Table 5 Staining Scores in SMPC, AMPC and nMPC lesions

Classification/Antibody	SMPC	AMPC	nMPC
Cellular adhesion molecules			
E-cadherin	215.3*	143.9	187.1
CD44	60.8‡	205.9¶	141.3
Laminin5γ2	69.4	36.9	60.3
Growth factor			
VEGF-C	294.4	296.4	282.1
Apoptosis-associated proteins			
bcl2	13.2	11.1	21.8
p53§	36.4	26.1	45.0
cleaved caspase-3§	0.3	0.2	0.4
Mucin-related proteins			
MUC1	169.7	182.5	202.1
MUC6	0.0	0.0	0.0
Hypoxia induced protein			
HIF-1α	1.8	2.4	2.9
Others			
TTF-1	267.9	289.3	248.6
SP-A	45.2‡	82.6	123.2
Vimentin	112.1	117.9	72.1
Ki-67§	22.6	16.9	16.2
LYVE1	98.9	107.2	101.9
c-Met	217.2	253.8	211.4
Phospho-c-Met	34.2‡	50.0	88.0

SMPC, stromal micropapillary component; AMPC, aerogeneous micropapillary component; nMPC, non-micropapillary component; VEGF-C, vascular endothelial growth factor-C; HIF-1α, hypoxia induced factor 1-α; TTF-1, thyroid transcription factor-1; SP-A, surfactant apoprotein A; LYVE1, lymphatic vessel endothelial hyaluronan receptor 1.

§ Positivity rate of positive tumor cells in 300 tumor cells (percentage).

* The difference in staining scores between SMPC and AMPC is statistically significant (*P* = 0.020).

‡ The differences in staining scores between SMPC and nMPC are statistically significant for CD44 (*P* = 0.011), and SP-A (*P* = 0.024) and phospho-c-Met (*P* = 0.011) expression.

¶ The difference in staining scores between AMPC and nMPC is statistically significant (*P* = 0.015).

tumors as well as AMPC(+) tumors are associated with several biological factors including tumor size, lymph node metastasis, advanced stage disease, and pleural and lymphovascular invasion. Univariate analysis also revealed the presence of SMPC and AMPC as a significant predictor of unfavorable outcome. However, the most remarkable finding was observed in multivariate analysis: among the patients in p-stage I, patients with not AMPC but SMPC showed a significantly poorer DFS than those without MPC. We used immunohisto-chemistry with monoclonal antibody D2-40 against lymphatic endothelium in TMA specimens and found that lymphatic vessels are involved within SMPC areas in 4 (21%) of 19 SMPC(+) tumors (data not shown). When compared with AMPC(+) tumors, SMPC(+) tumors

significantly more often showed pleural, lymphatic, and vascular invasion than AMPC(+) tumors (68% vs. 33%, *P* = 0.004; 74% vs. 30%, *P* < 0.001; 74% vs. 41%, *P* = 0.010, respectively). Therefore, these data suggest that a strong association between SMPC(+) tumors and pleural and lymphovascular invasion may in part explain their aggressive behavior.

Moreover, we investigated the immunohistochemical differences between SMPC and AMPC. In the study, we observed high E-cadherin expression and low CD44 expression in SMPC. Phospho-c-Met expression generally decreases in SMPC to a greater extent than in AMPC. Recently, it has been suggested that E-cadherin repression and CD44 expression are associated with the epithelial-mesenchymal transition (EMT), which was thought to lead to tumor invasion [19,20]. Additionally, Elliot et al. reported that hepatocyte growth factor (HGF) and c-Met signaling promotes EMT in breast cancer [21], and Orian-Rousseau et al. reported that CD44 is strictly required for c-Met activation by HGF in human carcinoma [22]. Consistent with these data, EMT may not occur in SMPC despite its existence in the stroma, or invasion of SMPC may occur through a different invasion mechanism from EMT. Our immunohistochemical findings of SMPC showed lower expression of SP-A than that of nMPC. Many studies have reported that SP-A deletion is correlated with patient survival, and reduced SP-A in MPC may be an excellent indicator for poor prognosis in small-size lung adenocarcinoma [23,24]. Reduced SP-A may contribute to an unfavorable outcome of SMPC(+) tumors.

Some studies have reported a significant association between the presence of MPC and *EGFR* mutations and effectiveness of EGFR tyrosine kinase inhibitor (EGFR-TKI) for MPC(+) tumors [25-28]. Since SMPC of lung adenocarcinoma may be associated with a high incidence of *EGFR* mutations, EGFR-TKI may be effective against SMPC(+) tumors. Patients with these pathological features of lung adenocarcinoma may benefit from EGFR-TKI as postoperative chemotherapy or first-line chemotherapy of relapsed lung adenocarcinoma.

In conclusion, we observed SMPC(+) adenocarcinoma. The incidence of SMPC(+) tumors is low, and SMPC(+) tumors have a different prognostic impact compared to AMPC(+) tumors. Particularly for the early stage tumors, SMPC(+) tumors have different pathobiological characteristics from AMPC(+) tumors, and SMPC(+) tumors frequently contain the *EGFR* mutation. Therefore, it is important to determine the presence of SMPC in lung adenocarcinoma, particularly p-stage I tumors, and the presence of SMPC should be noted in a pathology report to alert the clinician to the possibility of poor prognosis.
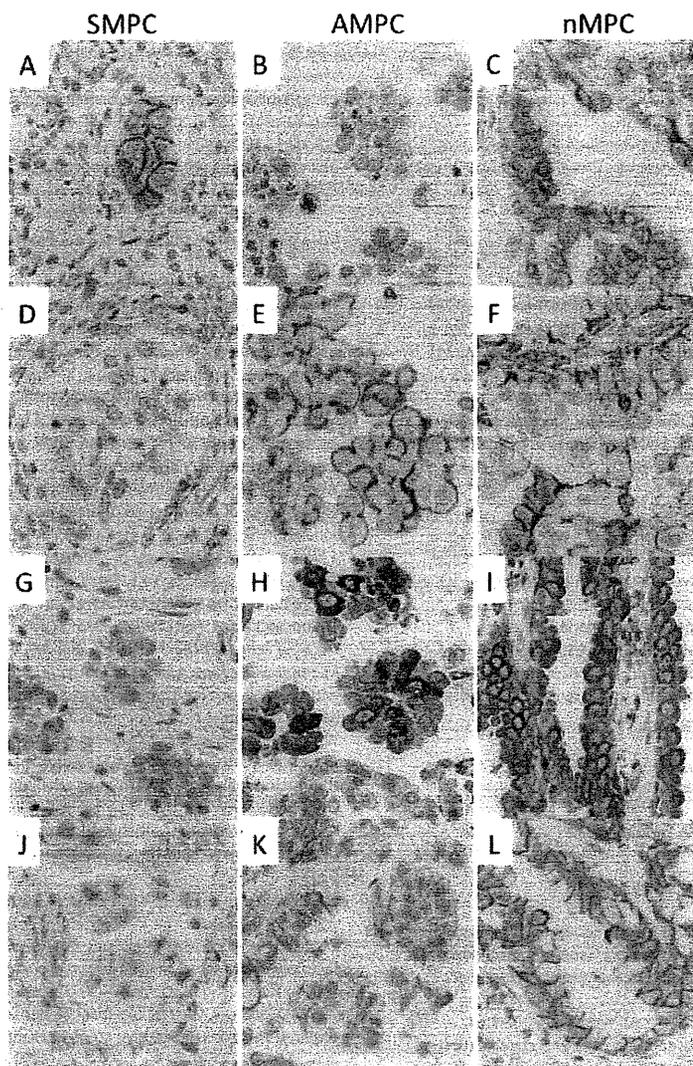
Figure 4 Photomicrographs of immunohistochemistry. E-cadherin (A-C); CD44 (D-F); SP-A (G-I); Phospho-c-Met (J-L). Compared with AMPC, increased E-cadherin and decreased CD44 membrane immunostaining were found in SMPC. Moreover, SP-A cytoplasm and Phospho-c-Met membrane immunostaining were decreased in SMPC (×400). SMPC, stromal micropapillary component, left panels; AMPC, aerogenous micropapillary component, middle panels; nMPC, non-micropapillary component, right panels.

Table 6 Mutation analysis

	total	%	SMPC(+) cases	%	SMPC(-) cases	%	*P* value
No.	33		19		14		
EGFR mutation							
Negative	13	39	5	26	8	57	0.076*
Positive	20	61	14	74	6	43	
ex19	7	35§	5	36§	2	33§	0.664*
ex21	13	65§	9	64§	4	67§	
KRAS mutation							
Negative	33	100	19	100	14	100	-
Positive	0	0	0		0		

No., number of patients; EGFR, epidermal growth factor receptor; SMPC, stromal micropapillary component
* Fisher's exact probability test
§ Rate of positive cases at ex19 and 21 in *EGFR* mutation positive cases, respectively.

