such as leptin, and HCC but the molecular mechanisms have not been clarified yet. Hepatocarcinogenesis is a multi-step process involving different genetic alterations that ultimately lead to malignant transformation of the hepatocyte [16,17]. One of the molecular events that underlie the multigenetic process of hepatocarcinogenesis is activation of human telomerase reverse transcriptase (hTERT)/telomerase which is normally suppressed in most human somatic tissues after birth [18,19].

In the present study we investigated, for the first time, the relationship between leptin, leptin receptors and hTERT mRNA expression in HCC. We also attempted to elucidate on the molecular pathways that may mediate this interaction by investigating the regulation of *hTERT* gene promoter by histone acetylation status as well as STAT3 and c-myc transcription factors. Finally, the biological effects of leptin in HCC progression through inflammatory cytokines such as IL-1, IL-6, TGF and MMPs were assessed.

Methods

Subjects

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the local Ethical Committee of the University Hospital of Larissa and by the Institutional Review Board (Institute of Medical Science, University of Tokyo). Specifically, control liver tissue specimens were obtained after oral informed consent from 23 patients (eleven male, twelve female; mean age 54.9 years, range 37-84 years) during an operation that was performed for cholelithiasis (cholecystectomy). All these individuals had apparently no evidence of chronic liver disease and normal ALT (alanine aminotransferase) values (26.6 ± 4.9 U/L), tested negative for HBsAg, anti-HCV and anti-HIV antibodies and denied ever having used hepatotoxic drugs, herbals, or having abused alcohol or injected drugs.

Twenty three liver tissue samples from HCC patients were used in this study, which were purchased from Biomax (US Biomax Inc, MD, USA) and were also provided from the University of Tokyo (thirteen male, ten female; mean age: 58.4 years; range: 45-75 years). Written informed consent was obtained from the patients. The diagnostic criteria for HCC were based on the conclusions of the Barcelona-2000 EASL conference while the histological diagnosis was made according to the AJCC/UICC classification system [20,21]. From the 23 HCC tissue samples, 8 were due to HBV-related cirrhosis and 15 were due to HCV-related cirrhosis.

Cell cultures, reagents and treatments

HepG2 hepatocellular carcinoma cells were used and were cultured in RPMI 1640 medium (Gibco, Paisley, Scotland, UK) supplemented with 10% fetal bovine serum (Gibco, Paisley, Scotland, UK), L-Glutamine 2 mM (Gibco, Paisley, Scotland, UK), penicillin 100 IU/ml and streptomycin 100 μg/ml (Gibco, Paisley, Scotland, UK), at 37°C in 5% CO_2. After 16 hours of serum starvation, the culture media were changed to serum free media containing leptin. Cultures were treated with human recombinant leptin at 25, 50, 100, 200 ng/ml (R&D Systems, Minneapolis, MN, USA). Cell culture supernates were removed, centrifuged and stored at -80°C until assayed. Leptin, TGF-b1, IL-6, IL-1b and IL-1a were measured using commercially available assays according to manufacturers' instructions (R&D Systems, Minneapolis, MN, USA).

RNA isolation and Real-time PCR

Each sample was homogenized and total cellular RNA was extracted, reverse transcribed to cDNA and real-time PCR was performed for leptin, OB-Rs, OB-Rl and telomerase, as previously described [22,23]

Immunohistochemistry for hTERT, leptin and OB-R

Immunohistochemical staining for hTERT and leptin expression was completed using antihuman hTERT antibody (PC563) (EMD Biosciences, Merck KGaA, Darmstadt, Germany), A20 leptin polyclonal Ab (pAb) (Santa Cruz Biotechnology, Santa Cruz, USA), or the M18 ObR pAb, (Santa Cruz Biotechnology) according to standard IHC procedures [24].

Cell viability

Cell viability was determined with the MTT assay using the TACS MTT kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. HepG2 proliferation was assessed in the presence of increasing concentrations of leptin (0-200 ng/ml) or in the absence of leptin (siRNA treatment against leptin). Cell proliferation was examined at 12h, 24h and 48h after addition of leptin.

TRAP assay

TRAP (telomeric repeat protocol assay) assay was performed using the TeloTAGGG telomerase PCR ELISA PLUS kit (Roche, Indianapolis, IN, USA) as previously described [25].

Small interfering RNA treatment

HepG2 cells were transfected with dsRNA oligonucleotides for leptin using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Different doses of siRNAs were administered at first for either 24, 48, 72 hours, in order to define the optimum dosage and time for a satisfying silencing, controlled by real time RT-PCR and ELISA

(cell culture supernates). Negative controls (scrambled) were used in order to verify the absence of toxicity for the different doses administered.

Chromatin immunoprecipitation

Chromatin Immunoprecipitation was performed using a ChIP assay kit (Upstate USA, Inc., Charlottesville, VA, USA). The immunoprecipitated DNAs were amplified by PCR with the primers indicated below. For leptin promoter (proximal promoter, forward: 5'-CCCTCTAA CCCTGGGCTTC-3'; reverse: 5'-ACTATGGCGCAAG-GACCAG-3'), for hTERT promoter (set 1 for STAT3, forward: 5'-CCAAACCTGTGGACAGAACC-3'; reverse: 5'-AGACTGACTGCCTCCATCGT-3', set 2 for STAT3, forward: 5'- GGGGTGTCTTCTGGGTATCA-3'; reverse: 5'-AAGGGCTGTGTTTGTGAATTG-3', proximal hTERT promoter, forward: 5'- TGCCCCTTCACCTTC-CAGCTC-3'; reverse: 5'- GTGGCCGGGGCCAGG GCTT-3').

Flow cytometry

Cell cycle distribution was determined by flow cytometry. At least 10.000 events were collected for each sample. Intracellular staining antibodies against MMP-1, MMP-9, proMMP-13 were used for cytometric analysis of HepG2 cells according to manufacturers instructions (R&D Systems, Minneapolis, MN, USA). Effect of leptin treatment (50, 200 ng/ml for 48 h and 100 ng/ml for 2 months) and leptin siRNA on MMP-1, MMP-9 and MMP-13 protein levels were evaluated.

Statistical analysis

Statistical analysis was performed as previously described [22].

Results

Leptin, OB-Rl and OB-Rs expression in liver tissues of HCC patients

In order to test the malignant dynamics of leptin in liver, we evaluated leptin and leptin receptors mRNA and protein expression using real-time RT-PCR and immunohistochemistry (IHC) respectively, in HCC and non-HCC liver tissues. Leptin was not expressed in any healthy liver tissue, but was expressed in 18 out of 23 HCC tissues as evaluated by RT-PCR or IHC (78.2%). More specifically, regarding real-time PCR data, mean leptin levels were $6.1 \pm 3.21 \times 10^{-2}$, while no difference in leptin expression levels was found between the HBV and HCV subgroups of the HCC group. Significant differences were observed between the mean OB-Rl and OB-Rs mRNA levels in HCC liver tissues (0.726 ± 0.155 and 0.227 ± 0.092, respectively,) and healthy tissues (0.0165 ± 0.0031 and 0.0292 ± 0.00194, respectively) ($p < 0.001$) (Figure 1).

Correlation of leptin expression with hTERT expression

Interestingly, taking into account our previous findings in chronic viral hepatitis and HCC (altered leptin and hTERT mRNA levels in HCC or chronic viral hepatitis liver samples compared to healthy liver samples), we proceeded to determine whether there is an association between leptin and hTERT mRNA expression [22,23]. We found a significant association between leptin and hTERT mRNA expression only in HCC livers ($r = 0.79$, $p < 0.05$).

Leptin affects hTERT expression levels and TA in HCC cells

The association between leptin and hTERT/TA in HCC samples prompted us to study the effect of leptin administration on hTERT in HepG2 cells. When HepG2 cells were treated with leptin concentrations of 50, 100, 200 ng/ml for 48 hours and 100 ng/ml for 2 months, we observed that hTERT mRNA levels and TA were significantly increased (Figure 2a, b). We then blocked leptin's expression in HepG2 cells using siRNA against leptin and transfection with liposomes and did not observe a significant decrease in hTERT mRNA levels and TA (Figure 2a, b).

The JAK/STAT3 pathway and the Myc/Max/Mad network are important for leptin-mediated up-regulation of hTERT expression

To gain insight into the mechanism underlying the leptin-mediated transactivation of hTERT promoter on HCC cells, we next examined signal transduction pathways possibly involved in mediating leptin's action. The presence of STAT3 binding sites in hTERT promoter and the role of STAT3 in leptin response, suggest that these sites may be involved in leptin's control of hTERT expression. Chromatin immunoprecipitation assays were performed with all putative STAT3 binding sites. In HepG2 cells, STAT3 was found to be associated with site 1 and 2 within hTERT promoter. Short and long term leptin stimulation (200 ng/ml for 48 h and 100 ng/ml for 2 months) of HepG2 led to the recruitment of STAT3 at the hTERT promoter (Figure 3a). In addition, using ChIP analysis we obtained direct evidence for the interaction between c-Myc, Mad1, Max and acetylated H3 with hTERT promoter. In untreated HepG2 cells an hTERT signal was observed in the Mad and Max immunoprecipitations, whereas in leptin treated cells (200 ng/ml for 48 h) a strong hTERT signal was ditected in the Myc/Max immunoprecipitations (Figure 3b). Interestingly, long term (two months with 100 ng/ml) leptin treatment of HepG2 attenuated the binding of Myc/Max to hTERT promoter. On the other hand acetylated H3 was found to bind on hTERT promoter only after long term leptin treatment (2 months) (Figure 3b).

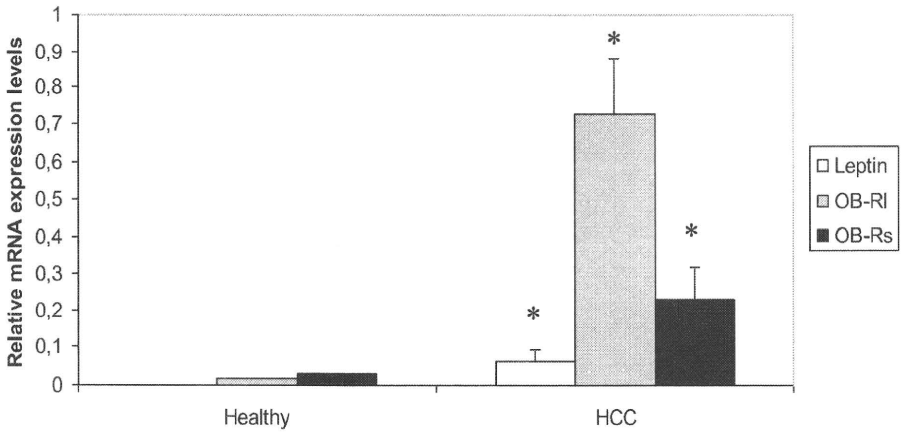


Figure 1 Leptin, OB-Rl or OB-Rs expression levels in healthy and HCC liver tissues. Comparison of liver tissues of healthy individuals and HCC patients with respect to mean leptin, OB-Rl, OB-Rs expression levels (leptin or OB-Rl or OB-Rs mRNA copies/PBGD copies) obtained after real time RT-PCR analysis. Bars, means ± standard deviation,*, $p < 0.05$ compared to healthy liver tissues.

