MC1R with Val92Met could be associated with higher susceptibility to non-ALM melanoma development in Japanese.

Key words: melanocortin 1 receptor, polymorphism, point mutation

INTRODUCTION

Many years have elapsed since the first alert was issued that the destruction of ozone layer would allow ultraviolet (UV) irradiation to reach the surface of the earth more than ever previously, resulting in an increase of harmful effects on the living world. Predictably, the incidence of skin cancer including melanoma has been increasing worldwide year by year (Lens et al., 2004) and, as is often noted, Caucasians are more predisposed to skin cancer due to their higher sensitivity to UV than other races with colored background. The lifetime risk of invasive melanoma in the United States was 1 to 1500 in 1935, 1 to 250 in 1980 and is estimated to be up to 1 in 50 in 2010 (Langley et al., 2003) and, although less significantly, an increasing tendency is also true for Japanese. It is believed that, in non-melanoma skin cancer, UV is one of the most significant factors to enhance tumorigenesis by inducing genomic mutations (Wikonkal et al., 1999). UV is also believed to be one of the causative agents of malignant melanoma, however, the fact that melanoma can develop on skin at any site, including non-exposed areas (Langley et al., 2003), makes its association with UV exposure more difficult to elucidate pathologically.

Melanoma is clinically and histopathologically classified into Clark's subtypes; superficial spreading melanoma (SSM), nodular melanoma (NM), acral lentiginous melanoma (ALM) and lentigo maligna melanoma (LMM), each of which has its own characteristics in terms of site of development, clinical features, overall prognosis and racial frequency. SSM accounts for 70% of the melanoma cases in Caucasians while only 17.5% in Japanese. On the other hand, ALM makes up no more than 10%

of Caucasian cases, whereas it accounts for 48.7% in Japanese (Langley et al., 2003; Ishihara et al., 2001). Since the most prevalent type of cutaneous melanoma in Asians is ALM, which occurs almost exclusively on the palms and soles, this type of melanoma appears to be unrelated to UV exposure. Very few studies have been done to elucidate this enigma, i.e., what makes the difference among these clinical features of melanoma subtypes and racial incidence.

To elucidate the relationship between skin and hair color of individuals and their sensitivity to UV and development of cutaneous melanoma, a number of studies have been carried out on melanocortin 1 receptor (MC1R). This molecule is a key receptor protein that binds α-MSH to initiate biosynthesis of melanin pigment in the melanocyte. In addition to the melanogenesis, MC1R exerts many biological influences on various kinds of cells; (1) modulation of inflammatory and immune systems (Hedley, 1998), (2) stimulation of melanocyte proliferation (Abdel-Malek et al., 1995), and (3) inhibition of melanoma cell proliferation (Robinson and Healy, 2002). MC1R has many genetic variants that differ slightly between human races or among individuals. MC1Rs with some amino acid variations have been reported to show uncommon phenotypes. Specific MC1R variations among Caucasians, including Arg151Cys, Arg160Trp or Asp294His are related to red hair and freckles (Schaffer and Bolognia, 2001) resulting from the interference with eumelanin synthesis (Thody et al., 1991), which is induced by inhibition of cAMP production (Frandberg et al., 1986). However, few studies on *MC1R* alleles of melanoma patients in non-Caucasian populations have been reported so far; thus further

analysis is required to elucidate the relationships between *MC1R* variations, UV sensitivity and development of melanoma.

To assess the association between MC1R amino acid sequence and susceptibility to melanoma, we determined the nucleotide sequence of *MC1R* gene of peripheral leukocytes in Japanese melanoma patients and non-melanoma populations. We also analyzed *MC1R* sequences of melanoma tissues and compared them with corresponding somatic cells.

MATERIALS AND METHODS

Subjects

A total of 95 melanoma patients were enrolled and studied (Table I). They were 44 males and 51 females whose ages ranged from 18 to 93 years old (mean age: 61.6 ± 17.6). The peripheral bloods were collected from three areas in Japan: Kumamoto University (southern area of Japan), Shinshu University (middle area of Japan) and Sapporo Medical University (northern area of Japan). All the melanoma cases were diagnosed clinically and pathologically in between 1989 and 2004, and applicable cases were classified into the four Clark's subtypes as shown in Table I. The control participants enrolled initially were 94 but the subjects whose DNA collected indeed were 92. They were selected randomly as healthy individuals or non-melanoma patients in the hospital, consisting of 55 males and 37 females ranging from 18 to 95 years old (mean age: 53.8 ± 22.2). Aside from 25 normal subjects, the non-melanoma controls consisted of chronic dermatitis (15), chronic

urticaria (2), bacterial or viral infection (13), collagen disease (2), drug eruption (1), burn (1), non-melanoma benign and malignant tumor of the skin (23), and others (10).