List of abbreviations

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Authors' contributions

MO and TY designed the study, performed clinical and pathological investigation, and wrote the drafts. YS and YM participated in pathological and genetical investigation. NO participated in statistical investigation. SO performed the histological and immunohistochemical evaluation. CH assisted the clinical investigation. HN participated in managing and operating the patients. YK assisted the pathological investigation. KY participated in collecting clinical data and images. TI participated in its design and coordination and helped to draft 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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NAD-dependent histone deacetylase, SIRT1, plays essential roles in the maintenance of hematopoietic stem cells

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ABSTRACT

Sir2 has been shown to be essential for transcriptional silencing and longevity provided by calorie restriction in *Saccharomyces cerevisiae* and *Caenorhabditis elegans*. In this study, we investigated the role for its mammalian homologue, SIRT1, in hematopoietic cells. SIRT1 inhibitor, nicotinamide (NA), promoted and its activator, resveratrol, inhibited the differentiation of murine bone marrow c-Kit^{high}Sca-1$^+$Lineage$^-$ (KSL) cells during the culture system *ex vivo*. To further clarify the roles of SIRT1 in hematopoietic cells, we isolated KSL cells from fetal liver of SIRT1 knockout (KO) mice and cultured them for 5 days, because SIRT1 KO mice die shortly after the delivery. In agreement with the results from the experiments using NA and resveratrol, KSL cells isolated from SIRT1 KO mice more apparently differentiated and lost the KSL phenotype than those from wild-type (WT) mice. Furthermore, in each of colony assay, replating assay, or serial transplantation assay, SIRT1 KO KSL cells lost earlier the characteristics of stem cells than WT KSL cells. In addition, we found that SIRT1 maintains prematurity of hematopoietic cells through ROS elimination, FOXO activation, and p53 inhibition. These results suggest that SIRT1 suppresses differentiation of hematopoietic stem/progenitor cells and contributes to the maintenance of stem cell pool.

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1. Introduction

Hematopoietic stem cells (HSCs) are characterized by two capacity: self-renewal and multipotency. To maintain the hematopoiesis all along the lifespan without exhaustion of HSCs, most of HSCs are in a quiescent state and are supported at the microenvironment in bone marrow, namely "Niche" [1]. Although a number of molecules to control the characters of HSC, we have never been able to maintain HSCs in steady state *ex vivo* even in the presence of any combination of cytokines and/or on stromal cells. Thus, identification of new players in the maintenance of HSCs in "Niche" will be very informative to understand normal and/or malignant hematopoiesis.

Recently, some papers have reported that hypoxia in bone marrow niche has a pivotal role in the quiescence and maintenance of undifferentiated states of HSCs [2]. In the understanding of energy metabolism, oxygen consumption and glucose metabolism cannot be taken in separately, which have close interaction to each other. Although hypoxia alone cannot explain the mechanism of quiescence of HSCs, crucial roles of microenvironment have gained recognition.

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Sirtuin 1 (SIRT1) is a key molecule in glucose metabolism, and has a nicotinamide adenine dinucleotide (NAD)-dependent deacetylase activity. SIRT1 expression is induced by calorie restriction, and activation of SIRT1 protein by fasting induces gluconeogenic genes and hepatic glucose production. In addition, SIRT1 deacetylates a number of substrates including histones and non-histone proteins that are involved in numerous biological functions. Now, SIRT1 is believed to play pivotal roles in cellular differentiation [3], apoptosis [4], autophagy [5], development [6], cancer [7], circadian rhythms [8], and metabolism [9].

In the present study, we found that SIRT1 regulates self-renewal and differentiation of HSCs. Together with possible molecular mechanisms, we discussed roles of calorie metabolism in HSC microenvironment.

2. Materials and methods

2.1. Cytokines and reagents

Recombinant human thrombopoietin (rhTPO) was provided by Kyowa Hakko Kirin (Tokyo, Japan). Recombinant murine stem cell factor (rmSCF) and recombinant murine Flt3 Ligand (rmFlt3L) were

purchased from R&D Systems (Minneapolis, MN). Pifithrin (, tamox-ifen, nicotinamide and resveratorol were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Separation of murine hematopoietic progenitors

Murine Lineage⁻Sca-1⁺c-Kit^{high} (KSL) cells were obtained as previously described [10]. Murine BM cells were flushed from both femora and tibiae. To isolate KSL (Lineage⁻Sca-1⁺c-Kit^{high}) cells, se-lected progenitors were stained with phycoerythrin-conjugated (PE-conjugated) monoclonal Abs against murine lineage markers [CD3e, CD45R/B220, Gr-1, CD11b and TER-119], fluorescein isothi-ocyanate(FITC)-conjugated anti-Sca-1 Ab (E13-161.7), and allo-phycocyanin(APC)-conjugated anti-CD117 Ab, and isolated by FACSAria Cell-Sorting System (BD Biosciences, San Jose, CA). All Abs were purchased from BD Biosciences.

2.3. Colony assays

Cells were plated at the indicated density in methylcellulose medium (MethoCult; Stem Cell Technologies, Vancouver, BC, Canada) supplemented with the indicated growth factors, and the numbers of colonies were counted after the indicated days.

2.4. Flow cytometric analyses

Flow cytometric analyses were performed using BD FACSCanto II (BD Biosciences). The data analyses were done with BD FACSDiva software (BD Biosciences).

2.5. In vitro immortalization assays for HPCs

Immortalization assays of HPCs in vitro were performed as pre-viously described [11]. In brief, 10^4 cells were plated in 1.1 ml of methylcellulose medium (Methocult M3434). After the 1 week of culture, colony numbers were counted, and single-cell suspensions of colonies (10^4 cells) were subsequently replated under identical conditions. Replating was repeated every week in the same way.

2.6. Semiquantitative RT-PCR analysis

Total RNA was isolated from 5×10^4 FACS-sorted GFP-positive cells using TRIzol reagent (Invitrogen). RT-PCR was performed using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The sequences of forward/re-verse primer sets are indicated in Supplementary Table. The PCR products were electrophoresed in agarose gels containing ethi-dium bromide.

2.7. Retrovirus transduction

The conditioned media containing high titer retrovirus particles were prepared as described previously [11]. Briefly, an ecotropic packaging cell line, 293gp, kindly provided by Dr. H. Miyoshi (RI-KEN BioResource Center, Tsukuba, Japan), was transfected with FKHRL/ERT loading retrovirus vector by the calcium phosphate coprecipitation method. After 12 h, the cells were washed and cul-tured for 48 h. Then the supernatant containing virus particles was collected. The pre-cultured murine BM cells were infected with each retrovirus in the RPMI1641 medium supplemented with the same medium containing protamine sulfate for 48 h in 6-well dishes coated with RetroNectin (Takara Bio Inc., Shiga, Japan).

2.8. Paired-daughter cell colony assays

Each single KSL isolated from fetal liver of SIRT1 KO or WT fetus was plated into each well of 98-well plate containing RPMI med-ium supplemented with SCF, TPO, and Flt3L, using FACSAria. When a single KSL was divided into two daughter cells, each cell was manipulated and moved to the other well containing methylcellu-lose medium supplemented with cytokines, IL-3, IL-6, SCF, TPO and EPO.

3. Results

3.1. The expression of SIRT1 in hematopoietic cells

Human cord blood or murine bone marrow cells were separated on the basis of the indicated surface phenotypes, and RNA samples from each cell population were subjected to RT-PCR to investigate SIRT1 expression. In samples from human cord blood, fragment of the amplified SIRT1 gene was detected in HSC population (CD34⁺CD38⁻) as well as lineage committed populations (CD33⁺ myeloid progenitors, CD3⁺ T-lymphocytes, CD19⁺ B-lymphocytes, glycopholin A⁺ erythrocytes, and CD41⁺ megakaryocytes) (Fig. 1A left). In murine bone marrow cells, SIRT1 gene expression was also detected in wide hematopoietic cells including KSL, a HSC-enriched popukation (Fig. 1A right). Therefore, SIRT1 was widely expressed in all of the hematopoietic lineages from primitive to mature cells.

3.2. The effects of NA and the deficiency of SIRT1 on the differentiation of hematopoietic stem and progenitor cells

The wide expression of SIRT1 led us to investigate its physiolog-ical roles on hematopoiesis. We utilized a SIRT1 inhibitor, nicotin-amide (NA), and a SIRT1 activator, resveratrol. When KSL cells isolated from murine bone marrow were cultured for 5 days in the medium containing TPO, SCF and Flt3L, approximately 30% of the cultured cells remained in the KSL fraction (Fig. 1B). While, the addition of NA resulted in the significant decrease of the KSL cells (approximately 5%). In contrast, the addition of resveratrol in-creased the proportion in this fraction up to approximately 37%. Similar results were observed in experiments to see granulocyte-specific terminal differentiation from KSL cells. By the addition of NA, under the granulocytes-inducing condition, the cultured cells bearing Gr1 were significantly increased and they morphologically showed granulocyte features (Fig. 1C). Furthermore, we examined the differentiation of KSL cells derived from SIRT1 KO fetal liver cells. At the point of extraction, embryonic day 14.5, the proportion of KSL cells were not different between WT and KO fetal liver cells (Fig. 1D upper). However, cultures of SIRT1 KO fetal liver cells showed significant decrease of the remaining KSL cells as com-pared with those of WT fetal liver cells.

Therefore, SIRT1 has an ability to inhibit differentiation of hematopoietic stem/progenitor cells.

3.3. The maintenance of stemness is inhibited in SIRT1 KO KSL cells

To analyze the characteristics of hematopoietic stem and pro-genitor cells, colony assays were performed. KSL cells isolated from SIRT1 KO or WT fetal liver at embryonic day 14.5 were plated in methylcellulose medium containing IL-3, IL-6, SCF, TPO and EPO. In both, similar numbers of colonies were generated (Fig. 2A), indi-cating that the number of existing hematopoietic stem/progenitor cells in fetal liver was not significantly different from each other.