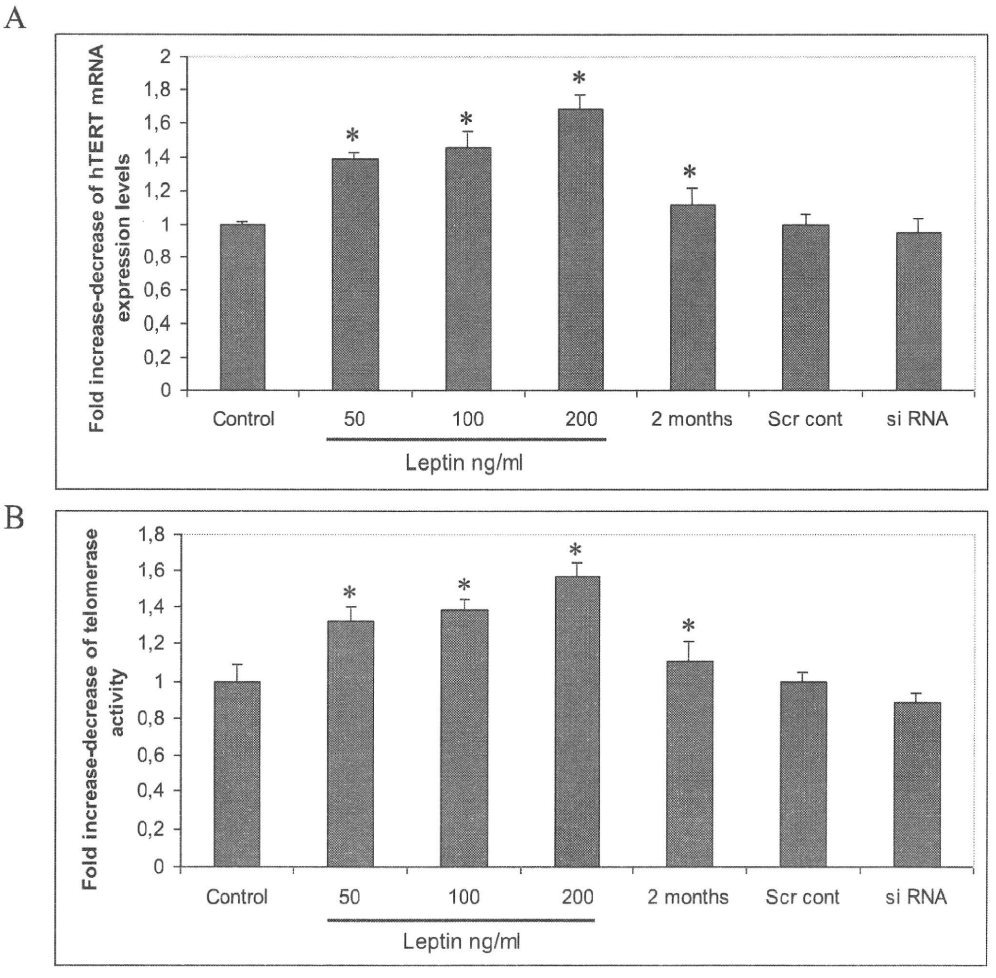


Figure 2 Leptin promotes hTERT expression and TA in HepG2 cells. (a) Total RNA was isolated and its expression was evaluated using real-time RT-PCR, to determine changes in the level of hTERT mRNA expression after normalization to PBGD expression. All data were presented as a fold induction relative to untreated cells. Columns, mean of three independent experiments done in triplicate; bars, SD; *, $p < 0.05$ compared to untreated cells, (b) Telomerase activity after leptin and leptin siRNA treatment as mentioned above.

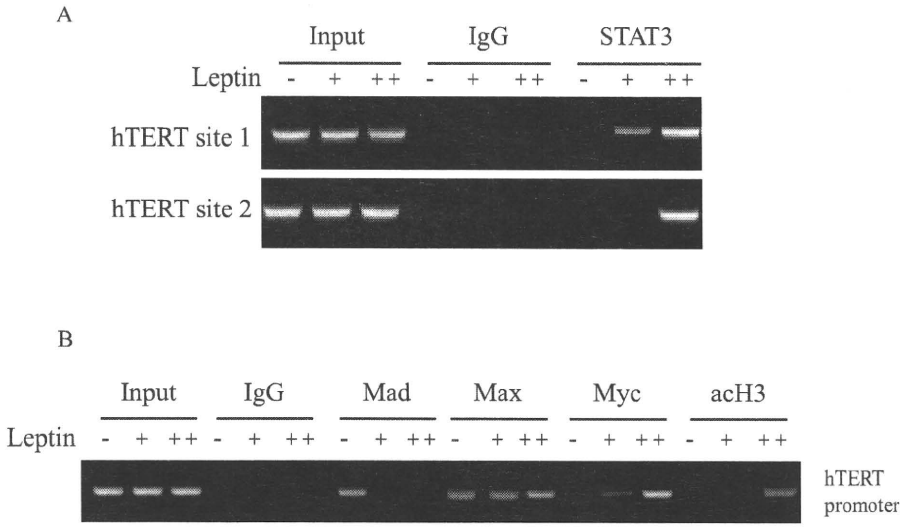


Figure 3 Leptin enhances the binding of STAT3, Myc, H3 to *hTERT* promoter. (a) ChIP assay was performed using anti-STAT3 antibody or irrelevant anti-immunoglobin G antibody as negative control. Input samples are total genomic DNAs used as control for the PCR. PCR primers covering the STAT3 binding sites of *hTERT* promoter region were used to detect promoter fragment in immunoprecipitates. Leptin (-), untreated cells, Leptin (+), 200 ng/ml for 48 h, Leptin (++), 100 ng/ml for 2 months. (b) ChIP assay was performed using anti-Mad1, anti-Max, anti-Myc and anti-acetylated H3 antibodies or irrelevant anti-IgG antibody as negative control. PCR primers covering proximal hTERT promoter were used. Leptin (-), untreated cells, Leptin (+), 200 ng/ml for 48 h, Leptin (++), 100 ng/ml for 2 months

Leptin administration affects cell proliferation and modulates the cell cycle of HCC cells

As leptin-mediated overexpression of hTERT might lead to tumorigenic growth and deregulated cell cycle, we investigated, next, the effect of leptin on HepG2 cells proliferation using the MTT assay. Leptin stimulated the growth of HepG2 cells in a time- and dose- dependent manner. Furthermore leptin's knockdown was correlated with a notable reduction in proliferation rate (Figure 4a). Additionally, we observed that treatment with leptin deregulated HepG2 cell cycle, as it increased the proportion of HepG2 in S and G2/M phase, while leptin's knockdown decreased the proportion of HepG2 in S and G2/M phase compared to untreated cells (Figure 4b).

Leptin could affect tumor progression and invasion dynamics in HCC

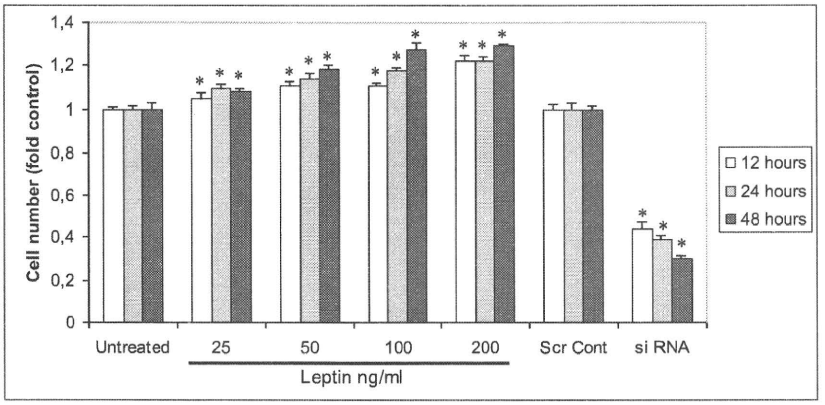
The possible role of the inflammatory cytokines in the development and spread of cancer cells led us to examine the involvement of leptin in the production of IL-1a, IL-1b, IL-6 and TGF-β1 by human HCC cells. We found that leptin enhanced only the production of IL-6, after 72 hours treatment and repressed the production of TGF-β1 in a time- and dose dependent manner (Figure 5a, b). Regarding IL-1a, there was no significant difference between stimulated with leptin and untreated HepG2 cultures (data not shown). Leptin siRNA treatment did not affect the production of the above mentioned cytokines (data not shown). As metalloproteinases (MMPs) have been linked with the promotion of tumor invasiveness, we next examined leptin's effect in the production of MMPs-1, -9 and -13 by HepG2 cells. We found that leptin decreased MMP-1 levels and increased MMP-13 and MMP-9 levels in a dose- and time- dependent manner (Figure 5c). siRNA treatment against leptin in HepG2 cells resulted in a significant induction of MMP-1 and reduction of MMP-9 and MMP-13 expression levels ($p < 0.001$).

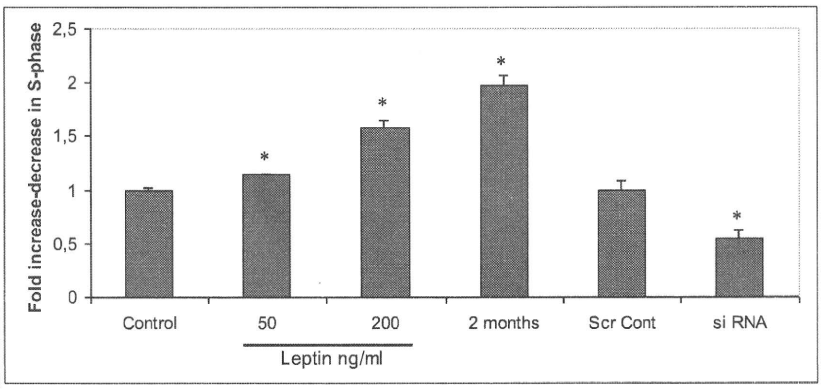
Histone H3 modifications contribute to leptin gene regulation in HCC cells

In order to investigate whether the amount of acetylated H3 interacting with leptin's proximal promoter was correlated with the regulation of leptin gene transcription, we used trichostatin A (TSA), an inhibitor of histone deacetylation. TSA treatment (200, 500, 1000 nM) of HepG2 cells increased leptin's mRNA expression in a dose dependent manner (~2, ~3.5, ~8 fold increase in leptin's mRNA expression respectively). The same treatment also upregulated leptin's protein expression, but not in the same pattern (Figure 6a). We tested the acetylation levels of histone H3 and found that in the absence of TSA, H3 binding on the promoter of leptin was undetectable, whereas in TSA treated (500 nM) HepG2 cells, a strong leptin promoter signal was detected in the acetylated H3 immunoprecipitations (Figure 6b).

