Table 4 Logistic regression models for predicting transfusion of red blood cell and platelet

Factor	RBC			PLT		
	Regression coefficient	Odds ratio	95% CI	Regression coefficient	Odds ratio	95% CI
Age*						
65–74	0·40	1·5	1·4–1·5	0·11	1·1	1·0–1·2
80+	0·75	2·1	2·1–2·2	−0·06	0·9	0·9–1·0
Female	−0·12	0·89	0·86–0·91	0·06	1·1	1·0–1·1
Haematopoietic disorders						
Acute leukaemia	3·85	46·9	42·4–51·8	5·74	310	278–346
Chronic leukaemia	2·33	10·3	8·1–12·9	3·26	26·2	19·2–35·6
Aplastic anaemia	3·12	22·7	19·4–26·6	3·52	33·6	27·3–41·4
Multiple myeloma	2·84	17·2	15·4–19·2	3·68	39·5	33·8–46·1
Malignant lymphoma	1·51	4·5	4·1–4·9	3·10	22·2	20·0–24·7
MDS	3·67	39·2	34·9–44·1	4·37	78·9	68·6–90·7
DIC	2·44	11·4	10·6–12·3	3·77	43·2	39·3–47·5
Obstetric bleeding	1·82	6·2	4·9–7·8	0·99	2·7	1·4–5·0
Severe trauma	2·65	14·1	12–16·6	2·08	8·0	5·9–11
Cardiovascular surgery						
Without CPB	3·56	35·0	32·0–38·3	3·84	46·5	40·8–52·9
With CPB	4·67	106·9	97·7–117	5·22	185	169–203
Malignant tumour						
Without chemotherapy	0·93	2·5	2·5–2·6	0·32	1·4	1·3–1·5
With chemotherapy	0·66	1·9	1·8–2	0·81	2·2	2·0–2·5
GI bleeding	2·58	13·2	12·8–13·7			
Hip fracture surgery	2·40	11·0	10·4–11·6			
Chronic renal failure	1·59	4·90	4·7–5·1			
Liver cirrhosis				1·25	3·5	3·0–4·0
Constant	−4·10			−5·78		

GI, gastrointestinal; DIC, disseminated intravascular coagulation; CPB, cardiopulmonary bypass; MDS. Myelodysplastic syndrome; RBC, red blood cell 95% CI, 95% confidence intervals for odds ratios.
*Patients aged below 65 years were used as reference.

system such as DRG can provide a means for inter-institutional benchmarking and cost comparison [14–16,37]. DRG is widely used for healthcare payments and analyses of hospital activities. The basic concept underlying the employment of DRG for reimbursement is that 'treatments for similar patients consume a similar degree of medical resources'. Therefore, patients within the same DRG are considered to have similar resource (e.g., blood product) utilization.

By the use of a patient classification system and identification of risk factors of transfusion, we can properly compare blood product use between different healthcare providers. Furthermore, such a method should be useful for identifying providers with extremely high levels of blood product use. If a simple risk adjustment model could be developed by analyzing large healthcare databases representing information obtainable with little labour and at low

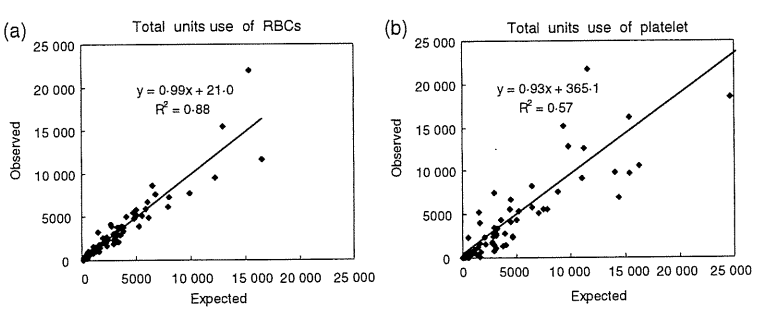

Fig. 1 The multiple linear regression model to predict total unit use of RBCs and platelets transfused at each hospital