To analyze the involvement of SIRT1 in self-renewal capacity of HSCS, we performed replating colony assays. At first, 1000 KSL cells from SIRT1 KO or WT fetal liver were plated into methylcellulose
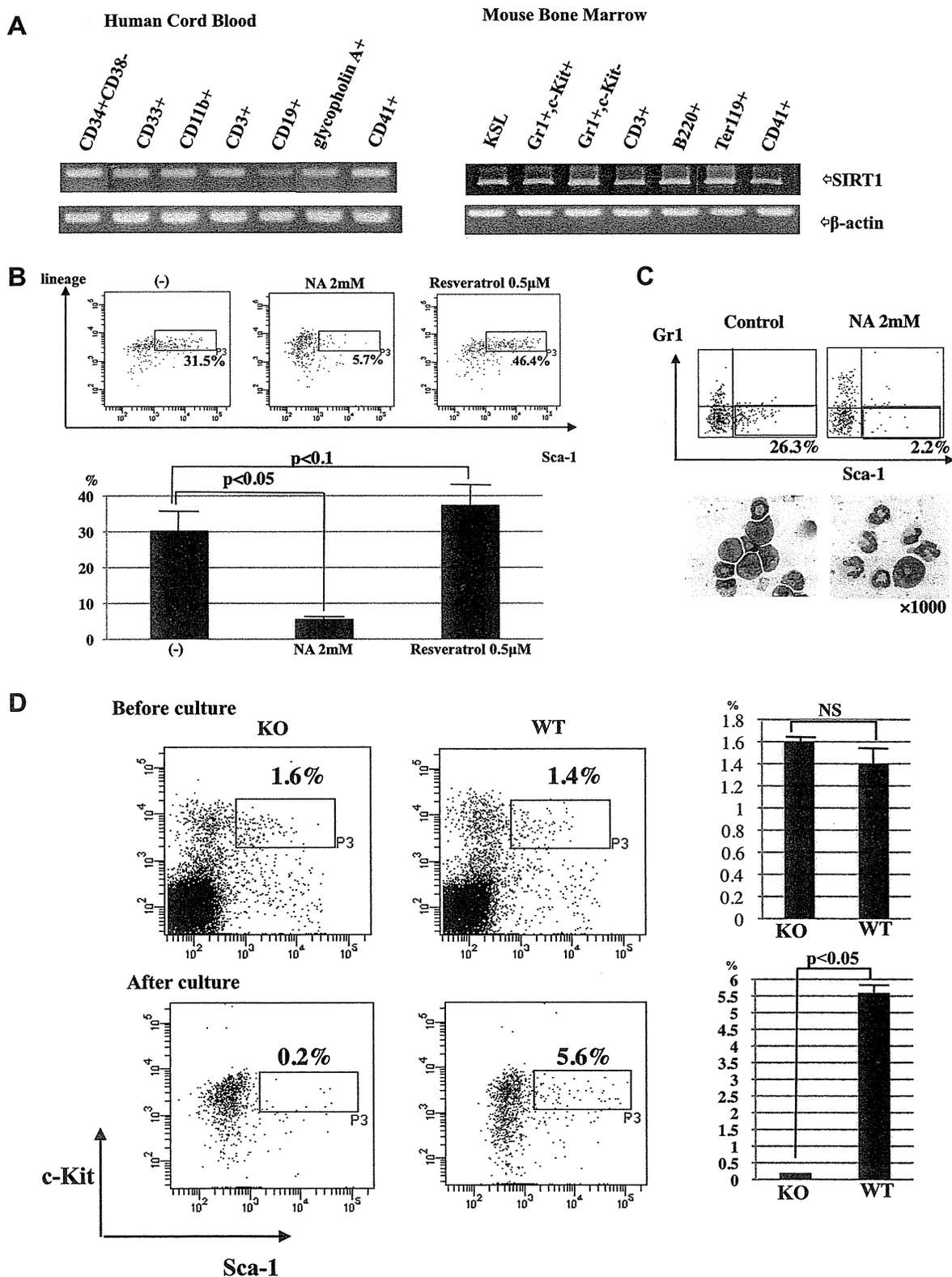
Fig. 1. SIRT1 expressions and its effects on hematopoietic stem/progenitor cells. (A) Hematopoietic cells of various lineages at the different stage of differentiation from human cord blood (upper panel) and murine bone marrow (lower panel) on the basis of indicated surface phenotypes, and RT-PCR was performed using mRNA samples. Human hematopoietic surface markers: $CD34^+38^-$: stem/progenitors, CD133, CD11b: granulocytes, monocytes, CD3: T-cells, CD19: B-cells, glycophorin A: erythrocytes, CD41: megakalyocytes. Murine surface markers: Lineage$^-$Sca-1$^+$c-Kit^{high} (KSL): stem/progenitors, Gr-1$^+$c-Kit$^+$: myeloid progenitors, Gr-1$^+$c-Kit$^-$: mature granulocytes, CD3: T-cells, B220: B-cells, Ter119: erythrocytes, CD41: megakalyocytes. (B) KSL cells isolated from murine bone marrow were cultured for 5 days in the medium containing TPO, SCF, and Flt3L, together with or without 2 mM NA or 0.5 μM resveratrol. Then cultured cells were stained with Abs for lineage markers, c-Kit, and Sca-1, and were subjected to FACS analysis. (C) Upper panel: fetal liver whole cells were subjected to FACS analysis, at the point of extraction. Lower panel: KSL cells were cultured with cytokines for 5 days and were subjected to FACS analysis. (D) Murine bone marrow KSL cells were cultured with G-CSF and SCF, together with or without 2 mM NA for 5 days. Upper panel: after the culture, cells were stained with indicated Abs, and were subjected to FACS analysis. Lower Panel: the cultured cells were concentrated on the slide glass with cytospin centrifuge, and were stained with May–Giemsa stain.

A

B

C

D
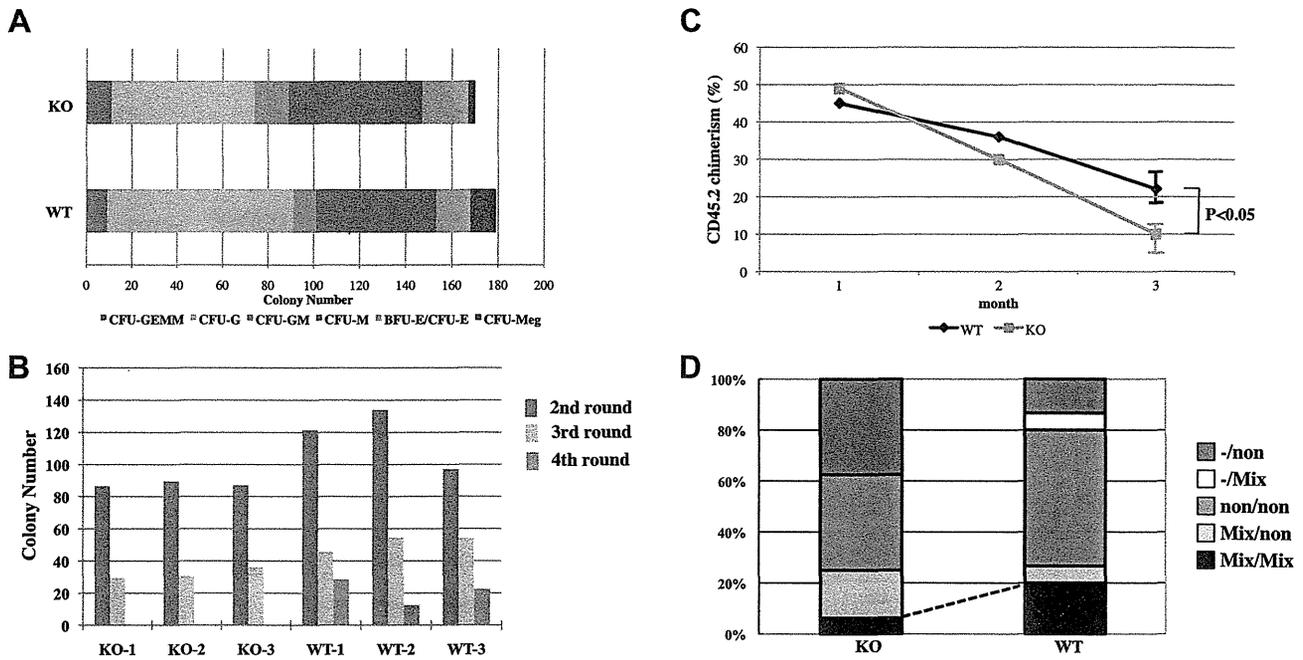
Fig. 2. Characteristics of SIRT1 deficient KSL cells. (A) KSL cells were seeded in methylcellulose medium containing SCF, IL-3, IL-6, TPO, and EPO. Colony numbers were counted after 9 days. Representative myeloid/erythroid colony numbers ($n = 3$) are shown. CFU-GEMM: multipotential colony-forming unit, CFU-G: colony-forming units-granulocytes, -M: -macrophages, and -GM: -granulocytes-macrophages, -E: -erythrocytes, -Meg: -megakaryocytes, and BFU-E: burst-forming units-erythroid. (B) First, 1000 fetal liver KSL cells from each indicated fetus were plated into methylcellulose plate. After 7 day cultures, 10,000 of colony-forming cells were suspended into the new methylcellulose plate, and after 7 days, colony numbers were counted. Replating methods were repeated every 7 days. (C) First, SIRT1 KO or WT fetal liver whole cells were injected intravenously in combination with 2×10^5 BM cells from CD45.1 mice into lethally irradiated 8–12 week-old CD45.1 (B6-Ly5.1) mice. Six weeks after, mice were sacrificed and donor chimerism (CD45.2$^+$) was analyzed. Then 5×10^3 of CD45.2 KSL cells derived from BM of each first transplanted mouse were serially transplanted into another irradiated CD45.1 mouse. This procedure was repeated one more time. The results are presented as means ± SEM. (D) When each single KSL cell was divided into two daughter cells during the liquid culture, each daughter cell was separated and moved to the other well containing methylcellulose medium supplemented with cytokines, IL-3, IL-6, SCF, TPO and EPO. After 7-day culture, colonies were observed and evaluated. "Mix" represents multipotential colony-forming unit, and "non" means not "Mix". Each square shows percentage of colony pairs from KO or WT fetal liver KSL cells.

plate. After 7 day culture, numbers of colonies were counted, and then 10,000 of colony-forming cells were re-suspended into the new methylcellulose plate. Replating methods were repeated every 7 days. As shown in Fig. 2B, SIRT1 KO KSL cells lost colony-forming ability within the 4th plating, while WT KSL cells could still make more than ten colonies. Self-renewal capacity of HSC was also compared between SIRT1 KO and WT fetal liver using serial transplantation assays. Donor chimerism (CD45.2$^+$) was more rapidly reduced in SIRT1 KO cells during serial transplantation compared with WT cells, with significant difference (Fig. 2C). These data suggest that self-renewal of SIRT1 deficient HSC was in part impaired.