Discussion

Numerous studies have established a relationship between obesity and various disease states including





	S-Phase	G2/M	G0/G1
Control	9,1 ± 1,6	23,7 ± 2,9	67,2 ± 2,6
50	10,4 ± 1,2	26,5 ± 2,1	62,2 ± 3,1
200	14,4 ± 0,9	29,7 ± 1,6	55,9 ± 2.1
2 months	16,4 ± 2,1	37,9 ± 1,3	45,6 ± 2,6
Scr Cont	11,6 ± 1,1	27,7 ± 2,5	60,7 ± 3,1
si RNA	6,5 ± 1,2	23,2 ± 1,2	70,3 ± 2,3

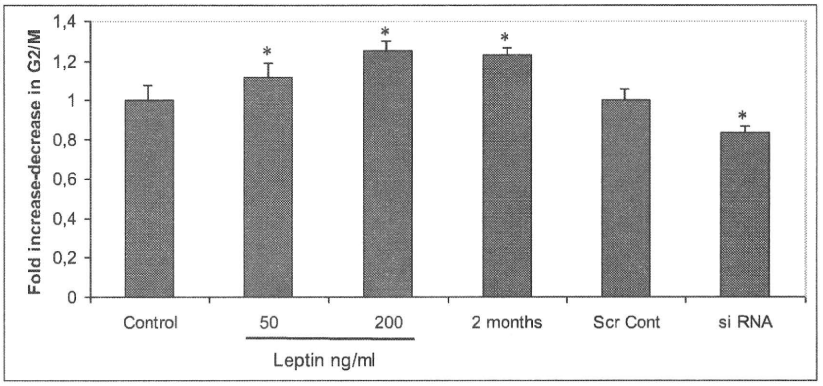


Figure 4 Leptin is mitogenic for HCC cells. (a) Effect of leptin on HepG2 cell proliferation. Cell number is expressed as percentage of control, i.e. cell cultures that was untreated. Columns, mean of three independent experiments done in triplicate; bars, SD, *, $p < 0.001$ compared to untreated cells (for siRNA experiment compared to siRNA control), (b) Leptin icreased the fraction of HepG2 cells in S and G2/M phases of the cycle. HepG2 cells were exposed to leptin (50, 200 ng/ml for 48 h and 100 ng/ml for to 2 months) and leptin siRNA. Columns, mean of three independent experiments done in triplicate; bars, SD, *, $p < 0.05$ compared to untreated cells (for siRNA experiment compared to siRNA control).

cancer. Obesity has been suggested as an important risk factor for both cirrhotic and non-cirrhotic hepatocellular carcinoma, which constitutes the third leading cause of cancer death worldwide [2,26]. It has also been suggested that there is a strong link between leptin and cancer growth and development, with increasing evidence on the involvement of leptin on breast, ovarian, endometrial, colon, and prostate cancer [27-33]. Recently, high leptin and leptin receptor expression levels were correlated with the degree of angiogenesis in human HCC [34]. In addition, leptin-mediated neovascularization showed an effective role of leptin in the development of hepatocarcinogenesis in non-alcoholic steatohepatitis [35]. In the present study, in order to determine the contribution of the leptin system in HCC progression, we investigated the expression of leptin and its receptors in HCC and normal liver tissues. The observed absence of leptin expression in normal liver

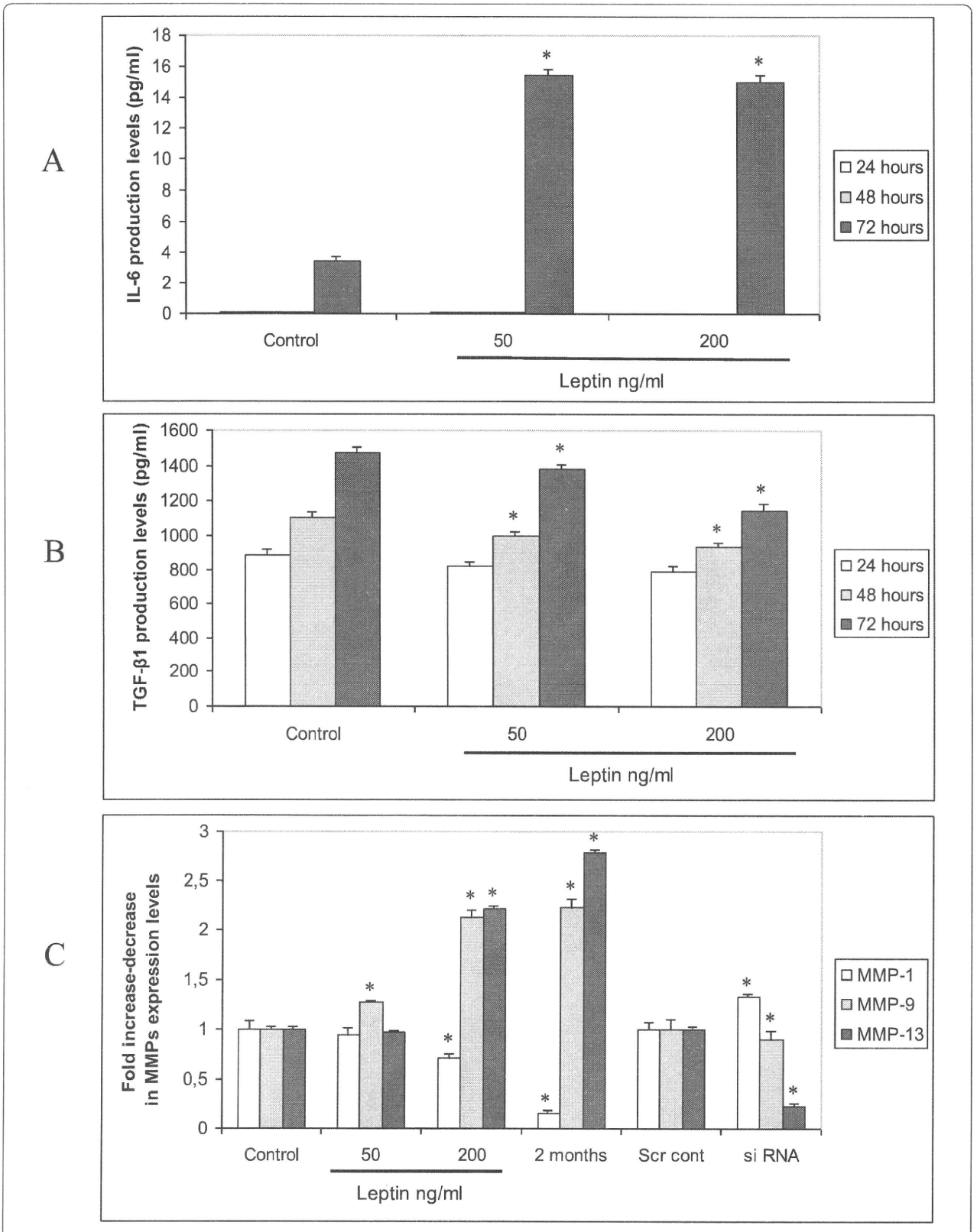


Figure 5 Leptin regulates inflammatory cytokines and MMPs production in HepG2 cells. (a, b) Effect of leptin treatment (200 ng/ml) at different times (24, 48, 72 h) on IL-6 and TGF-b1 production. Columns, mean of three independent experiments done in triplicate; bars, SD, *, $p < 0.001$ compared to untreated cells, (c) Effect of leptin treatment (50, 200 ng/ml for 48 h and 100 ng/ml for 2 months) and leptin siRNA on MMP-1, MMP-9 and MMP-13 protein levels.

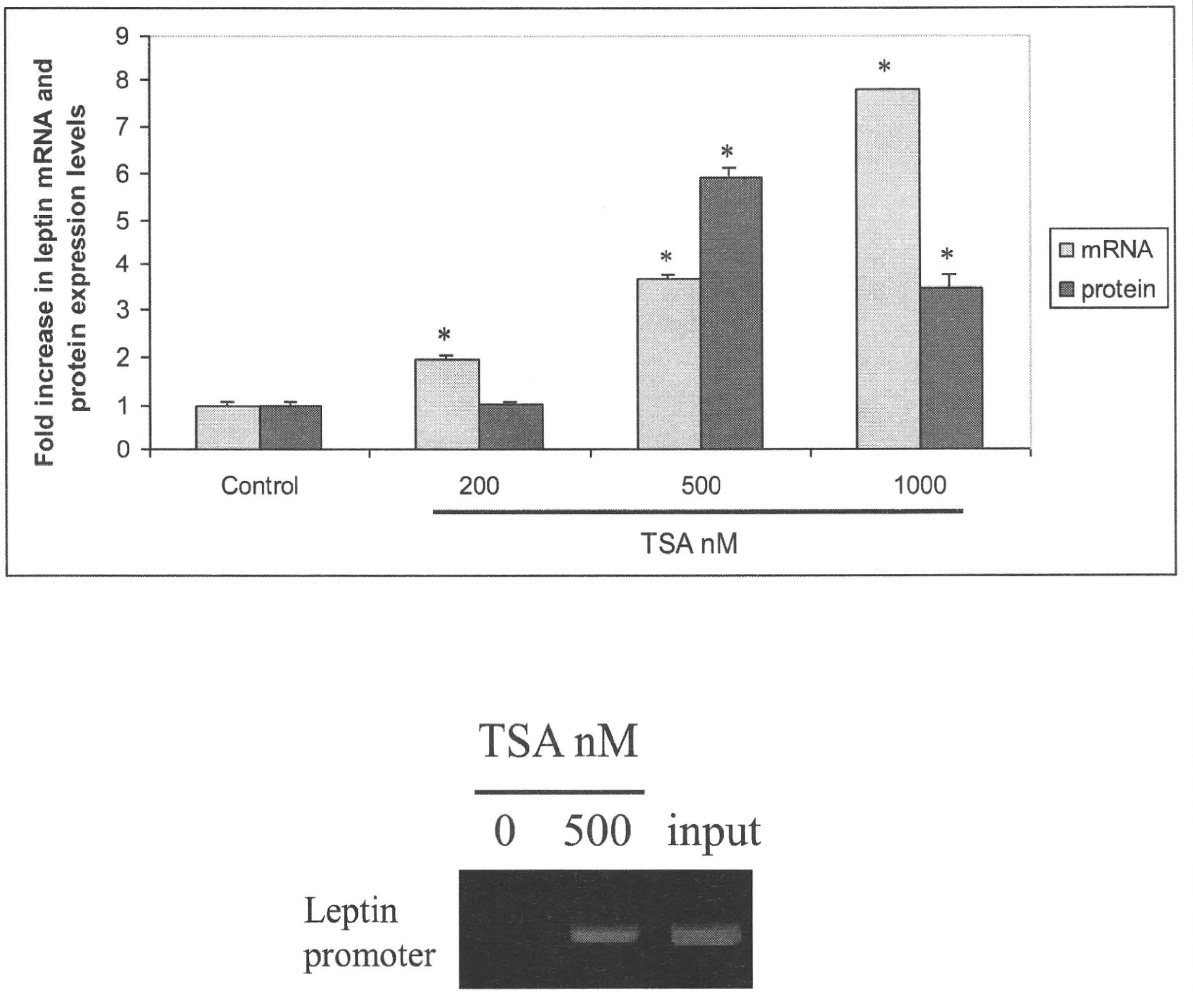


Figure 6 Histone acetylation regulates leptin expression in HepG2 cells. (a) TSA treatment (200, 500, 1000 nM) of HepG2 cells for 24 h increased leptin's mRNA and protein expression. Columns, means of three independent experiments done in triplicate; bars, SD, *, $p < 0.001$ compared to untreated cells, (b) After TSA treatment H3 was found acetylated in proximal leptin promoter.

tissues and it's remarkable presence (78.2%) in HCC liver, accompanied by the elevated OB-Rl and OB-Rs mRNA expression levels in HCC, support the role of leptin system in the development of HCC [36,37]. As the high expression of leptin and its receptors in HCC liver tissues was not found to be correlated with BMI we could assume that the production of leptin in HCC liver is not directly regulated by the adipose tissue deposit, but also reflects the intricate interactions taking place into the tumorigenic microenvironment.