Table 5 Results of the medical chart review (percentage of appropriateness) and O/E ratios calculated using the two prediction models

		Red blood cell			Platelet		
Patient	Hospital	% of appropriateness	O/E (Model 1)	O/E (Model 2)	% of appropriateness	O/E (Model 1)	O/E (Model 2)
Overall	A	72	1·09 (1·02–1·18)	1·11 (1·05–1·16)	35	1·54 (1·34–1·81)	1·60 (1·41–1·83)
	B	81	0·93 (0·89–0·93)	0·78 (0·75–0·81)	64	1·13 (1·03–1·25)	0·78 (0·75–0·89)
Haematopoietic malignancy	A	71	1·35 (1·11–1·72)	1·61 (1·40–1·90)	25	1·50 (1·19–2·00)	2·30 (1·91–2·89)
	B	91	1·09 (0·97–1·25)	0·90 (0·82–0·99)	78	1·17 (1·02–1·36)	0·78 (0·75–0·89)
Cardiovascular surgery	A	62	1·25 (1·08–1·48)	1·73 (1·52–2·01)	17	1·76 (1·35–2·53)	1·23 (1·04–1·52)
	B	71	0·96 (0·88–1·05)	0·86 (0·79–0·93)	83	0·99 (0·85–1·18)	0·95 (0·86–1·06)

cost, it would be possible to evaluate the frequency of blood product use by various healthcare providers. It should be noted, however, that such a method is not designed to replace the detailed analyses conducted by auditing committees. Instead of pinpoint evaluations on blood product utilization at the patient level, the purpose of our method is to provide continuous surveillance and routine general evaluations on a larger scale that allows for multi-institutional comparisons with a satisfactory level of accuracy.

We selected two indicators to evaluate hospital-wide use of blood products in Japan: (i) proportion of patients that received a blood transfusion and (ii) total amount (number of units) of blood products used. These are typical indicators used for assessment of hospital-level blood usage [9,16,25,26]. We developed two regression models to calculate risk-adjusted blood use. A logistic regression model was used to predict the percentage of transfused patients in each hospital. A multiple linear regression model was used to predict hospital-wide total units of RBCs or platelets transfused.

With a limited number of variables, the logistic regression model could effectively predict proportions of patients that received a blood transfusion. Our predictive model consisted of 19 variables that were easily collected from healthcare data in Japan. In the database of approximately 587 000 cases used for the study, the amount of blood products used for patients with any of these risk factors represented 80–90% of the total amount of blood products used. The multiple linear regression model used distribution of diagnostic groups to predict total unit use of RBCs and platelets transfused at each hospital. Although both models showed good prediction abilities, the logistic regression model better predicted RBC use than platelet use. Also, the multiple linear regression model better predicted RBC use than platelet use (Fig. 1).

The following issues should be noted when evaluating blood product use with O/E ratios. First, the mean value of the group is used as the reference value (expected value) when performing evaluations with O/E ratios, thus the evaluation is relative. For instance, if blood product use in the entire group is excessive, there is a possibility that the O/E ratio will be <1 in hospitals with high blood product use. Second, when O/E ratios are low, it is difficult to distinguish whether this is a result of appropriate blood product use, or under use. However, with blood transfusions, over use and misuse of blood product have been more of a problem than under use. Thus, when use is higher than average, there is a high likelihood that there has been inappropriate blood product use. On the contrary, when use is lower than average, there is a possibility that the healthcare provider is transfusing the bare minimum required.

Actually, O/E ratios calculated by use of the two models were very relevant to proportions of appropriate blood use (Table 5). Larger O/E ratios were associated with a smaller proportion of appropriate transfusions as judged by medical chart reviews. No particular difference was seen between the O/E ratio for proportion of patients receiving transfusions and that for the total amount (number of units) of RBC use. Both methods were considered to have successfully evaluated appropriate blood use.