The photosensitivity of skin types of enrolled cases was determined by interviews conducted by well-trained dermatologists. Each subject was classified into one of three subgroups of Japanese skin type (JST) (Satoh and Kawada, 1986); JST-I, burn easily and tan minimally; JST-II, burn moderately and tan moderately; JST-III, burn slightly and tan markedly, according to how their skin responds to the first exposure to the sun for about 1 hr at the beginning of the summer. This study was approved by the ethical and human genomic committees of Sapporo Medical University (No.216-5) and was conducted according to the Declaration of Helsinki Principles. All the subjects provided written informed consent.

Preparation of nucleic acids

We collected 6 mL peripheral blood from each subject to obtain RNA and synthesized cDNA as described previously (Jin et al., 2003). Briefly, blood collected in an EDTA-containing tube was applied on 3 mL of Ficoll-Paque™ PLUS (Amersham Biosciences, Uppsala, Sweden) and centrifuged at 14,000 x *g* for 30 min to separate the leukocyte layer. The collected leukocytes were lysed by adding 500 μL of ISOGEN (Nippon Gene, Tokyo, Japan) and 100 μL of chloroform followed by centrifugation, and RNA in the supernatant was precipitated after addition of 250 μL of isopropanol and centrifugation. cDNA was synthesized from 5 μg of RNA as

follows; the RNA was mixed with 1 μg of oligo $(dT)_{15}$, 1 mM dNTP mix, 20 mM DTT, 8 μL of 5X first-strand buffer and 400 U of Superscript II (Invitrogen life technologies, Carlsbad, CA) in a total volume of 40 μL. The reaction mixture was incubated at 45°C for 50 min, and at 70°C for 15 min in that order. Genomic DNA purified from peripheral blood collected at Shinshu University was directly processed for PCR using the same primers, because *MC1R* gene is intronless.

We extracted genomic DNA from some melanoma tissues using TaKaRa DEXPAT™ (Takara Bio, Otsu, Japan). Melanoma cells in approximately 5 μm thick paraffin-embedded tissue section were scraped off and transferred into an Eppendorf tube. Then, 0.5 mL of DEXPAT™ was added and incubated at 100°C for 10 min followed by centrifugation at 14,000 x *g* for 10 min at 4°C. The supernatant including genomic DNA was used for the sequence analysis.

Nucleotide sequencing

The whole coding sequence of the cDNA for human *MC1R* gene (1381-2331) (GenBank: XM047456) was amplified with polymerase chain reaction (PCR) using primers as follows. The N-terminal sequence from 1350 nucleotide position (n.p.) through 1910 n.p. was amplified by primers MC1R-1F: 5'-AAC GAC TCC TTC CTG CTT CC-3' and MC1R-1R: 5'-GTG CTG AAG ACG ACA CTG GC-3' and the C-terminal sequence from 1821 n.p. through 2361 n.p. by MC1R-2F: 5'-CTA CGC ACT GCG CTA CCA CA-3' and MC1R-2R: 5'-GCA CAC TTA AAG CCG CGT GCA-3'.

Genomic DNA prepared from paraffin-embedded sections was processed for amplification of short sequences that contain the alleles of interest; for 1601 n.p.-1780 n.p., MC1R-aF: 5'-ACT GCT TCA TCT GCT GCC TG-3' and MC1R-aR: 5'-AGC AGA GGC TGG ACA GCA TG-3', for 1791 n.p.-1910 n.p., MC1R-bF: 5'-CAT CGC CGT GGA CCG CTA CA-3' and MC1R-1R, for 1481 n.p.-1680 n.p., MC1R-cF: 5'-GGT GCC TGG AGG TGT CCA TC-3' and MC1R-cR: 5'-CAG GAG GAT GAC GGC CGT CT-3' and for 1711 n.p.-1926 n.p., MC1R-dF: 5'-GCG GTG CTG CAG CAG CTG GA-3' and MC1R-dR: 5'-GTA GGC GAT GAA GAG CGT GCT G-3'.