It has previously been reported that hTERT mRNA overexpression and elevation of TA might be some of the processes involved in tumour initiation and progression in the liver [17,23,38]. Our results demonstrate, for the first time to our knowledge, a strong correlation between leptin expression and hTERT levels in HCC liver tissues. Moreover, we found that leptin was capable of a direct beneficent action upon hTERT mRNA and TA in HepG2 cells. The fact that leptin's knockdown by siRNA did not decrease hTERT mRNA levels and TA, suggests that the basal hTERT levels are not only under the control of the leptin system. These findings are in accordance with a very recent study by Ren et al. in MCF-7 cells and reveal that hTERT is probably a target gene for leptin and strengthen the role of leptin as a pivotal factor in HCC [39].

Previous studies have shown that STAT3 is a key mediator of critical cancer cell processes, as it promotes cell cycle progression and survival, stimulates angiogenesis and generally promotes malignant transformation [13,14,40,41]. Very recently, hTERT has been identified as a direct downstream gene of STAT3 in both tumor

and normal cells [42]. Taking into account that STAT3 is downstream of leptin and upstream of hTERT, we investigated the hypothesis that the STAT3 signalling pathway plays a crucial role in leptin-mediated hTERT expression. Our findings showed a recruitment of STAT3 in two binding sites in hTERT promoter under leptin stimulation of HCC cells, supporting the key role of STAT3 signaling in leptin induced hTERT expression.

A number of interesting reports have proposed the identification of the Myc/Max/Mad network, as a molecular switch that either interacts with the core promoter to activate hTERT transcription (Myc/Max) or promotes down regulation of hTERT mRNA production (Mad/Max) [43-45]. In the present study we demonstrated, for the first time, an association between the switch from Mad1/Max to Myc/Max binding and activation of hTERT transcription after leptin treatment of HepG2 cells and additionally an expanded interaction of Myc/Max complex accompanied by an increase in H3 acetylation in hTERT proximal promoter after long term leptin treatment of HCC cells. As the long term leptin treatment of HepG2 cells did not extend further the mRNA production of hTERT and TA, we assume that leptin-mediated hTERT overexpression is also under the consistent control of post-transcriptional regulators.

HCC arises most frequently in the setting of chronic liver inflammation and moreover cytokines, such as IL-6, produced in the inflammatory tumor microenvironment stimulate the growth of cancer cells and tumor invasiveness [46]. In the present study, we demonstrated the ability of leptin to increase IL-6 secretion in HCC cells, suggesting that an alternative indirect and independent of the OB-R presence mechanism might be involved in leptin-mediated hTERT expression through JAK/STAT3 pathway. Furthermore, the fact that leptin repressed the production of TGF-b1, a known negative regulator of hTERT [47] represents one more step towards the understanding of the molecular mechanism of leptin action in HCC and the proof of power of leptin-hTERT axis in the tumorigenic processes. To gain insight into the biological effects of leptin's action in the progression and invasion of HCC, we next examined leptin's effect in the production of MMP-1, -9, 13 by hepatocarcinoma cells. Many secreted MMPs are nearly absent in healthy, resting tissues, although they are deregulated in active tissues, as in liver fibrosis and tumor metastasis [48]. In our study we observed, for the first time, that leptin is able to suppress MMP-1 expression and trigger MMP-9 and MMP-13 expression in HepG2 cells, and this could contribute to a more favourable environment for invasion and metastasis of HCC in the cirrhotic liver.

In order to elucidate the signalling cascades in liver cancer, the regulatory mechanisms of genes altered in HCC cells need to be determined. In our study, for the first time to our knowledge, we found that the amount of acetylated H3, in HCC cells, interacting with leptin proximal promoter was correlated with the regulation of leptin gene transcription. The importance of this finding lies in the fact that histone acetylation is reversible and thus may have therapeutic potential.

Conclusions

In conclusion, our data revealed, for the first time, that leptin up-regulates hTERT expression and TA and deciphered the molecular mechanisms responsible for their interaction in HCC, thus establishing a clearer view of leptin-mediated HCC cell proliferation and progression.

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Authors' contributions

NS carried out the molecular genetic studies and drafted the manuscript, VP participated in the design of the study and in the molecular genetic studies, YF and YN provided HCC liver tissue samples, AT conceived of the study, and participated in its design and coordination and helped to finalize the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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SMYD3 interacts with HTLV-1 Tax and regulates subcellular localization of Tax

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HTLV-1 Tax deregulates signal transduction pathways, transcription of genes, and cell cycle regulation of host cells, which is mainly mediated by its protein–protein interactions with host cellular factors. We previously reported an interaction of Tax with a histone methyltransferase (HMTase), SUV39H1. As the interaction was mediated by the SUV39H1 SET domain that is shared among HMTases, we examined the possibility of Tax interaction with another HMTase, SMYD3, which methylates histone H3 lysine 4 and activates transcription of genes, and studied the functional effects. Expression of endogenous SMYD3 in T cell lines and primary T cells was confirmed by immunoblotting analysis. Co-immunoprecipitaion assays and *in vitro* pull-down assay indicated interaction between Tax and SMYD3. The interaction was largely dependent on the C-terminal 180 amino acids of SMYD3, whereas the interacting domain of Tax was not clearly defined, although the N-terminal 108 amino acids were dispensable for the interaction. In the cotransfected cells, colocalization of Tax and SMYD3 was indicated in the cytoplasm or nuclei. Studies using mutants of Tax and SMYD3 suggested that SMYD3 dominates the subcellular localization of Tax. Reporter gene assays showed that nuclear factor-κB activation promoted by cytoplasmic Tax was enhanced by the presence of SMYD3, and attenuated by shRNA-mediated knockdown of SMYD3, suggesting an increased level of Tax localization in the cytoplasm by SMYD3. Our study revealed for the first time Tax–SMYD3 direct interaction, as well as apparent tethering of Tax by SMYD3, influencing the subcellular localization of Tax. Results suggested that SMYD3-mediated nucleocytoplasmic shuttling of Tax provides one base for the pleiotropic effects of Tax, which are mediated by the interaction of cellular proteins localized in the cytoplasm or nucleus. (*Cancer Sci* 2011; 102: 260–266)

Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of an aggressive leukemia known as adult T-cell leukemia (ATL).[1,2] The viral protein Tax plays a central role in the development of ATL in HTLV-1-infected carriers. Tax is known to enhance gene expression of HTLV-1 through target sequences in the U3 region of 5′LTR by interacting with cellular factors such as cAMP response element binding protein (CREB) and transcriptional co-activator CBP/p300.[3,4]

Tax also activates intracellular signal transactivation pathways that normally play a crucial role in cellular responses to various extracellular stimuli, resulting in the activation of several transcription factors, including nuclear factor (NF)-κB, serum response factor, and CREB. Consistent with both its cytoplasmic and nuclear activities, Tax was shown to be distributed in both compartments in HTLV-1-infected and Tax-transfected cells. Initial studies reported that Tax is localized predominantly in the nucleus and specifically accumulated in the nuclear speckled structures.[5,6] However, depending on the cell type, significant amounts of Tax have also been found in the cytoplasm.[7–9] The mechanism by which Tax was localized in cytoplasm was reported recently,[10–16] although the detailed mechanisms regulating Tax subcellular localization remain to be elucidated.

Epigenetic control of gene expression is mediated by chemical modifications of histone tails, such as acetylation, phosphorylation, and methylation, and DNA methylation such as CpG methylation, both leading to the regulation of chromatin structure and function.[17–19] Previous studies reported that Tax interacts with various histone modifying enzymes.[20–27] We previously reported that Tax directly interacts with SUV39H1.[27] The interaction of Tax with SUV39H1 was mediated by the SET domain of SUV39H1. The SET domain is shared among histone methyltransferases and possesses methyltransferase activity, and functions as lysine methylases that add multiple methyl groups to specific lysines in histones H3 and H4. As the SUV39H1 SET domain mediated the interaction with Tax, it was suggested that Tax is able to interact with a variety of histone methyltransferases.

SET and MYND domain-containing protein 3 (SMYD3) is unique in that it promotes di- and tri-methylation in H3-K4 and is frequently overexpressed in human colorectal, liver, and breast cancers where its enhanced expression is essential for the growth of cancer cells.[28,29] SMYD3 encodes a 428-amino acid protein containing a SET domain, a zf-MYND domain, and a SET-N region.

Thus, we speculated that Tax may interact with SMYD3 and the interaction may play roles in proliferation and immortalization of Tax-expressing T cells. In the present study, we examined direct interaction between Tax and SMYD3 and the subcellular localization of the Tax–SMYD3 complex.

Materials and Methods

Cell cultures. Jurkat, CEM, TIG1, Molt-4, HUT102, and MT-2 cells were cultured in RPMI-1640 supplemented with 10% FCS and antibiotics. HeLa, HEK293, and HEK293T cells were cultured in DMEM supplemented with 10% FCS and antibiotics. Peripheral blood mononuclear cells from healthy volunteers were prepared by centrifugation of peripheral blood with Ficoll-Paque (GE Healthcare UK Ltd. Buckinghamshire, UK) and used as control PBMC. Activated control T cells were prepared by stimulation of control PBMC with phytohemagglutinin (PHA) (10 μg/mL) for three days and cultured in RPMI-1640 containing 10% FCS, 1000 U/mL ampicillin, and 1 mg/mL streptomycin.

Antibodies. Lt-4 is a mAb that reacts with Tax.[30] Antibodies against SMYD3 (ab16027) were purchased from Abcam

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(Cambridge, UK). Anti-Flag antibody M2 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against IκBα (C-21) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against HA (6E2) and Phospho-IκBα (Ser32/36) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Plasmids. The wild-type Tax expression plasmid, pCG-Tax, pET3d/Tax, and a mutant Tax plasmid, TaxC29A, were kind gifts from Prof. Fujisawa (Kansai Medical University, Moriguchi, Japan) and Dr. Tsuji (National Institute of Infectious Diseases, Tokyo, Japan), respectively. The SMYD3 expression plasmid, p3xFLAG-CMV-SMYD3, was described previously.[28] Wild-type and mutant SMYD3 expression vectors were prepared by PCR using primers described below, and PCR products were cloned into pcDNA-HA or pGEX5X-1 (GE Healthcare UK Ltd. Buckinghamshire, UK). Forward primers: 5′-CCCGAATTCATGGAGCCGCTGAAGGTGGAAAAG-3′, 5′-CCCGAATTCTATCCTCCAGACTCCGTTCG-3′, 5′-CCCGAATTCGAGCGCCGGAAGCAGCTGAGG-3′, 5′-CCCGAATTCATTAACAAACTGACTGAAGATAAG-3′; reverse primers: 5′-CCCCTCGAGTCAGGATGCTCTGATGTTGGCGTC-3′, 5′-CCCCTCGAGTCACTCACTGGTCATCAGCATATC-3′, 5′-CCCCTCGAGTCATCTGGGTTTGCAGCTTTTAAG-3′.

Using pET3d/Tax plasmid, histidine-tagged wild-type Tax was bacterially expressed and purified as described previously.[27]

Quantitative RT-PCR analysis. Quantitative RT-PCR was carried out to analyze SMYD3 mRNA levels. Total RNA was isolated from the cells by Isogen (Wako Pure Chemical Industries, Osaka, Japan) and cDNA was synthesized using SuperScript2 (Invitrogen, Carlsbad, CA, USA), followed by real-time PCR using SMYD3 primers (5′-AGGGGTTCAAGTGATGAAAGTTG-3′, 5′-GCTGTGTTCTCTGCCATGTGT-3′). Levels of β-actin mRNA were measured as an internal control.