Based on these findings, we conclude that the assessment of blood product use employing O/E ratios can be used, not only as an index for valid and appropriate transfusions but also as an index for blood product use that takes patient risk into consideration. Additionally, our research strongly indicates that valid comparisons may be made across hospitals in Japan.

Evaluation of blood product use at the hospital level is important in several ways. Wide variation in blood product use exists among hospitals. By comparing blood product use in different hospitals, risk-adjusted assessment of blood product use has the potential to contribute towards appropriate use of blood products [7,10,25]. Because of the labour and cost involved in gathering data on blood product use, however, almost no previous attempts had been made to collect and analyse data from many hospitals in Japan. DPC data are advantageous, as it gathers into a unified format the clinical information and data on

treatment procedures for all hospitalized patients. Therefore, it is possible to use a shared assessment standard to compare conditions for blood product use between hospitals, and to engage in discussion about clinical standards.

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Absence of Oncogenic Mutations of *RAS* Family Genes in Soft Tissue Sarcomas of 100 Japanese Patients

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Abstract. *Background: Activating point mutations of genes of the RAS family (KRAS, HRAS and NRAS genes) are frequently found in carcinomas, but their prevalence in sarcomas varies considerably among ethnic groups. No extensive studies in Japanese patients have been performed. Materials and Methods: Mutation analyses of three RAS genes (KRAS, HRAS and NRAS) were performed using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analyses and PCR direct sequencing in one hundred cases of soft tissue sarcoma (STS) as well as six STS cell lines from Japanese patients. Results: No mutations were found in two hot spot regions (codon 12-13 and 61) of the three RAS genes. Conclusion: Activating mutations of the RAS gene family are uncommon events in soft tissue sarcomas in Japanese patients.*

The detection of oncogene mutations in tumors has not only led to a better understanding of tumorigenesis, but also aided in diagnosis and treatment. *RAS* genes are the most frequently found activating oncogenes in human carcinomas. They gain their oncogenic activity *via* a single amino acid substitution in codon 12, 13 or 61 of the *KRAS*-, *HRAS*- or *NRAS*-encoded p21 protein (1). Point mutations occurring in *RAS* genes give rise to proteins with reduced intrinsic GTPase activity associated with oncogenesis (2). Some reports have indicated that *RAS* oncogenes have no ability to transform primary cells and mutant Ras proteins can only transform cells that have undergone predisposing changes such as immortalization (3, 4). We have previously reported that an activated *HRAS* gene transformed mesenchymal stem cells only when they were immortalized beforehand by the introduction of a human telomerase catalytic subunit (*hTERT*) and *BMI1* gene (5).

Activated *RAS* genes have frequently been found in adenocarcinomas of the pancreas (90%), colon (50%), thyroid (50%) and lung (30%) (6). Soft tissue sarcomas (STSs) account for fewer than 1% of human malignancies and fewer than 2% of cancer deaths (7, 8). They present a heterogeneous group of tumors with respect to origin and morphological features. Previous studies have provided inconsistent results, with the frequency of *RAS* mutations in STS ranging from 0% to 44% (9-17). The inconsistency may be due to small sample numbers, incomplete sensitivity of the detection methods or the patients' ethnic origin. Moreover, no reports have investigated all codons 12, 13 and 61 of *KRAS, HRAS* and *NRAS* genes in a large number of samples.

In the present study, extensive analyses in codons 12, 13 and 61 of the *KRAS, HRAS* and *NRAS* genes were performed in one hundred STS samples and six STS cell lines from Japanese patients. To increase sensitivity, two methods were used to detect mutations: polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and PCR direct sequencing.

Materials and Methods

Tissue specimens and cell lines. Tumor tissues were obtained from 100 patients with STS tumors, which were obtained at either biopsy or resection in Kyoto University Hospital. The Ethics Committee of the Faculty of Medicine, Kyoto University, approved the procedure and informed consent was obtained from each donor. There were 60 female and 40 male patients and their median age was 52.5 years (10 to 90). The pathological diagnoses are described in Table I. Histological analyses were performed in all the samples, which showed that more than 90% of each tissue was composed of tumor cells.