For amplification, 125-250 ng of cDNA was added to the solution of 50 μL containing 5 μL of 10X buffer, 1.5 mM $MgCl_2$, 0.2 mM each of the four dNTPs, 1.25 U of Taq DNA polymerase (Promega, Madison, WI) and 30 pmol of each primer. The PCR condition was initially 95°C for 2 min, then 40 cycles of 94°C for 30 s, either 58°C for MC1R-1, -2, -a and -b or 62°C for MC1R-c and -d, for 1 min, and 72°C for 2 min, and finally 72°C for 5 min extension. The PCR products were purified by 1% agarose gel electrophoresis followed by electroelution, phenol extraction and ethanol precipitation. Then, they were processed to cycle sequencing on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan) using ABI Prism™ BigDye™ Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacture's specification.

Statistical analysis

To perform statistical analysis, one from among chi-scquare test, Fisher's exact

test or Mann-Whitney's U-test was chosen for each analysis depending upon which test was the most appropriate.

RESULTS

Variants of *MC1R* gene

MC1R is transcribed in various cells including leukocytes so that its nucleotide sequence can be determined from not only genomic DNA but also mRNA of blood leukocytes. As a result of analysis of a total of 187 samples, we detected five and two variant alleles with or without amino acid changes, respectively (Table II). The variant kinds found in the present study were far less than those in Caucasians reported previously (Kennedy et al., 2001) and all of the three variants detected from genomic DNA samples of Shinshu University were included in the five detected from cDNA samples of other institutes. Arg67Gln substitution was found in four cases (4.2%) and two controls (2.2%) ($p=0.683$), Leu85Gln in only one case (1.1%) ($p=1.000$) and Ile120Thr in five cases (5.3%) and one control (1.1%) ($p=0.212$). Every allele revealing a variant in these three was heterozygous with consensus allele.

Val92Met was exhibited in 22 cases (23.2%) whereas it was only in 11 controls (12.0%) ($p=0.045$, Odds ratio=2.2). On the other hand, the frequencies of this variant allele in terms of haplotype were 12.1% in cases and 7.1% in controls, revealing no statistical significance ($p=0.099$). Arg163Gln was detected in every subject studied except in one case (i.e. carried in 94 of 95, 98.9%) and in three controls (i.e. carried

in 89 of 92, 96.7%) (p=0.297). The frequency of this variant in haplotype was also remarkably higher both in cases and controls than other variants. Interestingly, 20 of 21 cases carrying heterozygous Val92Met possessed heterozygous Arg163Gln simultaneously and one case carrying homozygous Val92Met possessed no Arg163Gln substitution but consensus alleles (Table III). On the other hand, since those with homozygous consensus Val92 tended to demonstrate homozygous Arg163Gln, the correlation of haplotype between Val92Met and Arg163Gln in melanoma cases revealed marked significance (p<0.0001). However, this correlation between the two variants in controls also showed marked significance (p<0.0001), hence the serial feature penetrated to both groups and did not correlate to the melanoma development.

Two other variants, Ser83Ser (G249A) and Thr314Thr (A942G), were synonymous polymorphisms which do not cause alteration of amino acid sequence (Table II). Ser83Ser was seen in just one control (1.1%) (p=0.492), and Thr314Thr was seen in 20 cases (21.1%) and 16 controls (17.4%) (p=0.526). Each of these two variants showed no specific statistical finding by itself, however, A942G exhibited a strong association to coexist with Val92Met both in melanoma cases (p<0.0001) and in controls (p<0.0001) (Table III).

***MC1R* gene and clinical data**

The correlation of melanoma subtypes and the five nonsynonymous variants, Arg67Gln, Leu85Gln, Val92Met, Ile120Thr and Arg163Gln is summarized in Table IV.

Although Val92Met was found in all four subtypes, its ratio was not always the same in each; Val92Met was detected only in 14.9% (7/47) of ALM, whereas it was found in 33.3% of SSM (3/9) and NM (4/12), and in 36.4% of LMM (4/11). This tendency of Val92Met to occur more frequently in non-ALM cutaneous melanomas showed statistical significance (p=0.043, Fig 1). Arg163Gln was found in almost all the cases, thus no specific prevalence was observed for this variant. Arg67Gln and Ile120Thr were identified in three ALM each and in one with other origin than the skin (p=0.268), and one SSM (p=0.643), respectively. Leu85Gln was observed in only one SSM case (p=0.114). Therefore, with the exception of the relevance between Val92Met and non-ALM cutaneous melanoma, no other association between MC1R variants and melanoma subtypes became apparent.