***In vitro* transcription and translation.** For *in vitro* translation of the wild-type and mutant Tax proteins, the cDNA was amplified by PCR and cloned into pBluescript II SK (−). *In vitro* transcription and translation of the indicated cDNA was done using TNT Quick Coupled Transcription/Translation Systems (Promega, Madison, WI, USA) as described previously.[27]

GST pull-down assay. Wild-type and mutant GST-SMYD3 proteins (1 μg) bound to glutathione–Sepharose 4B (GE Healthcare and Biosciences) were mixed with *in vitro* translated wild-type and various mutants of Tax proteins. Binding reactions, SDS-PAGE, and visualization were carried out as described previously.[27] Relative intensities of the bands were determined using the NIH Image software (National Institutes of Health, Bethesda, MD, USA).

Co-immunoprecipitation and immunoblotting. For co-immunoprecipitation analyses, transfection was carried out by the standard calcium phosphate precipitation method. Cell lysates were prepared in TNE buffer (10 mM Tris–HCl [pH 7.8], 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA). When indicated, aliquots were removed for immunoblots of whole cell lysates. Immunoblots were carried out to detect co-immunoprecipitated or GST pull-down proteins, as described previously.[27] Primary antibodies used in this assay are described above. Alkaline phosphatase-conjugated anti-mouse immunoglobulin sheep and anti-rabbit donkey antibodies (both from Promega) were used as secondary antibodies.

Immunocytochemistry. HeLa (3×10^5/mL) were grown on cover slips in a 6-well plate for 1 day, and transfected with various expression plasmids using Lipofectamine 2000 (Invitrogen). Fixation and staining were done as described previously.[27] Fluorescence signals were detected using confocal microscopy (Radiance 2000; Bio-Rad, Hercules, CA, USA). HUT102 and MT-2 cells (1×10^6/mL) were cultured with RPMI-1640 containing 10% FCS. Fixation and staining were done as described previously.[27] Data were obtained using an immunofluorescence microscope (BX50; Olympus, Tokyo, Japan).

Reporter gene assays. HEK293 cells (1.6×10^5/mL) were transfected with reporter plasmids and expression plasmids using Lipofectamine 2000. After 24 h incubation, luciferase activity was measured with a luciferase assay kit (Promega). A control plasmid, RSV-Renilla, was prepared by inserting the RSV LTR to the Renilla reporter plasmid (Promega). The measured activities were standardized by the activities of Renilla luciferase, and transactivation was expressed as fold activation compared with the basal activity of p6κB-Luc without effectors. Another reporter plasmid, LTR-Luc, was described previously.[27]

shRNA-mediated SMYD3 knockdown. SMYD3 knockdown was established according to the manufacturer's protocol (Retrovirus Packaging kit Ampho; Takara Bio, Shiga, Japan) using the sense and antisense DNA oligomers as already described.[28,31] shRNA-mediated knockdown was done as described previously.[31]

Results

SMYD3 expresses in T cells and interacts with HTLV-1 Tax. To determine whether Tax can interact with SMYD3, we first carried out co-immunoprecipitation assays by transient expression for these proteins. The results clearly showed that immunoprecipitates of anti-Flag antibody contained Tax protein (Fig. 1a). Conversely, when the cell lysates were immunoprecipitated with Lt-4, the immunocomplex was shown to contain Flag-tagged SMYD3 (Fig. 1b). Taken together, these results indicated that Tax interacts with SMYD3 in cultured cells. We also showed that Tax protein directly interacts with GST-SMYD3 *in vitro* (Fig. 1c).

SMYD3 expression was originally reported in solid tumors such as hepatoma and breast cancer.[28,29] Thus, to examine whether SMYD3 is expressed in other cells, we carried out quantitative RT-PCR analysis and quantified SMYD3 gene expression in primary T cells, human fibroblast, and other cell lines. As shown in Figure 1(d), the level of SMYD3 transcription was higher in T-cell lines with or without HTLV-1 infection than those in others. Next we examined whether endogenous SMYD3 and Tax can interact with each other in HTLV-1-infected cell lines. Immunoprecipitates with anti-SMYD3 or Lt-4 were shown to contain Tax or SMYD3, respectively (Fig. 1e, top panels). This result indicated that endogenous Tax and SMYD3 interact with each other in T cell lines.

Analyses of domains responsible for interaction between SMYD3 and Tax. To define the domains within SMYD3 and Tax that are responsible for the interaction, we next carried out *in vitro* binding assays. First, we constructed various GST-fusion mutants of SMYD3 according to the domain structure[28] (Fig. 2a) and examined binding to the *in vitro* translated and S^{35}-labeled wild-type Tax. When C-terminal deleted series of SMYD3 were examined, the SMYD3-A showed a marked decrease in the intensity of the bound Tax. The SMYD3-B showed no binding activity to Tax. When N-terminal deletion series of SMYD3-mutants were tested, both the SMYD3-C and SMYD3-D showed binding activity to Tax. However, the SMYD3-E did not show any binding activity (Fig. 2b, upper panel). The relative levels of bound Tax compared with that of the wild-type Tax are shown in Figure 2(c). These results suggested that the region of amino acids from 248 to 428 of SMYD3 appears to be enough to show a significant level of affinity against Tax similar to that of the wild-type SMYD3.

We next analyzed the domains of Tax responsible for the interaction with SMYD3. In addition to the wild-type Tax, we used three kinds of mutants, TaxN180, TaxΔN108 and ΔCBP-B

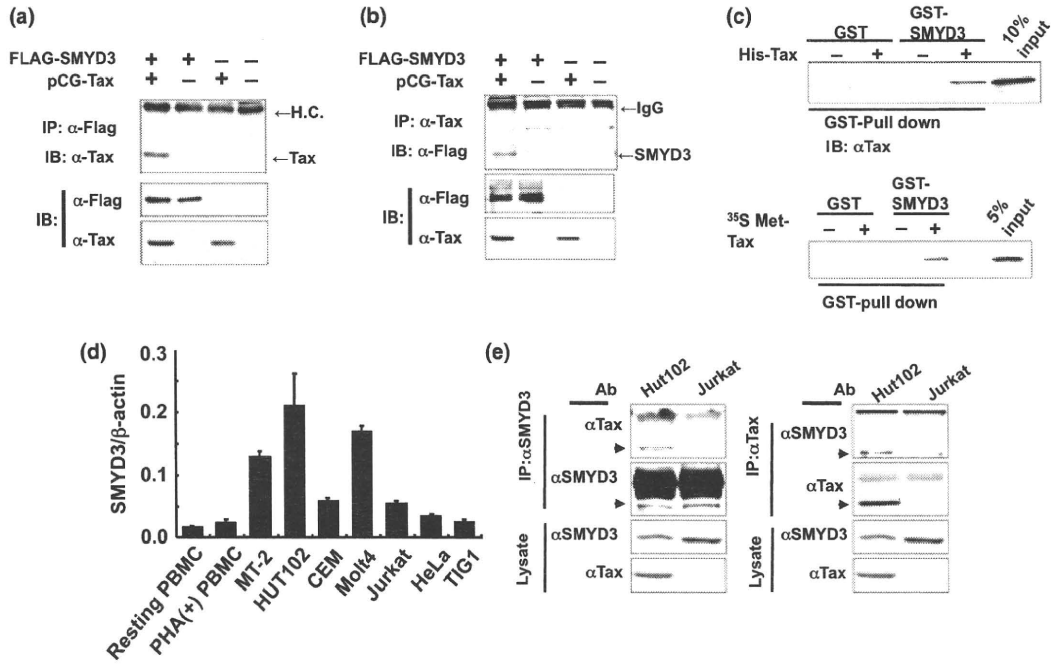

Fig. 1. SMYD3 expresses in T cells and interacts with Tax *in vitro* and *in vivo*. (a,b) HEK293T cells were transiently cotransfected with FLAG-SMYD3 and/or Tax. Cell lysates were immunoprecipitated (IP) with anti-FLAG or Lt-4. Tax or FLAG-SMYD3 were detected by immunoblot (IB) analyses with Lt-4 (a, top panel) or anti-FLAG (b, top panel). Expression of transduced proteins was confirmed by immunoblot analyses of whole cell lysates using respective antibodies (lower panels). GST-SMYD3 and GST were mixed with purified His-Tax or *in vitro* translated and radiolabeled Tax (c). Reactants were separated by SDS-PAGE and detected by immunoblot analysis with Lt-4 (upper panel) or autoradiogram (lower panel). As a control, an aliquot of purified His-Tax or radiolabeled Tax was run. Quantitative RT-PCR analysis of SMYD3 expression (d). RNA was extracted from primary T cells and human cell lines, and SMYD3 expression analyzed by quantitative RT-PCR. Error bars represent one standard deviation. Co-immunoprecipitation of endogenous SMYD3 and Tax. Cell lysates of HUT102 and Jurkat cell lines were immunoprecipitated with anti-SMYD3 or Lt-4 (e). The precipitates were blotted with anti-SMYD3 or Lt-4. Arrowheads indicate the position of Tax or SMYD3 (upper two panels). Whole cell lysates were immunoblotted with anti-SMYD3 or Lt-4, to confirm expression of SMYD3 and Tax (lower two panels). These experiments were repeated at least three times. Similar results were obtained in each experiment. H.C., heavy chain; IB, immunoblotting.

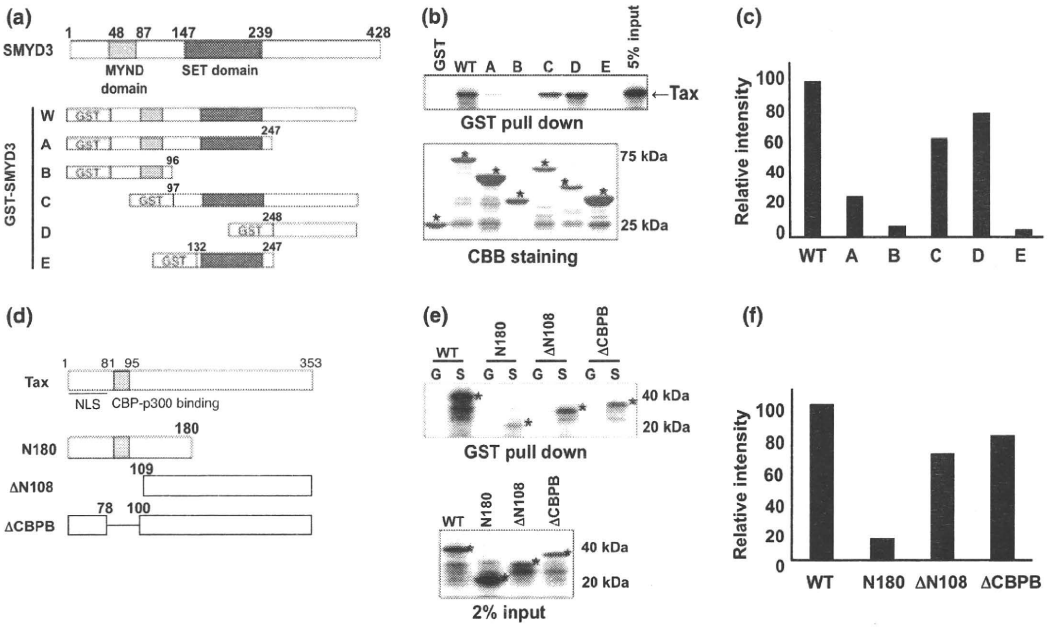

Fig. 2. Analyses of the interacting domains of SMYD3 and Tax. (a,d) Schematic descriptions of the structures of wild-type (WT) and various mutants of SMYD3 and Tax. Results of the pull-down assays are shown in the upper panels (b,e). The bottom panels show Coomassie Brilliant Blue (CBB) stained gels of the various mutant SMYD3 proteins and autoradiograms of the various mutant Tax proteins. Asterisks indicate wild-type and various mutant Tax proteins. (c,f) The graphs show the results of measurement of the bands by NIH Image software. These experiments were repeated at least three times. Similar results were obtained in each experiment.