To estimate the symmetric or asymmetric division of HSCs, which indicates the state of self-renewal or differentiation of HSCs, we performed paired-daughter cell colony assays, using SIRT1 KO or WT KSL cells. Single cells were isolated from fetal liver KSL cells and plated into each well. When a single KSL was divided into two daughter cells, each cell was moved into the other well containing methylcellulose. After 7 days, the character of each colony was evaluated. As shown in Fig. 2D, some daughter cells lost capacity to produce multi-lineage types of colonies. It is noteworthy that Mix/Mix colony-pairs were apparently reduced in SIRT1 KO KSL cells compared with those in WT KSL cells (5% versus 20%, respectively). These data also suggest that SIRT1 is involved in self-renewal capacity of HSCs.

3.4. Possible molecular mechanisms of SIRT1-mediated suppression of HSC differentiation

To clarify the molecular mechanisms underlying SIRT1-related self-renewal capacity of HSCs, we examined the effects of NA on

the expression of several genes, which regulate self-renewal of HSCS. When NA was added into the culture for 24 h, the gene expression of HOXB4, E2F-1, and PML was slightly down-regulated and that of $p16^{INK4a}$ and $p19^{ARF}$ was obviously up-regulated (Fig. 3A). Because the accumulation of ROS is known to induce $p16^{Ink4a}$ expression in HSCS [12], we evaluated levels of ROS in SIRT1 KO KSL cells using REDCC1. SIRT1 KO KSL cells showed higher accumulation of ROS than SIRT1 WT KSL cells (Fig. 3B upper). Moreover, the addition of an anti-oxidant agent, N-acetyl cysteine (NAC), reduced ROS in SIRT1 KO KSL cells to almost the same level as in WT KSL cells. In parallel with ROS accumulation, SIRT1 KO KSL cells differentiated more rapidly than WT KSL cells, which were canceled by the treatment with NAC. After 5 day culture, although the population of remaining KSL fraction was smaller in SIRT1 KO cells (0.2%) compared with those in WT (5.8%), NAC addition in KO cells increased this fraction up to the same level as seen in WT (4.8%) (Fig. 3B, lower). These data indicate that SIRT1 regulate the level of ROS that promotes differentiation of HSCS.

SIRT1 is also reported to deacetylate FORKHEAD proteins, thereby enhancing or inhibiting their activity according to the cellular context [13]. Thus, we evaluated the involvement of FOXO3a, a member of FORKHEAD family, in SIRT1-mediated suppression of HSC differentiation. We introduced a retrovirus vector MSCV-neo loading FKHRL1/ERT [14] into KSL cells, which can induce the FOXO3a activity by supplement of tamoxifen. Although, as described above, SIRT1 deficient KSL cells rapidly lost Sca-1 expression under tamoxifen null condition, of importance, tamoxifen-induced FOXO3a activity clearly restored the maintenance of KSL phenotypes in SIRT1 KO cells (Fig. 4A). These data suggest that the activity of FOXO3a can improve the insufficient capacity to maintain HSC phenotypes in SIRT1 deficiency.
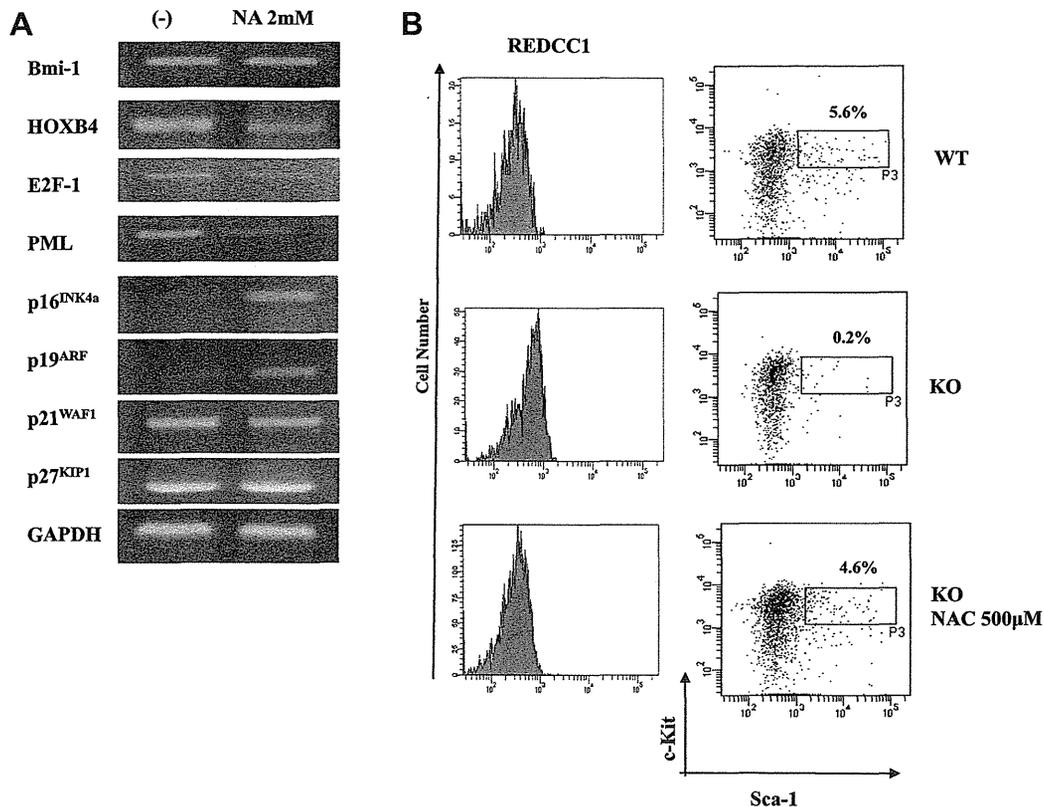
Fig. 3. Gene expression and ROS accumulation in SIRT1 KO KSL cells. (A) KSL cells isolated from murine bone marrow were cultured for 5 days in the medium containing TPO, SCF, and Flt3L, together with or without 2 mM NA. Messenger RNAs from the cultured cells were subjected to RT-PCR. (B) WT KSL cells were cultured, and SIRT1 KO cells were cultured with or without N-acetyl cysteine (NAC) for five days with TPO, SCF, and Flt3-L. Then ROS accumulation was evaluated by FACS analysis using REDCC1, and remaining population of KSL fraction was examined.

SIRT1 is also reported to suppress the p53 activity through its deacetylation [4]. Thus, we examined the effects of a p53 inhibitor (Pifithrin α) in HSCS culture systems. As shown in Fig. 4B left, although the KSL fraction was more rapidly reduced in SIRT1 KO cells compared with WT cells during cultures without Pifithrin α, the addition of Pifithrin α restored the number of the cultured SIRT1 KO KSL cells to the same level as that of WT (12.0% and 10.8%, respectively) (Fig. 4B). We also examined the effects of NA on the differentiation of KSL cells derived from p53 KO mice. In loosing Sca-1 expression during cultures, p53 KO, but not WT, KSL cells were resistant to the treatment with NA (Fig. 4B right). These data indicate that p53 has critical roles in the SIRT1-mediated suppression of HSCS differentiation.

To analyze the effects of MAPK/ERK and p38 pathways, SIRT1 KO or WT fetal liver KSL cells were cultured with MEK inhibitor, PD98059, or p38 inhibitor, SB203580. However, both compounds could not change the population of KSL cells in SIRT1 KO and WT cell cultures (data not shown).

Therefore, the capacity of SIRT1 to maintain immature state of hematopoietic stem/progenitor cells is likely to be related to the ROS, FOXO3a, and p53 pathways.

4. Discussion

Besides important roles in calorie metabolism, several reports have indicated that SIRT1 is a multi-functional protein involving in stress responses and aging [15]. In the present study, we found that SIRT1 has an ability to inhibit the differentiation of HSCs and to maintain self-renewal of HSCs. We also showed the possible mechanism showing that ROS, FOXO3a, and p53 pathways are involved in the SIRT1-mediated inhibition of HSC-differentiation.