Six STS cell lines derived from Japanese patients were also analyzed in this study (Table I). YaFuSS (synovial sarcoma) was established in our laboratory (18). HS-SY-II (synovial sarcoma) was a gift from H. Sonobe (Kochi Medical School, Japan) (19), SYO-1

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Key Words: RAS, mutation, soft tissue sarcoma, Japan.

Table I. *Pathological diagnoses of STS patients and STS cell lines.*

Diagnoses	No. of tumor tissues	No. of cell lines
Liposarcoma	20	0
well-differentiated	2	
myxoid	12	
pleomorphic	4	
dedifferentiated	2	
MFH	14	0
Synovial sarcoma	10	4
Leiomyosarcoma	11	0
MPNST	9	1
ARMS	5	1
Solitary fibrous tumor	4	0
Epitheliod sarcoma	3	0
Fibrosarcoma	2	0
Myxofibrosarcoma	2	0
ASPS	2	0
Clear cell sarcoma	2	0
PNET	1	0
DFSP	1	0
Malignant hemangiopericytoma	1	0
UDS	13	0
	100	6

MFH, malignant fibrous histiocytoma; MPNST, malignant peripheral nerve sheath tumors; ARMS, alveolar rhabdomyosarcoma; ASPS, alveolar soft part sarcoma; PNET, primitive neuroectodermal tumor; DFSP, dermatofibrosarcoma protuberans; UDS, undifferentiated sarcoma.

(synovial sarcoma) was from A. Kawai (Okayama University, Japan) (20), Fuji (synovial sarcoma) was from S. Tanaka (Hokkaido University, Japan) (21), NMS-2 (malignant peripheral nerve sheath tumor) was from A. Ogose (Niigata University, Japan) (22), and KP-RMS-DM (alveolar rhabdomyosarcoma) was from H. Hosoi (Kyoto Prefectural University of Medicine, Japan) (23). For the positive controls in mutation analyses, cell lines known to have oncogenic *RAS* gene mutations were used: HT1080 (fibrosarcoma), SW480 (colon carcinoma), EJ (bladder carcinoma) and MOLT-4 (leukemia), all of which were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA).

DNA extraction. High molecular weight DNA was isolated from the tumor tissues and cell lines by standard phenol-chloroform extraction methods (24).

Positive and negative controls for analyses. For the positive control in the PCR-SSCP analyses, PCR fragments containing specific mutations were amplified using cell lines with each mutation (25) (Table II). Because no cell lines containing mutations in codon 61 of the *KRAS* and *HRAS* genes were available, mutant fragments were created according to the literature (25) for each mutation: Codon 61 of the *KRAS* gene in PR310 (lung carcinoma) and codon 61 of the *HRAS* in SK-2 (melanoma). Briefly, corresponding PCR fragments of the *KRAS* and *HRAS* genes were amplified from primary human skin fibroblasts, and cloned into pCR2.1 vector (Invitrogen, Carlsbad, CA, USA). Each mutation was introduced into the

Table II. *Point mutations in positive control cell lines.*

Gene	DNA sequences at target codons			Cell line with each mutation	
	12	13	61	Name	Tumor type
Wild-type					
KRAS	GGT	GGC	CAA		
HRAS	GGC	GGT	CAG		
NRAS	GGT	GGT	CAA		
Mutant					
KRAS	G**T**T	GGC	CAA	SW480	colon carcinoma
KRAS	GGT	GGC	CA**T**	PR310 *	lung carcinoma
HRAS	G**T**C	GGT	CAG	EJ	bladder carcinoma
HRAS	GGC	GGT	C**T**G	SK-2 *	melanoma
NRAS	G**A**T	GGT	CAA	MOLT-4	leukemia
NRAS	GGT	GGT	**A**AA	HT1080	fibrosarcoma

*Plasmids containing mutant fragments were used to replace cell lines.

fragment by site-directed mutagenesis using a PrimeSTAR Mutagenesis Basal Kit (TAKARA Bio Inc, Otsu, Japan). The plasmids containing each mutant fragment for *KRAS* and *HRAS* were designated Mut-PR310 and Mut-SK-2, respectively and used as template DNA for the PCR-SSCP analyses.