(Fig. 2d).[27] The results showed that the wild-type Tax and all of these mutants could bind to SMYD3 (Fig. 2e, upper panel). The relative levels of bound Tax compared with that of the wild-type Tax are shown in Figure 2(f). Generally the bands of pulled-down Tax mutants were significantly weaker in intensity than that of the wild-type Tax, with the weakest being that of

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TaxN180 (Fig. 2e,f). These results suggested that amino acids 109–353 of Tax are more important to bind with SMYD3 than N-terminal 108 amino acids.

Co-localization of Tax and SMYD3 *in vivo*. When SMYD3 alone was expressed in HeLa cells, SMYD3 localized in the cytoplasm or nucleus, whereas singly expressed Tax localized in the nuclei in most of the transfected cells (Fig. 3a). However, when Tax and SMYD3 were simultaneously expressed in HeLa cells, Tax was colocalized with SMYD3 in the cytoplasm in a part of the cotransfected cells (Fig. 3b, lower panel). To evaluate the effects of co-expression quantitatively, we counted the cell numbers that show Tax and SMYD3 localization in the nucleus and cytoplasm. The frequency of the cells where Tax is localized in cytoplasm increased with co-expression of SMYD3, when compared with those where Tax is expressed alone (Fig. 3c), suggested a tethering of Tax by SMYD3 to the cytoplasm in these cells. To confirm whether the same results were obtained in T cell lines, we analyzed the subcellular localization of endogeneous SMYD3 and Tax in MT-2 and HUT102 cells. The results showed that Tax colocalized with SMYD3 in most of the cells (Fig. 3d). Taken together, these results suggested that Tax was tethered to the cytoplasm by SMYD3.

To examine this possibility, we used two Tax mutants, neither of which enter the nuclei because of deletion of the N-terminal region containing the nuclear localization signal (TaxΔN108)[27] or a point mutation in the amino acids of the nuclear localization signal (Tax-C29A).[32] TaxΔN108 and Tax-C29A interacted with SMYD3 (Fig. 4a), and a clear cytoplasmic localization when expressed alone (Fig. 4b,c, top panels). However, TaxΔN108 and Tax-C29A were found in the nuclei in a part of the transfected cells, and in the other cells they were colocalized with SMYD3 in the cytoplasm when co-expressed (Fig. 4b,c, lower panels).

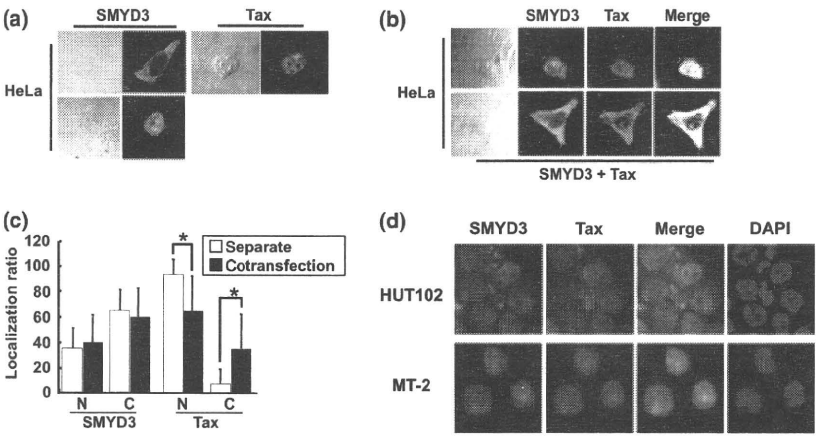

Fig. 3. Immunofluorescence microscope analysis of Tax and SMYD3. (a) Subcellular localization of SMYD3 and Tax when transfected alone. (b) Subcellular localization of SMYD3 and Tax when cotransfected. (c) Percentage of cells with Tax or SMYD3 in the cytoplasm (C) or nucleus (N). The results were expressed as the mean ± SD(error bars). *Significant difference ($P < 0.05$) determined by a Fisher's protected least-significant test. (d) Subcellular localization of Tax and SMYD3 in HUT102 and MT-2 cell lines.

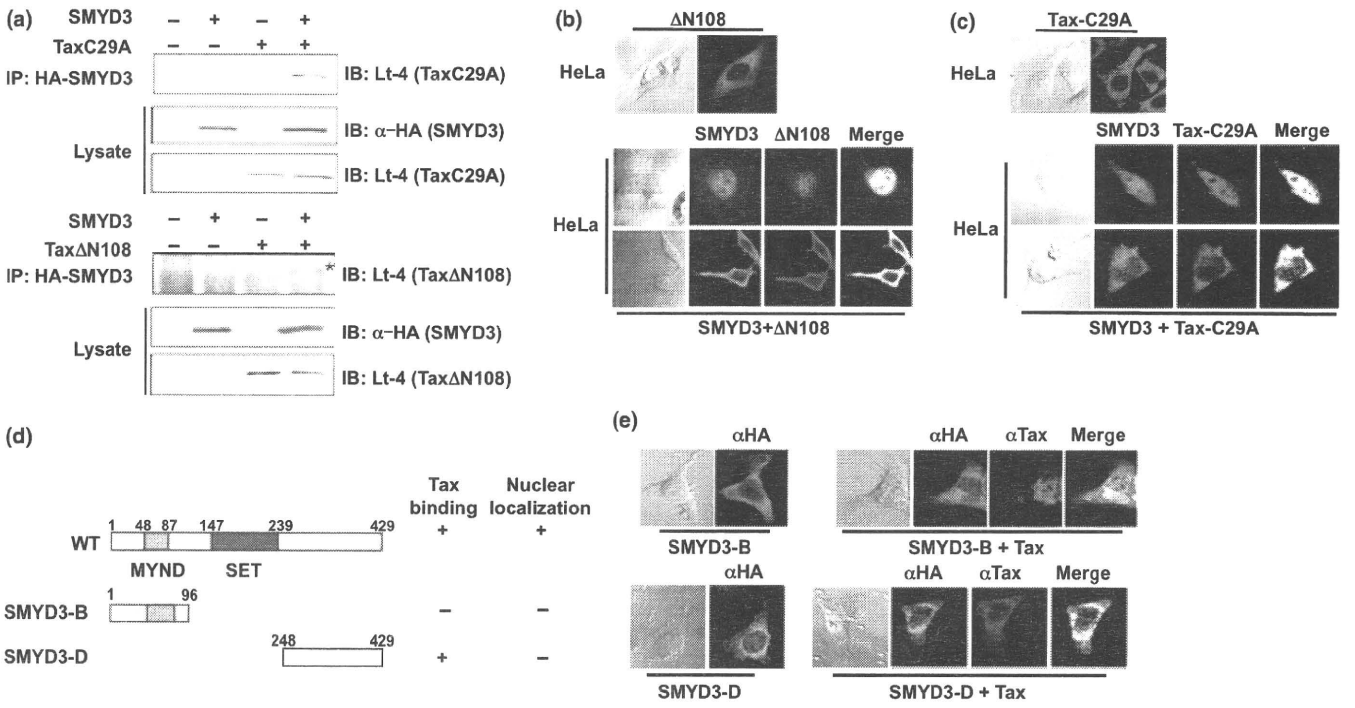

Fig. 4. Analysis of subcellular localization of SMYD3 mutants and Tax mutants. (a) Interaction between SMYD3 and Tax mutants. *Position of TaxΔN108. Subcellular localization of SMYD3 and Tax ΔN108 (b) or Tax-C29A (c). Top panels show localization of Tax mutant expressed alone. Lower panels show the localization of SMYD3 and Tax mutants. (d) A schematic presentation of two SMYD3 mutants. (e) Subcellular localization of Tax co-expressed with SMYD3 mutants (right panels), or of SMYD3 mutants expressed alone (left panels). IB, immunoblotting; IP, immunoprecipitation; WT, wild-type.

Next we studied effects of SMYD3 mutants on subcellular localization of Tax, using two mutants that are localized in the cytoplasm but cannot enter the nuclei. One of the mutants, SMYD3-B, does not interact with Tax and the other, SMYD3-D, can interact with Tax (Fig. 4d). When transfected alone, SMYD3-B and SMYD3-D are mostly localized in the cytoplasm (Fig. 4e, left upper and lower panels). When SMYD3-B was co-expressed with Tax, it was localized in the cytoplasm, whereas Tax was localized in the nucleus (Fig. 4e, right upper panels). However, Tax was localized in the cytoplasm when co-expressed with SMYD3-D (Fig. 4e, right lower panels).

SMYD3 enhances Tax-mediated NF-κB activation. To examine whether NF-κB activation by Tax is enhanced in the presence of SMYD3, we carried out reporter gene assays using the p6κB-Luciferase assay system to measure the activity of NF-κB. The results showed that expression of Tax induced 15-fold activation of NF-κB in HEK293 cells. The luciferase activity was enhanced up to 25-fold in these cells when co-expressed with SMYD3 (Fig. 5a, upper panel). In contrast, expression of SMYD3 alone showed no significant activation of p6κB-Luc (Fig. 5a, lower panel). To study the effects of co-expression of SMYD3 and Tax in another system, we next studied the phosphorylation of IκBα as an indicator of IKK complex activity. Immunoblot analysis of the cell lysates from Tax-transfected HEK293 cells showed increased levels of phosphorylation of IκBα Ser32/36 and the level appeared to be further enhanced when co-expressed with SMYD3 (Fig. 5b, upper panel). However, no change was observed in the levels of IκBα Ser32/36 phosphorylation when SMYD3 alone was expressed. Intensities of the detected bands are shown in the graphs (Fig. 5b, lower panel).

To examine the effect on LTR promoter activity, we carried out another luciferase assay using pLTR-Luc as a reporter. Tax-mediated transactivation of LTR-Luc was suppressed by co-expression of SMYD3 down to approximately half of the activity without SMYD3 (Fig. 5c, left panel). However, SMYD3 alone did not show any significant effects on LTR activity (Fig. 5c, right panel).

Next, we tested whether the suppression of SMYD3 expression may influence the Tax localization and NF-κB activation in MT-2 and HUT102 cell lines (Fig. 6). Quantitative RT-PCR and Western blot analysis showed a significant reduction of endogenous SMYD3 expression in SMYD3-shRNA expressing cells compared with cells infected with a mock retrovirus (Fig. 6a,b). Tax was less localized in cytoplasm when SMYD3-shRNA was expressed in MT2 and in HUT102 (Fig. 6c). Furthermore, 6κB-Luc activity was significantly suppressed in SMYD3-shRNA expressing MT-2 (Fig. 6d). These results suggested that SMYD3-mediated cytoplasmic localization of Tax results in efficient activation of the NF-κB signaling pathway.