Subsequently, the variant Val92Met was analyzed further with the clinical data of the melanoma cases, because Val92Met alone showed statistical significance. Categorization into JSTs, as a research item, was not done at the beginning of this study and therefore information about JST was not available for every subject. None of the characteristics consisting of age, gender, JST, clinical stage and tumor thickness, however, had any statistical significance with Val92Met (data not shown).

MC1R gene of melanoma cells

We carried out the same procedures both for genomic DNA extracted from paraffin-embedded melanoma and for mRNA from untreated melanoma tissue as for cDNA synthesized from blood leukocytes, and then compared their *MC1R*

sequences from the same individual. The cDNA obtained from untreated melanoma tissues were of three primary lesions and five metastatic lesions, two samples each of which were from the same cases. According to the DNA from paraffin-embedded tissue, some samples did not provide proper DNA so the DNA finally collected were five, which were from three primary and two metastatic lesions. Of the eight cDNAs from untreated melanoma tissues, no difference in the *MC1R* sequence whatsoever was disclosed compared with that from blood cells. Of the five DNA extracted from paraffin-embedded melanomas, however, three samples revealed obvious differences from cDNA synthesized from leukocytes in the same subjects. The alterations were confined to the 92nd codon where two cases showed changes from heterozygous G274A of leukocytes to consensus G274, whereas another exhibited the opposite behavior, i.e., consensus G274 of leukocytes to heterozygous G274A of melanoma cells (Fig 2). The other samples of melanoma tissues showed no difference anywhere in the sequence from that obtained from leukocytes.

DISCUSSION

MC1R belongs to a melanocortin receptor subfamily comprising five members. They are distributed to various kinds of cells. Aside from MC1R, MC2R is expressed almost exclusively on adrenal gland cells, MC3R on central nervous cells or macrophages, MC4R on central nervous cells, in association with regulation of appetite, and MC5R on many kinds of exocrine glands including foreskin, lacrimal, sebaceous and eccrine glands (Bohm and Luger, 2000). Besides melanocytes,

MC1R has been found on various kinds of cells such as keratinocytes, microvascular endothelial cells, human dermal fibroblasts, mast cells (Bohm and Luger, 2000), macrophages (Luger et al., 1999), follicular epithelia, sebaceous glands and secretory and ductal epithelia of sweat glands (Bohm and Luger, 2000). MC1R responds mainly to α -MSH comparably to or rather more potently than adrenocorticotropic hormone (ACTH) followed by β-MSH and γ-MSH (Suzuki et al., 1996). Interaction of MC1R with MSH promotes adenylcyclase to upregulate cAMP, leading to melanogenesis and proliferation of melanocytes via activation of protein kinase A and/or C (Park et al., 1996), CREB and microphthalmia-associated transcription factor (MITF) (Tachibana, 2000).

Finally, MC1R contributes distinctly to individual skin or hair color. The diversity of coat color of mammals represented by mouse hair or human skin and hair refers to the MC1R function in terms of the difference in quality and quantity of melanin production. Eumelanin is a black-brown pigment while pheomelanin is a yellow-red one and the proportion of these two kinds of melanin determines the coat color (Jimbow et al., 1983; Rees, 2004). In mice, the synthesis of pheomelanin is under the regulation of *agouti* gene coding for the agouti signaling protein, the physiological antagonist of α-MSH (Rees, 2004). Although neither agouti gene nor agouti signaling protein has been found yet in human, the diversity of melanin production has been confirmed to depend partly on MC1R potential. Approximately half of African populations have consensus *MC1R* sequence and most of the other half have only Thr314Thr (A942G) (Rana et al., 1999; Harding et al., 2000), whereas

more than 65 variants of *MC1R* gene have been reported, mainly from Caucasians so far (Rees, 2004), some of which had a strong association with skin and hair color.