To maintain hematopoietic homeostasis and thus protect against the exhaustion of HSC population, a majority of HSCs are in a quiescent state. Particular microenvironment for HSCs, termed "Niche", provides these stimuli to HSCs and regulates not only the homeostatic self-renewal and the differentiation of HSCs but also hematopoiesis under pathological conditions such as aging and hematological malignancies. As widely believed, the proliferation and differentiation of HSCs are regulated by various cytokines, stromal cells, and extracellular matrix. Of importance, genetic manipulation in mice has provided several key molecules for self-renewal and pluripotentiality of HSCs. Indeed, mice lacking ataxia telangiectasia mutated have a defect in the self-renewal of HSCs and exhibit a progressive decline of HSCs in parallel with age. This abnormality is restored by inhibition of ROS accumulation or by downregulation of p16^{Ink4a} expression. Our results indicate SIRT1 as a new regulator of HSCs.

Chow et al. [16] speculated that stem cells are located at the region with very low pO$_2$ levels (almost anoxic), and Parmer et al. [17] showed that regionally defined hypoxia plays a fundamental role in regulating stem cell function. In a hypoxic condition, ATP production depends mainly on a glycolytic pathway, which may increase lactate and decrease the ratio of NAD/NADH within the cells. As SIRT1 is an NAD-dependent protein, the activity of SIRT1 is supposed to be reduced under hypoxic condition. However, in the serum starved in vitro or the ischemic in vivo cardiac myocytes, the expression of SIRT1 is reported to upregulated dependent on
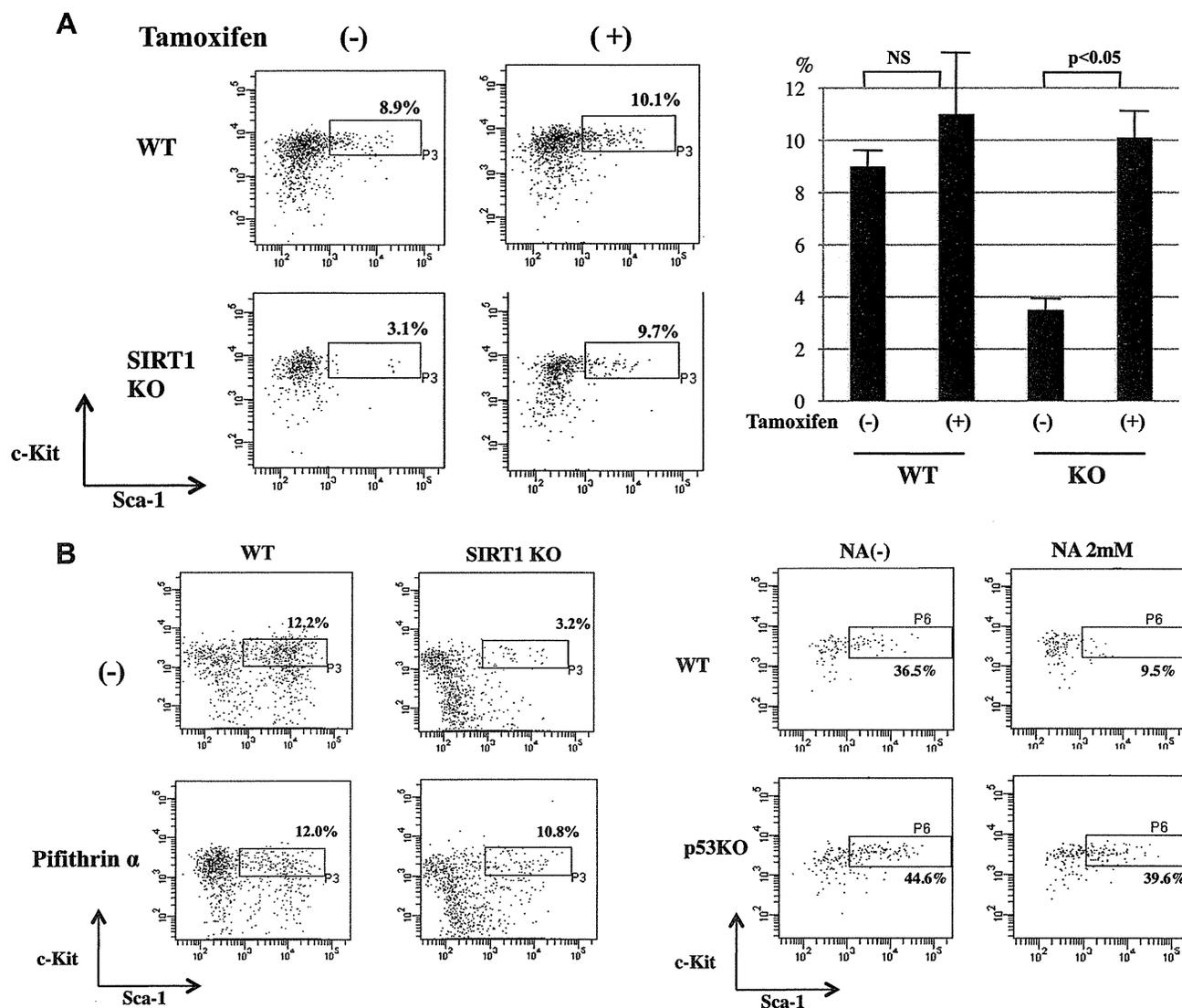
Fig. 4. Roles of FOXO3a and p53 in the inhibition of differentiation of KSL cells by SIRT1. (A) KSL cells from KO and WT fetal liver were transfected with retrovirus loading FKHRL/ERT or MOCK. Two days after, the transfected cells in KSL fraction were sorted again and cultured with tamoxifen or vehicle only for 5 days. KSL population was evaluated by FACS analysis (left panel). The results are presented as means ± SEM in the bar graph (right panel). (B) KSL cells from SIRT1 KO or WT fetal liver were cultured with or without p53 inhibitor, Pifithrin 〈, and the population of KSL fraction was evaluated by FACS analysis after 7 day culture (left panel). p53 KO or WT BM KSL cells were cultured with or without 2 mM NA. After 3 day culture, the population of KSL fraction was evaluated (right panel).

the intracellular accumulation of NAD [18] [19]. Thus, the circulation failure causes calorie lack as well as hypoxia. If the hypoxia in BM niche should be caused by inadequate circulation, it seems quite likely that BM niche is in calorie-restricted condition, which induces NAD accumulation followed by SIRT1 activation in HSCs. SIRT1 is known to inhibit ROS production in human umbilical vein endothelial cells with H_2O_2-induced oxidative stress [20]. SIRT1-deficient hepatocytes show high ROS levels during glucose toxicity [21]. Thus, SIRT1 acts to eliminate ROS in a variety of cells. SIRT1 deacetylates forkhead box transcription factors, resulting in varying effects on FOXO-induced gene expression, ranging from activation to repression [13]. p53 is also a non-histone deacetylation target of SIRT1, and overexpression of SIRT1 attenuates p53-dependent apoptosis upon DNA-damage and oxidative stress [22]. The reports correspond well with our data showing that SIRT1 eliminates ROS, and control FOXO and p53 pathways. Notably, many groups reported links between ROS and p53 and FOXO transcriptional activity, and FOXO transactivates antioxidant defending enzymes to reduce ROS levels, suggesting a ROS–FOXO feedback-signaling loop. Through the modification of this complex

intracellular network, SIRT1 seems to influence a number of modulators for cell growth and differentiation of HSCs, including p16^{Ink4a} and p19^{Arf}. We showed in this paper that SIRT1 has an important role in the maintenance of HSCs through the regulation of ROS, FOXO3a, and p53 pathways, which indicates that the calorie metabolism, as well as, or in corporate with, the hypoxic state of BM niche, may have a pivotal role in the resistance to oxidative stress and the determination of HSCs' fate.

Activating SIRT1 by resveratrol improves health and survival of mice on a high-calorie diet [23], and SIRT1 is now believed to act as an anti-senescence factor *in vivo*. ROS, FOXO, p53, p16^{Ink4a} and p19^{Arf}, which we described as SIRT1-related molecules in HSCs, are well-known to control senescence. For example, p16^{Ink4a} increases with age and regulates age-associated HSC functions [24]. Thus, our results are likely to suggest that calorie metabolism plays a role in controlling of senescence as well as self-renewal and differentiation of HSCs.

Our data obtained from replating colony assay, daughter cell colony assay, and serial transplantation assay strongly indicated that SIRT1 is involved in the self-renewal of HSCs. In addition,

the inhibitory effects of SIRT1 on HSC differentiation are likely to be in part useful to avoid the exhaustion of HSCs.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2012.01.109.

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Oncology

A Phase I Study of Infusional 5-Fluorouracil, Leucovorin, Oxaliplatin and Irinotecan in Japanese Patients with Advanced Colorectal Cancer Who Harbor UGT1A1*1/*1, *1/*6 or *1/*28

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Abstract
Objective: To evaluate the safety and efficacy of combination chemotherapy with 5-fluorouracil (5-FU), leucovorin, irinotecan and oxaliplatin (FOLFOXIRI) in Japanese patients with advanced colorectal cancer. *Methods:* This phase I dose-finding study was designed to determine the maximum tolerated dose (MTD), recommended dose (RD) or both of FOLFOXIRI. Patients with *UDP-glucuronosyltransferase (UGT) 1A1*6/*6, *28/*28* and *6/*28 genotypes were excluded, because these *UGT1A1* genotypes are linked to severe neutropenia in Japanese. *Results:* A total of 10 Japanese patients with advanced colorectal cancer were studied. The MTD of FOLFOXIRI in these Japanese patients was 165 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin and 2,400 mg/m^2 5-FU. Accordingly, the RD of FOLFOXIRI was determined to be 150 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin and 2,400 mg/m^2 5-FU. Toxic effects, evaluated until the completion of 4 cycles, were manageable. Grade 3–4 neutropenia occurred in 27% of cycles, but there was no febrile neutropenia. Among the 9 assessable patients, the objective response rate was 89%. *Conclusions:* We thus determined the RD of FOLFOXIRI in Japanese patients with advanced colorectal cancer who do not have *UGT1A1*28/*28, *6/*6 or *6/*28* genotypes. Our results indicate that FOLFOXIRI is a well-tolerated regimen for these Japanese patients.