Discussion

Our results of *in vitro* analyses indicated that the major interacting domain of SMYD3 with Tax was the C-terminal region of approximately 180 amino acids that does not contain the SET domain of SMYD3 (Fig. 2). The results were different from our previous observation that SUV39H1 interacts with Tax by the SET domain,[27] suggesting another mechanism. Although we were not able to clarify the binding domain in Tax, determination of the amino acids of SMYD3 and Tax that are involved in the interaction between these proteins will provide additional information for understanding the mechanisms of Tax interaction with host cellular proteins. Although we have not examined the effect of Tax interaction on the histone methyltransferase activity of SMYD3, the complex formation of Tax and SMYD3 may lead to changes in SMYD3 function. Another possibility will be that complex formation of Tax–SMYD3 may result in a new target specificity of SMYD3, because Tax may recruit proteins that are not original targets of SMYD3. These possibilities remain to be studied in the future.

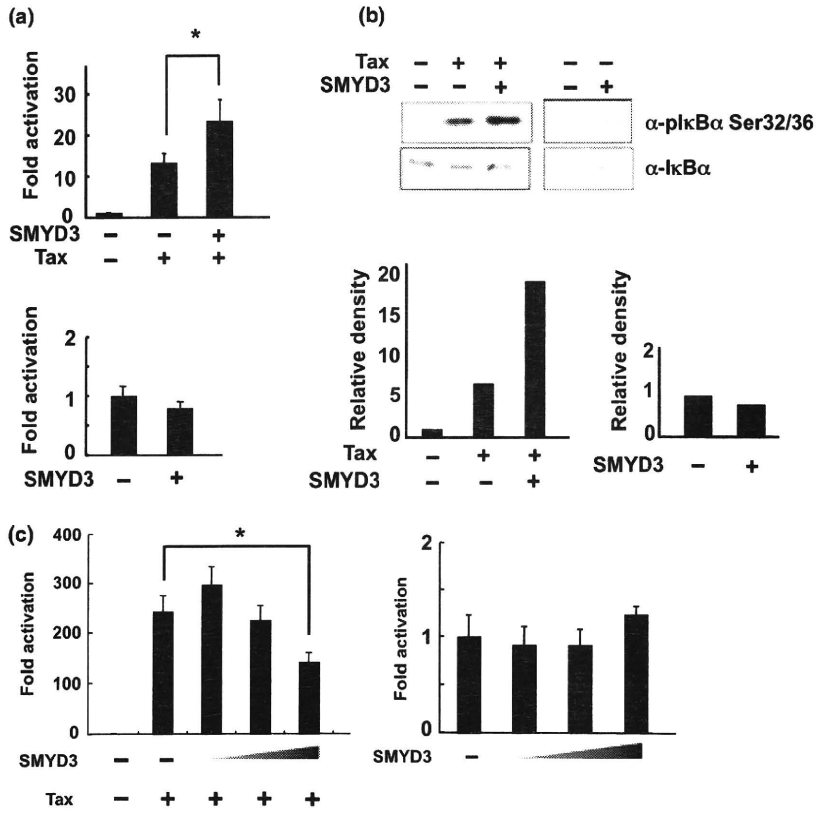

Fig. 5. Effects of SMYD3 on transcriptional activities of Tax. (a) Transactivation of nuclear factor (NF)-κB-driven luciferase activity by Tax with SMYD3 in HEK293 cells. Expression plasmids indicated below the graph were transfected into HEK293 cells (50 ng 6κB-Luc, 100 ng pCG-Tax, and 200 ng pcDNA-HA-SMYD3). (b) Enhanced phosphorylation of IκBα by co-expression of Tax and SMYD3. Expression plasmids indicated above the top panels were transfected into HEK293 cells. The cells were harvested and analyzed by immunoblotting with indicated antibodies. The intensities of the bands were measured and shown in the graphs at the bottom. Relative levels of intensities are shown compared with the intensity of untransfected cells. (c) Transactivation of LTR-driven luciferase activity by Tax in the presence of SMYD3 in HEK293K cells. LTR-Luc and Tax expression plasmids (5 ng each) were transfected with increasing amounts of pcDNA-HA-SMYD3 (2.5, 5.0, and 10.0 ng).

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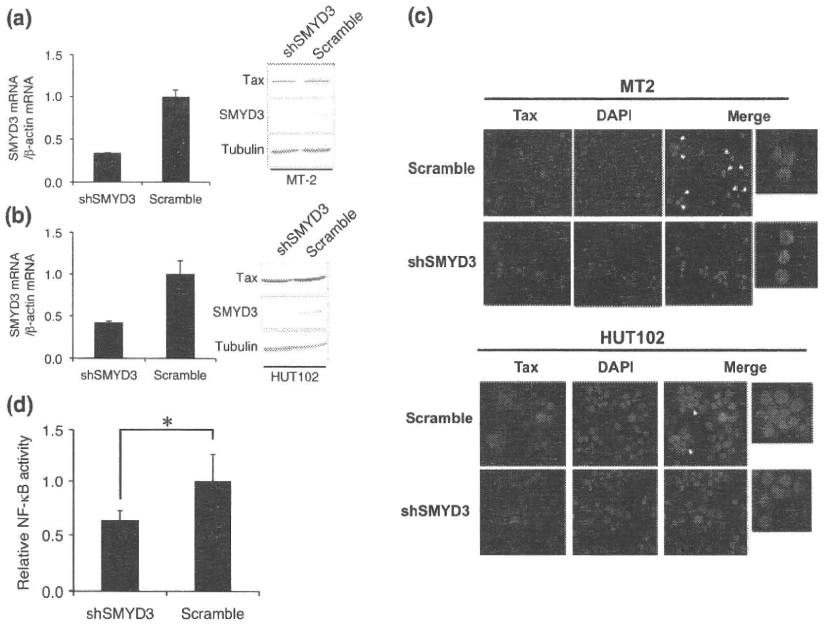

Fig. 6. Effects of SMYD3 knockdown on Tax localization and Tax-mediated nuclear factor (NF)-κB activation. Effect of stable expression of SMYD3-shRNA in MT-2 (a) and HUT102 (b) cells. Suppression of SMYD3 expression in shSMYD3 expressing cells was confirmed by quantitative RT-PCR (left panels) and Western blot analysis (right panels). (c) Subcellular localization of Tax in MT-2 and HUT102 cells. Arrowheads indicate the positions of cytoplasmic localization of endogenous Tax. In the merge panels, the left panels show overviews, and the right panels show enlarged images. Nuclei were stained with DAPI. (d) Transactivation of NF-κB reporter plasmid (6κB-Luc) in the presence or absence of SMYD3-shRNA in MT-2 (mean ± SD). $*P < 0.05$ by a Fisher's protected least-significant difference test.

As for the intracellular distribution of Tax, initial studies indicated nuclear localization with little or no Tax localized to the cytoplasm, using laboratory cell lines including HeLa, COS, and 293T.[3,4,6,33–35] However, subsequent studies have reported the localization of Tax to the cytoplasm in a number of cell types including Tax-transfected and HTLV-1-infected cells of both cell line and primary cell origin.[7,8,36] Thus, the regulatory mechanisms of Tax subcellular localization have been a focus of research interest. A number of studies have reported nucleocytoplasmic shuttling of Tax and functional effects of subcellular localization of Tax, although the regulatory mechanisms of subcellular localization remain to be clarified.[37] The result of our study clearly showed that the interaction between Tax and SMYD3 regulates subcellular localization of Tax, depending on the shuttling of SMYD3 (Figs 3,4). Tax was localized in the cytoplasm and the nucleus (Fig. 3). The shuttling of Tax was observed in HTLV-1-infected T cell lines as well as in the cell lines transiently transfected with the SMYD3 expression vector (Fig. 3). Accordingly, it is concluded that the subcellular localization of Tax can be changed in the presence of SMYD3, but the detail of the mechanism is not clear and remains to be studied.

A number of published studies have indicated that Tax functions both in the cytoplasm and nucleus. The results showed nucleocytoplasmic shuttling of Tax, at least in some of the Tax-expressing cells, which was associated with changes in functions of Tax. We observed enhanced activation of Tax-mediated NF-κB activation with SMYD3 co-expression (Fig. 5), which was eliminated by SMYD3 knockdown, probably due to the decreased amount of Tax in the cytoplasm as observed by immunocytochemistry (Fig. 6). These observations are consistent with the idea that many aspects of Tax function depend on its subcellular localization. Our study revealed for the first time the interaction between Tax and SMYD3 and the apparent tethering of Tax by SMYD3 between the nucleus and cytoplasm. The results suggest that SMYD3-mediated nucleo-cytoplasmic shuttling of Tax provide bases for pleiotropic effects of Tax, which are mediated by interaction of cellular proteins localized in the cytoplasm or nucleus. Continued studies are required to understand how Tax–SMYD3 interaction contributes to the unique phenotype of HTLV-1-infected T-cells and the pathogenesis of HTLV-1-related diseases.

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Disclosure Statement

The authors have no conflict of interest.

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BJD
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Three-base deletion mutation c.120_122delGTT in *ATP2A2* leads to the unique phenotype of comedonal Darier disease

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Darier disease (DD; Darier–White disease; OMIM 124200) is an autosomal dominant inherited disorder.[1] Clinically, it is characterized by recurrent and multiple hyperkeratotic papules or nodules affecting the trunk and flexural aspects of the extremities.[1] Characteristic histopathological features are dyskeratotic cells in the form of corps ronds and grains, suprabasal acantholysis forming suprabasal lacunae and irregular upward proliferation into the lacunae of papillae lined with a single layer of basal cells, the so-called villi.[2] The causative gene is *ATP2A2* (OMIM 108740) on chromosome 12, which encodes the sarco/endoplasmic reticulum calcium pump ATPase (SERCA2).[2]

Clinical variants include the hypertrophic, vesiculobullous, hypopigmented, cornifying, zosteriform and linear subtypes, and the rare subtype comedonal Darier disease (CDD).[1,3–6] CDD tends to appear in seborrhoeic areas. The characteristic morphological features are prominent follicular involvement, sometimes associated with keratotic plugs, and the presence of greatly elongated dermal villi and papillary projections.[4] There have been no conclusive reports on the aetiology of CDD and it is still controversial as to whether or not CDD is a variant of DD, and if it is caused by *ATP2A2* gene mutations, although a combination of CDD and classic DD was reported in one patient.[7] The present study identifies a previously unreported three-base deletion mutation in *ATP2A2* in a patient with CDD.

Patient and methods

A 22-year-old Japanese man presented with acne-like comedonal lesions on the face and chest, most densely distributed on the forehead, cheeks, back, axillae and chest. The comedonal lesions had first appeared in his teens and had gradually increased in number. Physical examination showed open comedones, closed comedones, red papules, nodules, cysts and ice-pick scars (Fig. 1a,b). His parents were clinically healthy, without any skin problems. He had been treated with oral biotin, Korean ginseng, an antihistamine, topical bufexamac ointment, calcipotriol ointment and betamethasone butyrate propionate ointment without any improvement. Histopathological observations revealed suprabasal acantholytic clefts and numerous dyskeratotic cells (corps ronds) in the outer root sheath in the affected follicular infundibulum, which was surrounded by plasma cells and lymphocytes (Fig. 1c,d). We made a diagnosis of CDD. Oral etretinate 10 mg daily combined with adapalene gel remarkably improved most of the skin lesions, except those on the forehead.

Polymerase chain reaction (PCR) amplification and direct sequencing of the entire coding region and exon/intron boundaries of *ATP2A2* were performed using the proband's and his parents' genomic DNA samples and genomic DNA samples from 50 healthy Japanese individuals as controls. A detected mutation was verified by mutant allele-specific amplification analysis[8] with mutant allele-specific primers carrying the substitution of two bases at the 3′ end, a PCR product band derived from the mutant allele.