Of the many *MC1R* gene variants, Arg151Cys, Arg160Trp and Asp294His have been described as reliable candidates for the responsibility for red hair and fair skin in Caucasian (Smith et al., 1998; Palmer et al., 2000; Bastiaens et al., 2001). As for other variants such as Val60Leu, Asp84Glu and Arg142His, the correlation with hair or skin color is controversial or rather weak, if any (Palmer et al., 2000; Valverde et al., 1996; Ichii-Jones et al., 1998; Schioth et al., 1999). Other frequent variants, Val92Met, Arg163Gln and Thr314Thr may have prevalence in specific regions or populations, but no significant association of these variants with light hair and skin colors and/or susceptibility to melanoma development has been reported (Rana et al., 1999; Sturm, 2002; Na et al., 2003). Since MC1R plays a key role in pigmentation, it was interesting to consider whether MC1R is involved in pigmentary phenotypes including freckles, photosensitivity, development of pigment cell nevus and carcinogenesis. Some active MC1R variants were reported to be associated with skin types (Bastiaens et al., 2001; Healy et al., 2000), freckles, melanoma (Palmer et al., 2000) and basal cell carcinoma (BCC) or squamous cell carcinoma (SCC) (Bastiaens et al., 2001; Box et al., 2001), whereas no variant was reported to be associated with the number of moles (Palmer et al., 2000). In our study, the control group included malignant skin tumors such as three cases of BCC, four SCC, three extramammary Paget's disease, three Bowen's disease and so on, though only one with extramammary Paget's disease had Val92Met, Arg163Gln and Thr314Thr

(A942), and only one with SCC had Ser83Ser and Arg163Gln, while all the other individuals with malignant skin tumors carried only Arg163Gln.

In the present study, we have found that (1) Val92Met showed no statistical significance in haplotype of melanoma cases (p=0.099), but did reveal a statistical significance in individual of melanoma cases (p=0.045), (2) cutaneous melanoma with Val92Met occurred preferentially with non-ALM subtype (p=0.043) and (3) no variants described previously as susceptible to melanoma in Caucasian were detected in Japanese melanoma. No variant in the seven detected was novel but some variants had characteristic penetrance (Table II). Arg163Gln was observed in 97.9% (183/187) of the total subjects, indicating that this variant must be a wild-type allele in Japanese. As similar results on the frequency of Arg163Gln were reported previously in Korean (Na et al., 2003) and in American Indian subjects (Rana et al., 1999), this variant may form a peculiar distribution in ethnic groups according to genetic evolution (Rana et al., 1999).

Because of the small number of reports on *MC1R* of Japanese, the frequency of Val92Met of Japanese population is disputable; Rana et al (Rana et al., 1999) showed 23% in East and Southeast Asian population and Harding et al (Harding et al., 2000) reported 6.7% in Japanese haplotype. In our study, Val92Met substitution did not show higher frequency in melanoma cases than in the report of Rana et al (Rana et al., 1999), but it did show lower frequency in controls, thus consequently its statistical analysis in individual reached to significance. On the contrary, the statistical analysis in haplotype revealed no significance despite of higher frequency

of this variant in cases than in controls. Considering the number of subjects with homozygous variant (only 1 case and 2 controls), these results may indicate that Val92Met, even in heterozygote, may play a role in melanoma development in Japanese independently on haplotype. Furthermore the occurrence of Val92Met in association with non-ALM cutaneous melanoma revealed a statistical significance indeed (Fig 1), implying that this variant could be involved with UV-associated melanoma in Japanese through the pathway like in Caucasians. However, the number was not sufficient to sustain strongly this tendency and, moreover, since no other characteristic features could be linked with Val92Met, the background information and conclusions with respect to this variant cannot be definitely elucidated as yet.

Val92Met has been found to a certain degree in populations around the world and, with the exception of a few studies, has been described not to affect the pigmentation-related phenotypes. Ichii-Jones et al (Ichii-Jones et al., 1998) explained that Val92Met may correlate to melanoma limited to skin type III or IV, and Na et al (Na et al., 2003) stated that a higher ratio of Val92Met was carried in vitiligo patients than in controls (no statistical significance, though). Furthermore, the argument about the effect of Val92Met on MC1R *in vitro* is controversial; Koppula et al (Koppula et al., 1997) and Scott et al (Scott et al., 2002) concluded that Val92Met did not cause loss-of-function of MC1R whereas Xu et al (Xu et al., 1996) and Ringholm et al (Ringholm et al., 2004) reported that it had decreased affinity to α-MSH, resulting in red hair and burning tendency of skin type I and II. Our result, however, may be