PCR-SSCP analysis. The prevalence of the *RAS* mutations in the 100 STS samples and cell lines was investigated by PCR-SSCP, as previously described (26, 27). A previous study had shown 97% of mutations to be detected in 100- to 300-base-long strands (28). Genomic DNA (200 ng) was amplified using 20 pmol of sense and antisense primers (Table III) in a 50 μl reaction mixture with a final $MgCl_2$ concentration of 1.5 or 2.0 mM using AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA). All the PCR programs included an initial denaturation for 5 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at the annealing temperature (Tm) for each PCR primer pair and 1 min at 72°C, with a final extension at 72°C for 7 min. The PCR products were electrophoresed through a 2.0% agarose gel with ethidium bromide to confirm the quality of amplification. Aliquots of the reaction mixtures denatured at 95°C for 5 min in a dilution buffer (0.1% SDS and 10 mM EDTA) were loaded onto a 15% polyacrylamide gel (acrylamide/bisacrylamide 19:1) with 10% glycerol and run for 3 to 4 h at 30 W and 10°C. The bands were visualized with a DNA Silver Staining Kit (Bio-Rad, Richmond, CA, USA).

Direct DNA sequencing. Six sets of PCR primers were designed to cover the entire coding region of the *RAS* gene and small introns (Table III). In each PCR, 200 ng of genomic DNA was used as a template and amplification was performed in a 50 μl reaction mixture with 20 pmol of each primer, 1 unit of AmpliTaq Gold DNA Polymerase and 1.5 mM $MgCl_2$, using GeneAmp PCR System 9700 (Applied Biosystems). The efficiency of the reaction was confirmed based on an analysis of the products by electrophoresis in a 1.5% agarose gel. The products were then purified using the QIAquik PCR purification kit (Qiagen, Tokyo, Japan). The purified PCR products were sequenced with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) on an automated DNA

Table III. *Primers used for the PCR-SSCP and direct sequencing.*

Gene	Target codon	Primers for PCR-SSCP			Primers for direct sequencing		
		Name	Sequence	Length of amplified fragment (bp)	Name	Sequence	Length of amplified fragment (bp)
KRAS	12-13	K1a	ACTGAATATAAACTTGTGGTAGTTGGAGCT	135	K1-SF	TGGAGGAGTTTGTAAATGAAG	533
		K1b	TAATATGCATATTAAAACAAGATTTACCTC		K1-SR	ACCCTGACATACTCCCAAG	
KRAS	61	K2a	TTCCTACAGGAAGCAAGTAG	128	K2-SF	CTATTGATGATGTTGAGCATC	514
		K2b	CACAAAGAAAGCCCTCCCCA		K2-SR	ATAAAACAGGGATATTACCTAC	
HRAS	12-13	H1a	AGGAGACCCTGTAGGAGGACC	126	H1-SF	GGCACGCTGCAGTCCTTG	587
		H1b	TGGTTCTGGATCAGCTGGATG		H1-SR	CCTGCTTCCGGTAGGAATC	
HRAS	61	H2a	TCCTGCAGGATTCCTACCGG	194	H2-SF	CGAATACGACCCCACTATAG	649
		H2b	GGTTCACCTGTACTGGTGGA		H2-SR	CGAGTCCTTCACCCGTTTG	
NRAS	12-13	N1a	GACTGAGTACAAACTGGTGG	118	N1-SF	GTGTGAGGCCGATATTAATC	446
		N1b	GGGCCTCACCTCTATGGTG		N1-SR	TGTTCTCTATAAACACGTTAAG	
NRAS	61	N2a	GGTGAAACCTGTTTGTTGGA	103	N2-SF	TTAGCAATTTGAGGGACAAAC	557
		N2b	ATACACAGAGGAAGCCTTCG		N2-SR	CAAGCTTCACTTATGTATTATC	

sequencer (3130/3130xl Genetic Analyzer, Applied Biosystems). A DNA sequence analysis was performed with 3130/3130xl Genetic Analyzer Data Collection Software v3.0 (Applied Biosystems).