This study was approved by the Hokkaido University Medical Ethics Committee and conducted according to the principles of the Declaration of Helsinki. All clinical samples were obtained with informed consent.

Results and discussion

Direct sequencing of *ATP2A2* in the proband's genomic DNA revealed a heterozygous three-base deletion c.120_122delGTT in exon 2, which causes deletion of leucine at the 41st amino acid residue from the amino terminus (p.Leu41del). This mutation was not detected in his parents nor in 100 normal unrelated alleles from 50 healthy individuals (Fig. 2). No other pathogenic mutations were detected within *ATP2A2* in the patient's DNA. By mutant allele-specific amplification ana-

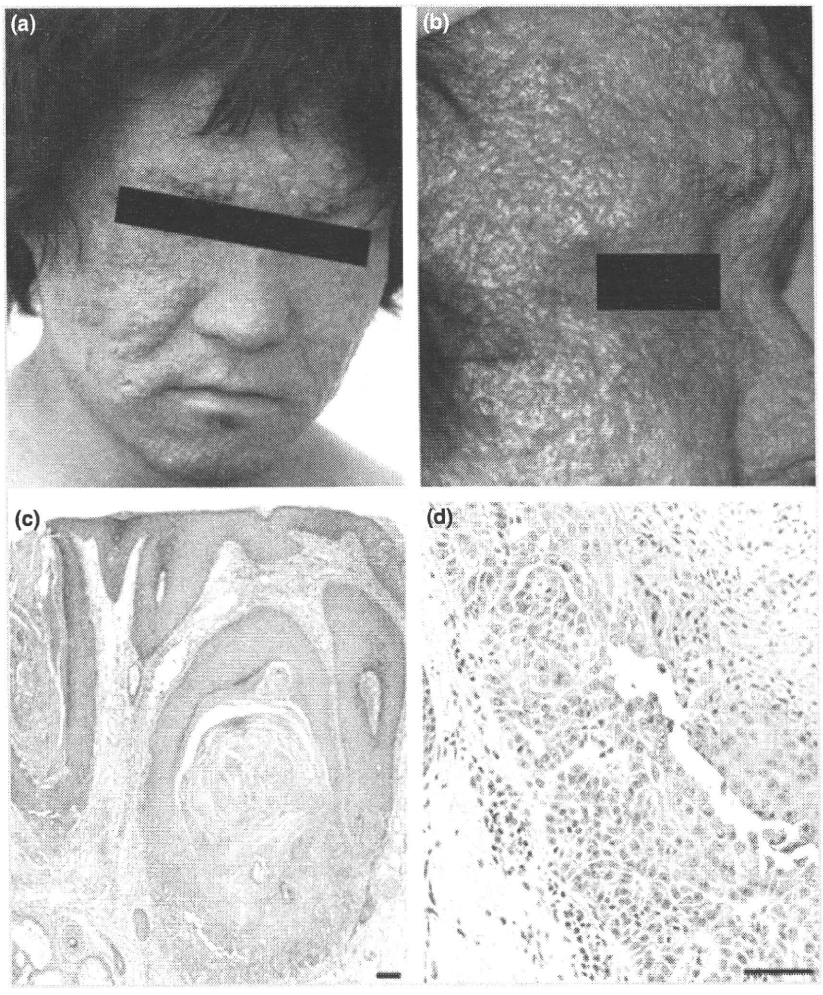

Fig 1. Unique clinical and histological features of comedonal Darier disease in the patient. (a, b) Open comedones, closed comedones, red papules, nodules, cysts and ice-pick scars are present on the face. (c, d) Histology of the comedonal lesions. Dilated, cystic hair follicles with keratin plugs are seen in the dermis (c). Suprabasal acantholytic clefts and numerous dyskeratotic cells in the form of corps ronds are apparent in the outer root sheath in the affected follicular infundibulum (d). Bars = 100 mm.

lysis,[8] a PCR product band derived from the mutant allele was amplified from the patient's genomic DNA, but not from either parent nor from the control DNA samples.

Since the first description of CDD by Derrick *et al.*,[4] only seven cases including the present one have been reported. The present case is the first in which a causative mutation has been identified. *ATP2A2* gene mutations in DD have been reported to result in alterations in calcium signalling during keratinocyte differentiation, causing acantholytic dyskeratosis.[2,9,10] The function of SERCA2 is to pump calcium from the cytosol to the endoplasmic reticulum and to excite oscillation of calcium spikes in the cytosol.[11–13] The mutation site in our patient localized to the first stalk (S1) of SERCA 2. The S1 region adjacent to transmembrane helices is considered to be highly conserved at the amino acid level by many species.[2] p.Leu41-del in S1 of SERCA2 is considered to impair calcium-binding sites in the α-helix of the region that contains a signal for sarco/endoplasmic reticulum localization, and to change the conserved alignment of five glutamic acid residues.[11,14] Several mutations in the S1 region of *ATP2A2* in DD have been reported.[2,10,14–16] In addition, the dephosphorylation process of SERCA2 is thought to be important for Ca^{2+} ion release into the lumen by SERCA2 and, recently, Miyauchi *et al.*[17] reported that both p.Leu41del and p.Pro42del mutations inhibit the dephosphorylation process. The present c.120_122delGTT mutation has not been reported, although a heterozygous deletion of the identical leucine residue, c.121_123delTTA (p.Leu41del), was reported in one patient.[14] The patient showed severe hypertrophic scar formation in addition to common DD skin manifestations and had severe emotional problems and a family history of suicide.[14] Our patient with CDD had a quite different skin phenotype and showed no mental problems. We do not know exactly why the phenotype differs between our case and the previously reported cases. There is a possibility that a silent mutation or allelic variant of *ATP2A2* may have affected the phenotypic expression in our patient and/or the previously reported cases. Certain environmental factors, such as mechanical trauma, sun exposure, heat and sweating often define a phenotype of DD,[2] and such factors may be related to the formation of the CDD phenotype in our patient, because the face is more frequently affected than other body sites by environmental factors. Further functional studies are required to elucidate the pathomechanisms of CDD, a unique phenotype of DD. Interestingly, an *ATP2A2* mutation was reported to underlie two cases from one British family with another unique phenotype, acrokeratosis verruciformis (AKV), providing evidence that AKV and DD are allelic disorders.[18] On the other

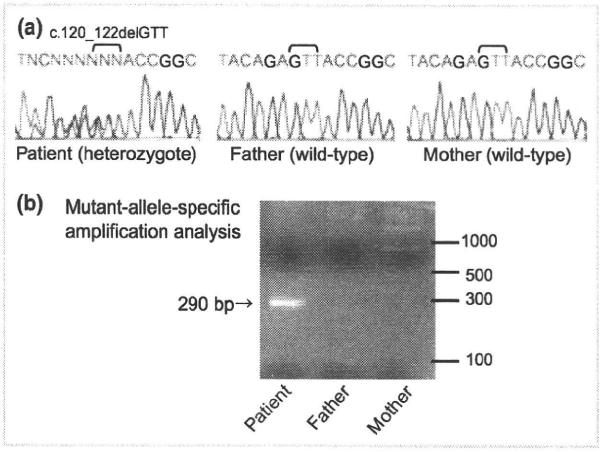

Fig 2. A heterozygous in-frame three-nucleotide deletion mutation c.120_122delGTT in *ATP2A2* was detected. (a) Direct sequencing of *ATP2A2* exon 2 polymerase chain reaction products by a reverse primer revealed that the patient was heterozygous for the three-nucleotide deletion mutation c.120_122delGTT. This mutation was not detected in genomic DNA samples from the patient's parents. (b) Mutant allele-specific amplification analysis shows the amplification band from the mutant allele as a 290-bp fragment only from the DNA sample of the patient, confirming the mutation c.120_122delGTT in the patient.

hand, mutational analysis showed no mutation in *ATP2A2* and genotyping and linkage analysis results revealed no linkage evidence to the locus including *ATP2A2* in a large Chinese family with AKV.[19] Thus, AKV might be a genetically heterogeneous disorder. In any case, further accumulation of cases with molecular genetic assessment is needed to improve understanding of the pathogenesis of variant phenotypes of DD such as CDD and AKV.

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Medical genetics

An Indian family with Sjögren-Larsson syndrome caused by a novel *ALDH3A2* mutation

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Abstract

Sjögren-Larsson syndrome is an autosomal-recessive hereditary disorder characterized by congenital ichthyosis, mental retardation and spastic diplegia or tetraplegia. It is known that mutations in the fatty aldehyde dehydrogenase (FALDH) gene (*ALDH3A2*) underlie SLS. We report two Indian sisters showing typical clinical features of SLS. Direct sequencing of the entire coding region of *ALDH3A2* revealed a novel homozygous mutation, c.142G>T (p.Asp48Tyr) in exon 1, in both patients. Their parents harbored the mutation heterozygously. Mutant-allele-specific amplification analysis using PCR products as a template verified the mutation in the patients. The aspartic acid residue at the mutation site is located in the C-terminal portion of the second a-helix strand, a2, of N-terminal four helices of FALDH and the FALDH amino-acid sequence alignment shows that this aspartic acid residue is conserved among several diverse species. Until now, a number of mutations in *ALDH3A2* have been shown to be responsible for SLS in Europe, the Middle East, Africa, and North and South America. However, in Asian populations, *ALDH3A2* mutations have been identified only in Japanese SLS patients. Here we report an *ALDH3A2* mutation for the first time in SLS patients in the Asian country other than Japan. The present results suggest that *ALDH3A2* is a gene responsible for SLS in Asian populations. We hope *ALDH3A2* mutation search will be globally available including many Asian countries in the future.

Case

Two sisters were born in an Indian nonconsanguineous family. The patient was a 1.5-year-old girl. She had had severe ichthyosis on the entire body since birth, especially prominent on the bilateral lower limbs (Fig. 1a–c). She showed mental retardation and spastic tetraplegia. Ocular fundus evaluation revealed white dots in the maculae. The elder sister also had icthyotic lesions all over the body at birth and had global developmental delay. She had had seizures since 2.5 years of age that had been controlled with multiple antiepileptic medications. At the age of four, severe hyperkeratosis appeared on the chest, back, axillae and predominantly over the limbs (Fig. 1d,e). She has hypertelorism, dolichocephalic head, large low-set ears, long eyelashes and short 3rd, 4th, and 5th metatarsals. Neurological evaluations revealed severe spastic tetraplegia with persistent ankle clonus and complete head lag. She showed serious mental retardation. She had severe photophobia, and ocular fundus evaluation showed white glistening dots in the maculae bilaterally. Severe auditory startle reaction was a characteristic feature. Magnetic resonance imaging of the brain showed bilateral symmetrical diffuse white matter at high intensity in T2-weighted images in the frontal, temporal, and parietal regions. Both sisters were diagnosed with Sjögren-Larsson syndrome (SLS) from these clinical features and laboratory data.

Fatty aldehyde dehydrogenase (FALDH) gene (*ALDH3A2*) mutational analysis was performed on the affected girls and their parents, as previously described.[1,2] In the patients, a novel homozygous mutation, c.142G>T (p.Asp48Tyr) in exon 1, was identified. Their parents harbored the mutation heterozygously (Fig. 2a). This mutation was not found in 200 normal unrelated alleles (100 individuals) by direct sequence analysis. Mutant-allele-specific amplification (MASA) analysis verified the mutation in this family (Fig. 2b).

Discussion

Sjögren-Larsson syndrome (MIM# 270200) is an autosomal-recessive hereditary disorder characterized by congenital ichthyosis, mental retardation and spastic diplegia or