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Introduction

The key cytotoxic antitumor drugs for colorectal cancer are 5-fluorouracil (5-FU), irinotecan and oxaliplatin. Exposure to all three of these active cytotoxic drugs during the course of treatment has been associated with longer overall survival (OS) [1, 2]. However, several studies have demonstrated that only 60–80% of patients can receive second-line treatments in sequential strategies and therefore are not exposed to all three agents [1]. These fac-

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tors have fostered attempts to develop potentially more active first-line regimens combining 5-FU with both irinotecan and oxaliplatin.

A combination of 5-FU with leucovorin (LV), irinotecan and oxaliplatin (FOLFOXIRI) has been demonstrated to be more effective than 5-FU-LV and irinotecan (FOLFIRI) in randomized clinical trials performed in Western countries [3, 4], suggesting that first-line treatment with FOLFOXIRI might improve survival in metastatic colorectal cancer. FOLFOXIRI has produced a higher response rate (RR) of 66% and longer OS of 23.4 months than any other regimen evaluated in randomized studies of metastatic colorectal cancer reported to date [5–10]. FOLFOXIRI might also thus facilitate the radical surgical resection of metastases initially considered unresectable [11, 12]. The FOLFOXIRI regimen is considered an initial therapy for advanced colorectal cancer and is included in the National Comprehensive Cancer Network guidelines version 2.2012. However, no study has reported on the safety and efficacy of FOLFOXIRI in Japanese patients with advanced colorectal cancer.

Polymorphisms of the UDP-glucuronosyltransferase (UGT) 1A1 gene have been established to underlie irinotecan-related toxicity [13, 14]. Asians have a lower allele frequency of UGT1A1*28 than Whites, although this polymorphism is seen in both ethnic groups (16% in Asians and 39% in Whites) [15]. In contrast, a single-nucleotide polymorphism in exon 1 of the UGT1A1 gene, UGT1A1*6, which is related to decreased catalytic activity for SN-38 glucuronidation [16], occurs at a relatively high allele frequency in Asians (approximately 20%), but not in Whites [17–19]. Since UGT1A1*28 and *6 are separately located on two different alleles, UGT1A1*6/*6, *28/*28 and *6/*28 genotypes are found in Japanese patients with cancer at a frequency of approximately 10% [17]. These UGT1A1 genotypes have been linked to severe neutropenia [18, 19]. Because FOLFOXIRI is a potent regimen with a relatively high frequency of grade 3–4 neutropenia, occurring in 35–50% of patients [3, 4], it might be prudent to exclude patients with these UGT1A1 genotypes to avoid irinotecan-related severe neutropenia. To gain further insight into these issues, we performed a dose-finding phase I study to assess the safety and efficacy of FOLFOXIRI in Japanese patients with advanced colorectal cancer who harbor UGT1A1*1/*1, *1/*6 or *1/*28. The primary endpoint of our phase I study was to determine the maximum tolerated dose (MTD), the recommended dose (RD) or both. The secondary endpoint was to clarify the objective RR.

Table 1. Dose adaptation schedule

Level	Irinotecan mg/m^2	Oxaliplatin mg/m^2	5-FU mg/m^2
2	180	85	3,200
1	165	85	3,200
0	165	85	2,400
−1	150	85	2,400
−2	120	85	2,400

Patients and Methods

Patient Selection

Enrolled patients were required to meet the following eligibility criteria: histologically confirmed adenocarcinoma of the colon or rectum, unresectable recurrent or metastatic disease, age 20–70 years, an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1, measurable disease according to the Response Evaluation Criteria in Solid Tumors, version 1.0 [20], leukocyte count $\geq 3,500/mm^3$, neutrophil count $\geq 1,500/mm^3$, platelet count $\geq 100,000/mm^3$, serum creatinine ≤ 1.5 mg/dl, serum bilirubin ≤ 1.2 mg/dl and serum aspartate aminotransferase and alanine aminotransferase ≤ 5 times the respective upper limits of normal. Previous adjuvant or palliative chemotherapy with 5-FU with or without LV was allowed. Exclusion criteria were previous chemotherapy with irinotecan or oxaliplatin, symptomatic cardiac disease, myocardial infarction, uncontrolled arrhythmias, active infections and inflammatory bowel disease. Patients with UGT1A1*6/*6, *28/*28 or *6/*28 genotypes were excluded. The study was approved by the Institutional Review Board of the Saitama Medical University, and patients were informed of the investigational nature of the study and provided their written informed consent before registration (trial registration ID: UMIN000000883).

Dose Adaptation Schedule

The dose adaptation schedule is shown in table 1. If the MTD was not reached at the initial dose level (level 0: 165 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin and 2400 mg/m^2 5-FU), the next group of patients received an escalated dose level (level 1: 165 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin and 3,200 mg/m^2 5-FU). If the MTD was also not reached at level 1, subsequent patients received level 2 (180 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin and 3,200 mg/m^2 5-FU). If the MTD was not reached at level 2, no further dose escalations of any drugs were planned because the respective doses of irinotecan and oxaliplatin at level 2 were approximately their RDs as single agents or in combination with 5-FU. This level was defined to be the RD. If the MTD was reached at level 0, the dose could be reduced down to level −2 (level −1: 150 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin and 2,400 mg/m^2 5-FU; level −2: 120 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin and 2,400 mg/m^2 5-FU). If level −2 was the MTD, the study was concluded.

Dose-Limiting Toxicity and MTD

Dose-limiting toxicity (DLT) was defined as any grade 3 or 4 nonhematologic toxicity, except for nausea, vomiting, anorexia,

A Phase I Study of FOLFOXIRI in Japanese

Table 2. Patient characteristics

Characteristic	
Total patients	10
Median age (range), years	54 (33–69)
Male/female	9/1
ECOG PS	
0	10
1	0
Primary tumor site	
Colon/rectum	6/4
Previous surgery on primary tumor	7
Number of metastatic sites	
Single	4
Multiple	6
Metastatic sites	
Lung	6
Liver	6
Lymph nodes	4
Others	1
Previous chemotherapy	
Adjuvant	3
Palliative	0

Table 3. Maximum toxicity per cycle observed in a total of 37 cycles

Event	NCI-CTC grade					
	1^a	2^a	3^a	4^a	$1-4^b$	$3-4^b$
Leukopenia	7	11	1	0	51	3
Neutropenia	8	7	7	3	68	27
Anemia	12	0	1	0	35	3
Thrombocytopenia	3	0	0	0	8	0
Fatigue	13	1	0	0	38	0
Anorexia	17	3	1	0	57	3
Nausea	17	3	1	0	57	3
Vomiting	10	0	1	0	30	3
Diarrhea	3	3	0	0	16	0
Constipation	5	0	0	0	14	0
Mucositis	4	0	0	0	11	0
Alopecia	19	1	–	–	54	–
Neuropathy (sensory)	13	0	0	0	35	0
AST	8	0	0	0	22	0
ALT	12	1	0	0	35	0
Infection with normal ANC	0	0	1	0	3	3
Febrile neutropenia	–	–	0	0	–	0

Maximum toxicity in each patient was evaluated until the completion of 4 cycles. A total of 37 cycles were completed. NCI-CTC = National Cancer Institute Common Toxicity Criteria, version 3.0; – = not defined in the National Cancer Institute Common Toxicity Criteria, version 3.0; AST = aspartate aminotransferase; ALT = alanine aminotransferase; ANC = absolute neutrophil count.
[a] Values represent numbers of cycles.
[b] Values represent percentages.

Toxicity

Toxicity was assessable in all patients until the completion of 4 cycles (table 3). The most common toxicities were neutropenia, anorexia, nausea, vomiting and alopecia. However, grade 3 and 4 toxicities were uncommon, except for neutropenia. In particular, 27% of cycles were associated with grade 3–4 neutropenia, although no patient had febrile neutropenia. When we evaluated the maximum toxicity per patient over 4 cycles, 5 patients (50%) had at least one episode of grade 3–4 neutropenia. No patient required hospitalization or died because of toxicity.

The causes of treatment discontinuation were PD in 4 patients, delayed recovery from toxicity such as neuropathy and nausea in 2 patients, allergic reaction to oxaliplatin in 2 patients, conversion therapy in 1 patient and DLT in 1 patient.