Results

PCR-SSCP analyses. To confirm the quality of the PCR-SSCP analyses, fragments encompassing either codons 12-13 or codon 61 of each *KRAS, HRAS* and *NRAS* gene were amplified from the cell lines listed in Table II as well as the normal fibroblasts and electrophoresed on non-denaturing gel. The plasmids containing a mutant fragment (pMut-PR310 or pMut-SK-2) were also used as a DNA template for the positive control. Clear mobility shifted bands were detected in all cases (Figure 1A). Contamination from normal cells in tumor tissues may cause false negative results. To investigate the sensitivity of our PCR-SSCP method, DNA was extracted from mixtures of cells with each mutation and normal fibroblasts and analyzed. In the case of mutations in codons 12-13 of *HRAS*, clear mobility shifted-bands were detected if the fraction of tumor cells (EJ) made up more than 10% of all the cells (Figure 1B). Similar results were obtained in the other regions (data not shown), proving the quality and sensitivity of the PCR-SSCP method used in this study.

PCR-SSCP was then performed using DNA samples from the surgical specimens of STSs and the cell lines. The results of five samples in six regions are shown in Figure 2. The positive control samples showed clear mobility-shifted bands in each region, whereas no such shifted bands were detected in the STS samples. None of the 100 samples and six cell lines showed mobility shifted-bands in any of the six regions.

Direct sequencing. To confirm the results of PCR-SSCP analyses, each target region was analyzed by direct sequencing. Figure 3 shows the results for six regions in one STS sample. Positive control samples derived from either cell lines or plasmids showed the expected point mutations as previously described, whereas only the normal sequence was found in the sarcoma samples. No mutation was detected in these 6 regions of the 100 STS samples and six STS cell lines.

Discussion

STSs are defined as malignant tumors arising in mesenchymal tissues except bone and cartilage, and have a wide variety of types. There are several ways to classify STSs, for example, based on morphology (small round cell or spindle cell), grade of differentiation (well- or poorly-differentiated), or grade of malignant phenotype (high, intermediate, or low grade malignancy). In terms of molecular genetics, STSs can be divided into two categories; tumors with defined genetic alterations and tumors with various genetic alterations (9) The former group consists of tumors with oncogenic fusion genes created by reciprocal translocations such as the *SYT-SSX* gene in synovial sarcomas (30) and tumors with point mutations in specific genes such as the *KIT* mutation in gastrointestinal stromal tumors (31). In more frequently observed latter group, histological diagnoses varied and a considerable portion of tumors had no specific features and were designated undifferentiated sarcoma (UDSs). Mutations of tumor suppressor genes such as the *RB* and p53 genes were found frequently, but not always (32, 33). Among the 100 tumors analyzed in this study, 28 belonged to the former group and 72 to the latter group.