Efficacy

Response was assessable in 9 patients. Response was not assessed in 1 patient because of DLT (infection) occurring during the first cycle of treatment. One of the 9 patients (11%) had a CR, and 7 (78%) had PRs, resulting in an objective RR of 89% (95% confidence interval 56–98%; table 4). The location of responses were metastases of abdominal lymph nodes in a patient who had a CR and

metastases of lung (n = 2), liver (n = 2) or both (n = 3) in patients who showed PRs. The median number of cycles required to reach PR was 3.5 (range 2–6). Residual liver metastases were surgically removed after chemotherapy in 1 patient whose metastases were initially considered unresectable. The median PFS was 11.6 months, after a median follow-up of 34.7 months, as calculated by the Kaplan-Meier method with the use of JMP software, version 6 (SAS Institute Inc., Cary, N.C., USA). The median dose intensities of irinotecan, oxaliplatin and 5-FU during the first 4 courses of treatment were 73 mg/m²/week (88% of planned), 37 mg/m²/week (88% of planned) and 1,066 mg/m²/week (88% of planned), respectively, among the 3 patients given level 0, and 59 mg/m²/week (79% of planned), 33 mg/m²/week (79% of planned) and 950 mg/m²/week (79% of planned), respectively, among the 6 patients given level –1.
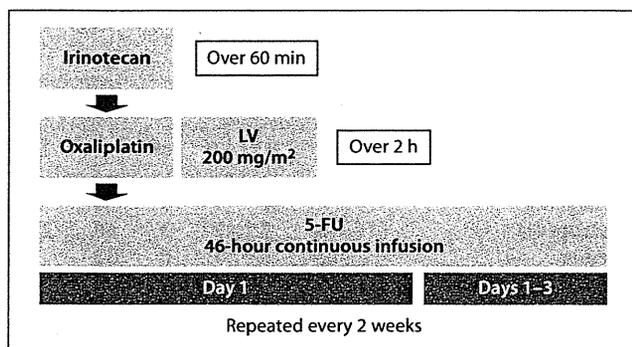
Fig. 1. Treatment schedule for FOLFOXIRI.

fatigue and constipation, any grade 4 neutropenia lasting more than 4 days or associated with fever ($\geq 37.5°C$) and any grade 4 thrombocytopenia or grade 3 bleeding tendency in the first cycle of treatment, as defined by the National Cancer Institute Common Toxicity Criteria, version 3.0. DLT was also defined as toxicity precluding administration of the fourth cycle of treatment within 9 weeks from day 1 of cycle 1, or toxicity precluding administration of the next cycle of treatment within 4 weeks from the completion of the previous cycle. If DLT occurred in 1 of the first 3 patients assigned to a given dose level, 3 additional patients were assigned to the same dose level. The MTD was defined as the dose level associated with DLT in at least 2 of 3 or 2 of 6 patients. The RD was defined as the dose level one rank below the MTD.

Treatment Plan

Irinotecan in 5% dextrose (250 ml) was administered over the course of 60 min and was followed immediately by a concomitant infusion of oxaliplatin in 5% dextrose (250 ml) and 200 mg/m² LV in 5% dextrose (250 ml), given over the course of 2 h through a Y connector, which was then followed immediately by a continuous infusion of 5-FU over the course of 46 h (fig. 1). Treatment was repeated every 2 weeks until disease progression. The maximum number of cycles was not specified.

Treatment was delayed if patients had any of the following findings on the planned day of treatment: neutrophil count <1,500/mm³, hemoglobin <8.0 g/dl, platelets <100,000/mm³, peripheral neuropathy >grade 2 or nonhematologic toxicity >grade 1, except for alopecia, nausea, vomiting, anorexia, fatigue and constipation. To prevent nausea and vomiting, a 5-hydroxytryptamine-3 antagonist plus dexamethasone was administered intravenously before chemotherapy. Prophylactic use of granulocyte colony-stimulating factor was prohibited.

Toxicity and Efficacy Assessments

Pretreatment evaluations included disease history, ECOG PS, white blood cell counts with differential and platelet counts, complete blood profile, carcinoembryonic antigen, CA19-9, urinalysis, electrocardiogram, chest radiograph, computed tomographic scan and any other appropriate diagnostic procedures to evaluate metastatic sites. During treatment, the following examinations were performed every week until the completion of cycle 4: physical examination, complete blood cell count, blood profile and urinalysis.

Toxicities were monitored weekly until cycle 4 and were scored according to the National Cancer Institute Common Toxicity Criteria, version 3.0.

Responses were evaluated every 2 cycles according to the Response Evaluation Criteria in Solid Tumors, version 1.0 [20], as complete response (CR), partial response (PR), stable disease or progressive disease (PD). The duration of response was calculated from the date of starting treatment to the date of the first confirmation of PD or the last examination. Progression-fee survival (PFS) was calculated from the date of starting therapy to the date of its discontinuation because of PD or death.

UGT1A1 Genotyping

Genomic DNA was extracted from 200 µl of peripheral blood, which had been stored at $-80°C$ until analysis, with the use of a QIAamp Blood Kit (Qiagen GmbH, Hilden, Germany). The UGT1A1*6 polymorphism was analyzed by the polymerase chain reaction-restriction fragment length polymorphism method as described elsewhere [21]. The UGT1A1*28 polymorphism was determined by direct sequencing as described by Fujita et al. [21].

Results

Patients

A total of 10 patients with advanced colorectal cancer were enrolled in this study from October 2007 through April 2009. Median age was 54 years (range 33–69), and ECOG PS was 0 in all patients. Six patients (60%) had liver metastases, 6 (60%) had multiple metastatic sites and 3 (30%) had received previous adjuvant chemotherapy with 5-FU-LV (table 2). In this study, a total of 111 cycles of chemotherapy were administered, with a median of 8.5 cycles per patient (range 1–36).

Dose Adaptation Results

The first patient was assigned to dose level 0 and had DLT (grade 3 infection with normal neutrophils). On day 8 after the first cycle of chemotherapy, the patient had severe diarrhea with a body temperature of 40°C and a C-reactive protein level of 27 mg/dl and therefore received antibiotics by intravenous infusion. Therefore, 3 additional patients were assigned to receive dose level 0. The first additional patient had DLT (a treatment delay of 14 days due to neutropenia). Because 2 of 4 patients had DLT at dose level 0, the next 3 patients were given dose level -1. One of these patients had DLT (a treatment delay of 6 days due to neutropenia). Therefore, 3 additional patients received dose level -1. Only 1 of the 6 patients who received dose level -1 had DLT. On the basis of these results, dose level 0 and dose level -1 were determined to be the MTD and RD, respectively.

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Table 4. Objective responses

Response	Level 0 (n = 4)	Level –1 (n = 6)	Total
Complete response, n	0	1	1
Partial response, n	3	4	7
Stable disease, n	0	1	1
Progressive disease, n	0	0	0
Not evaluable, n	1	0	1
RR, %			89

Responses were assessed by computed tomography or magnetic resonance imaging according to the Response Evaluation Criteria in Solid Tumors.

Discussion

This is the first study to demonstrate the feasibility and activity of FOLFOXIRI in Japanese patients with advanced colorectal cancer who harbor UGT1A1*1/*1, *1/*6 or *1/*28. The RD of FOLFOXIRI in these Japanese patients was determined to be 150 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin and 2,400 mg/m^2 5-FU. The FOLFOXIRI regimen showed a manageable safety profile.

In the present study, patients with UGT1A1*1/*1 and UGT1A1*1/*28 or *1/*6 were enrolled. We reported previously that the efficacy and toxicity of irinotecan did not differ significantly between patients with UGT1A1*1/*1 and *1/*28 or *1/*6 [22]. Exclusion of patients with UGT1A1*28/*28, *6/*6 or *28/*6 might be one of the reasons for the manageable toxicities in our study, since these UGT1A1 genotypes have been linked to severe irinotecan-induced toxicity [18, 19].

In this study, we used the treatment schedule described by the Gruppo Oncologico Nord Ovest (GONO). Two randomized phase III trials of FOLFOXIRI have been performed in Western countries. In the GONO study, median PFS and OS obtained with FOLFOXIRI as first-line treatment in patients with metastatic colorectal cancer were significantly longer than those obtained with FOLFIRI [3]. Furthermore, an updated analysis of the GONO study showed that FOLFOXIRI was associated with a clinically significant improvement in long-term outcomes as compared with FOLFIRI [23]. In contrast, the Hellenic Oncology Research Group (HORG) failed to demonstrate statistically significant benefits of FOLFOXIRI as compared with FOLFIRI, although some improvements in RR, time to disease progression and OS were obtained with FOLFOXIRI [4]. There was a major

difference between the GONO and HORG studies with regard to the regimen of FOLFOXIRI used. Intravenous bolus 5-FU was included in the treatment regimen used by HORG but not that used by GONO. Consequently, the HORG study used lower doses of oxaliplatin and irinotecan than those used in the GONO study. We therefore decided to use the treatment schedule adopted by GONO. In our study, the doses of irinotecan and oxaliplatin at the initial dose level were the same as those used in the GONO study, and that of 5-FU was the approved dose (2,400 mg/m^2) in Japan. This may have contributed to the high RR and long PFS obtained in our study.

In this dose-finding study, 2 criteria for severe DLT were met during the search for the optimal RD of FOLFOXIRI: (1) the fourth cycle of treatment was not administered within 9 weeks from day 1 of cycle 1 and (2) the next cycle was not started within 4 weeks after completion of the prior cycle. When FOLFOXIRI was administered at the RD determined in the initial phase I–II study performed by GONO, 35% of cycles required dose reductions of at least one drug and 16% of cycles were delayed by at least 1 week because of toxicities [24]. GONO therefore performed another phase II study using slightly lower doses of irinotecan, oxaliplatin and 5-FU to improve the dose intensity. Use of the modified RD resulted in lower incidences of both hematologic and nonhematologic toxic effects, and the median dose intensity increased from 78 to 88% [25]. We considered the initial RD found in the phase I–II study by GONO to be too high to maintain adequate dose intensity. We therefore used the severe DLT criteria described above to determine the optimal RD that allowed a high dose intensity to be delivered safely, potentially enhancing treatment effectiveness.

First-line FOLFOXIRI might reduce the efficacy of second-line treatments, since all three key cytotoxic drugs used for the management of colorectal cancer (irinotecan, oxaliplatin and 5-FU) are used simultaneously. However, the GONO study showed that the FOLFOXIRI regimen did not negatively affect the outcomes of patients who received second-line chemotherapy [26]. Among patients given second-line treatment, median PFS and OS have been shown to be better in those who initially receive FOLFOXIRI than in those who initially receive FOLFIRI [23]. In our study, all of the 9 patients in whom response was assessable (excluding the 1 patient with a CR, who received FOLFOXIRI for the duration of treatment) received second-line treatments (5-FU-LV and oxaliplatin plus bevacizumab in 2, FOLFIRI plus bevacizumab in 2, infusional 5-FU plus bevacizumab in 2,

FOLFOXIRI in 1, 5-FU-LV and oxaliplatin in 1 and FOLFIRI in 1). We obtained a RR of 44% and median PFS of 9.1 months, similar to the results of the GONO study. We did not observe any toxicities which were life-threatening or grade 3 or worse neurotoxicity with second-line treatment. Our study also showed no evidence suggesting a negative impact of first-line FOLFOXIRI on treatment benefits in patients who subsequently received second-line chemotherapy.

Although our results suggested that FOLFOXIRI is a promising regimen for Japanese patients with advanced colorectal cancer, it remains unclear whether first-line treatment with three cytotoxic agents is better or worse than that with two cytotoxic agents plus one monoclonal antibody. Because the FOLFOXIRI regimen has a high RR, it might facilitate the radical surgical resection of metastases in patients with initially unresectable metastatic colorectal cancer [11, 12]. Although excessive chemotherapy can cause liver injury and further increase perioperative morbidity and mortality, one study assessing hepatic toxicity reported no treatment-related liver damage in patients who underwent surgery for metastases after they received FOLFOXIRI [12]. These results have suggested that FOLFOXIRI is a 'conversion therapy' for patients in good general condition who have potentially resectable disease [23]. The addition of cetuximab to FOLFOXIRI [27] may be a promising treatment strategy to increase the RR in patients without *Kras* mutations. Phase II studies of FOLFOXIRI combined with cetuximab should be performed to improve the surgical resection rate of metastases in Japan.

In conclusion, we determined the RD of FOLFOXIRI in Japanese patients with advanced colorectal cancer who do not have *UGT1A1*28/*28, *6/*6* or *6/*28* genotypes. Our results showed that FOLFOXIRI is a well-tolerated regimen even in Japanese. Exclusion of patients with *UGT1A1*28/*28, *6/*6* or *6/*28* might have contributed to the good tolerance in our study. FOLFOXIRI was also shown to be a very active regimen for the initial treatment of colorectal cancer.

Acknowledgements

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CLINICAL ARTICLE

Clinical features of patients bearing central nervous system hemangioblastoma in von Hippel-Lindau disease

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Abstract

Background Central nervous system (CNS) hemangioblastoma (HB) is one of the most common manifestations in von Hippel-Lindau disease (VHL), but large-scale studies on clinical features of CNS HB in VHL are scarce.
Methods On the basis of the results of a questionnaire, we collected data of VHL patients with CNS HB.
Results The total number of CNS HBs in 111 VHL patients (male 59, female 52) was 264 with the following distributions: cerebellar, 65.4 %; brainstem, 9.9 %; spinal cord, 23.9 %; and pituitary, 1. 1 %. The follow-up period was 0.6 to 39.2 years, with the mean 12.5 years. Patients bearing brainstem or spinal cord HB also had another HB significantly more frequently than those bearing cerebellar HBs ($P<0.05$). The mean onset age of CNS HB was 29.1 years, and that of patients bearing a single HB (mean 34.4 years) was significantly greater than that of multiple HBs (mean 25.7 years). Patients with multiple HBs under 40 years are more dominant than those with a single HB. The distribution rate of brainstem HB is significantly smaller in patients below 30 years than patients above 29 years. Although ECOG PS score increased along with number of operations, the onset age decreased with increasing number of operations. The mean ECOG PS score of patients below 20 years is significantly smaller than patients above 19 years.
Conclusions When the onset age of CNS HB is under 40 years, and CNS HB is located at the brainstem or spinal cord HB, the probability of multiple occurrence can be predicted. Since patients with an onset age under 20 years old preserve a high performance status, early detection of CNS HB would be important. In addition, since a multiple operations aggravate performance status, number of operations should be reduced.

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Introduction

Von Hippel-Lindau disease(VHL) is a familial neoplasia disorder with an autosomal dominant pattern of inheritance that results from a germline mutation in the *VHL* gene, and which is characterized by a predisposition to develop

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multiple neoplastic lesions, including central nervous system (CNS) hemangioblastomas (HBs), retinal HBs, renal cell carcinomas (RCCs), pheochromocytomas (PhCs), pancreatic tumors (PTs), endolymphatic sac tumors (ELSTs), and epididymal cystadenomas (ECs) [9, 11–13]. VHL gene, located at chromosome 3p25 region, was isolated by a positional cloning approach [7] and shown to be mutated in the germline of VHL patients, as well as in sporadic tumors including CNS HBs [5] and RCCs [16]. Common CNS lesions in affected individuals include cerebellar, spinal cord, brainstem HBs, as well as retinal HBs, pituitary stalk HBs, and ELSTs [3, 4, 8–10, 13]. The annual incidence of VHL is approximately one in 36,000 live births and has over 90 % penetrance by 65 years of age [9, 11–13]. The earliest feature among manifested lesions in VHL patients is usually a retinal or cerebellar HB. At present, metastases from RCC and neurological complications from CNS HBs are the most common causes of death. Although the median survival of VHL patients previously did not reach 50 years, the prognosis of VHL patients has been improved and the complications related to VHL-associated lesions were reduced by comprehensive serial screening and routine follow-up, earlier diagnosis of lesions in VHL patients by computed imaging and laboratory studies including DNA tests, progress in treatment such as micorosurgery or radiotherapy for CNS HB and tumor enucleation for RCC, and increased knowledge of VHL [9]. At present, even small asymptomatic HBs in VHL patients are detected on an MRI without difficulty [11]. Since most VHL patients with a CNS HB also have other CNS HBs and/or visceral tumors, these patients might have opportunities to undergo treatment for other CNS HBs or visceral tumors in the future. Large-scale epidemiological studies involving over 100 patients have rarely been done, and the clinical features of CNS HBs in VHL disease are not yet fully defined. In addition, the previous studies mostly have treated a smaller number of only cerebellar or spinal cases; and therefore the clinical aspects and the quality of life of CNS HBs in VHL patients have not yet been fully elucidated [1, 17]. Here, focusing particularly on onset age of CNS HB, we describe the clinical features and quality of life of HBs in the CNS of VHL patients.

Materials and methods

VHL patients were gathered by the Japanese VHL Study Group in the Japanese Health & Labor Ministry during 2009–2010 via the results of a questionnaire (Table 1) answered by Japanese neurosurgeons at 1020 hospitals approved as training facilities for neurological surgery in Japan. The clinical diagnosis for VHL was made on the basis of the following criteria [9]. In the

presence of a positive family history, VHL can be diagnosed clinically in a patient with at least one typical VHL tumor, retinal or CNS HB; RCC; PhC; and PT. ELSTs and multiple pancreatic cysts suggest a positive carrier. In contrast, in patients with a negative family history of VHL-associated tumors, diagnosis of VHL can be made when they exhibit two or more CNS HB or a single HB in association with a visceral tumor such as RCC, PhC, and PT. All CNS HBs or visceral tumors were diagnosed on the basis of clinical histories, symptoms, histories, laboratory studies, and radiological findings made by CT or MR. Among the collected VHL patients, those bearing CNS HBs were investigated with respect to the following: patients' characteristics, location of CNS HB, onset age of CNS HB, follow-up period, number of operations for CNS HB, and radiation therapy. In addition, performance status (PS) at the point based on the results of the questionnaire was assessed according to the following Eastern Cooperative Oncology Group (ECOG) PS [14]. The relationship between the number of operations and the PS or onset age of CNS HB was also examined. Exclusion criteria were as follow: incomplete description about location of tumor, onset age or performance status. For statistical analysis, we applied the non-parametric Mann-Whitney's U test or Spearman's correlation coefficient rank test. Statistical significance was set at $P<0.05$. This study was conducted with the approval of the ethics committee of Kochi University School of Medici,ne which is the center of this study.

Results

According to the criteria described in the Methods section, 294 patients were defined as VHL according to results of the questionnaire. Among them, 200 (68.1 %) bore CNS HBs. Among these 200 patients, their tumor locations, onset age, ECOG PS,and follow-up period were clarified in 111 patients. Summary of data on CNS HBs in VHL patients is shown in Table 2. Among 111 patient, 2 died due to CNS HB at 44 and 47 years, and remaining 109 patients were living.

Among the 111 patients bearing CNS HBs, those bearing cerebellar ones were 92 (82.9 %); brainstem, 22 (20.7 %); spinal cord, 43(38.7 %); pituitary, 3 (2.7 %). Fifty-three patients had only cerebellar HBs (30, single tumor; 23, multiple ones). Ten patients had only spinal cord HB (seven, single tumor; three, multiple ones); and three patients had only brainstem HBs (two, single tumor; one, multiple ones). In addition, the result of the present study revealed that 83.7 % of the patients with spinal cord HB had another CNS HB at