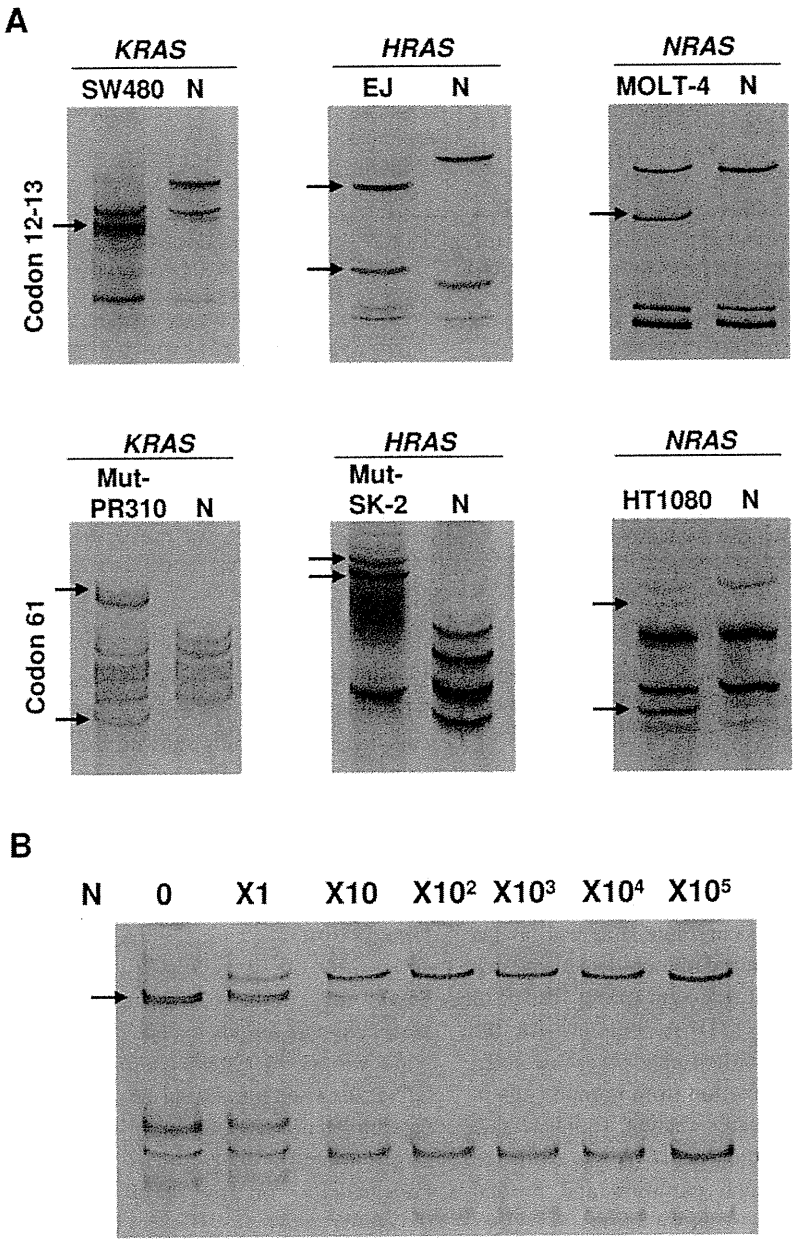

Figure 1. *Detection of mutant fragments by PCR-SSCP. A, Positive controls. PCR fragments encompassing codons 12-13 (upper panel) and codon 61 (lower panel) were amplified using DNA extracted from cell lines or plasmids containing each mutation. Arrows indicate mobility shifted bands. N, Normal skin fibroblast. B, Sensitivity of detection. Normal skin fibroblasts (N) were mixed with EJ cells at the indicated ratio, and DNA extracted from cell mixtures were analyzed by PCR-SSCP using primers for codons 12-13 of the HRAS gene. Arrow indicates mobility shifted bands.*

The detection sensitivity of the PCR-SSCP assay was estimated at 88 to 98% and that of PCR direct sequencing at 97% (34). Despite combining these two methods and using a large number of samples, no mutations were detected in any of three *RAS* genes in the STS samples. The frequencies of *RAS* mutations in STSs varied according to the investigators from 0% to 44% (Table IV). Bohle *et al.* (10) showed that 28% out of 32 malignant fibrous histiocytoma (MFH) samples from German patients contained a point mutation in codon 12 of *HRAS*. However, Rieske *et al.* (12) demonstrated that none out of 35 samples of MFH tissue from patients in Poland contained a point mutation or any other change within or around codons 12 and 13 of the *HRAS* gene. Rieske *et al.* suggested the differences in results could be connected with the ethnic and genetic heterogeneity of the study population. Yoo *et al.* evaluated 45 STS samples from Korean patients

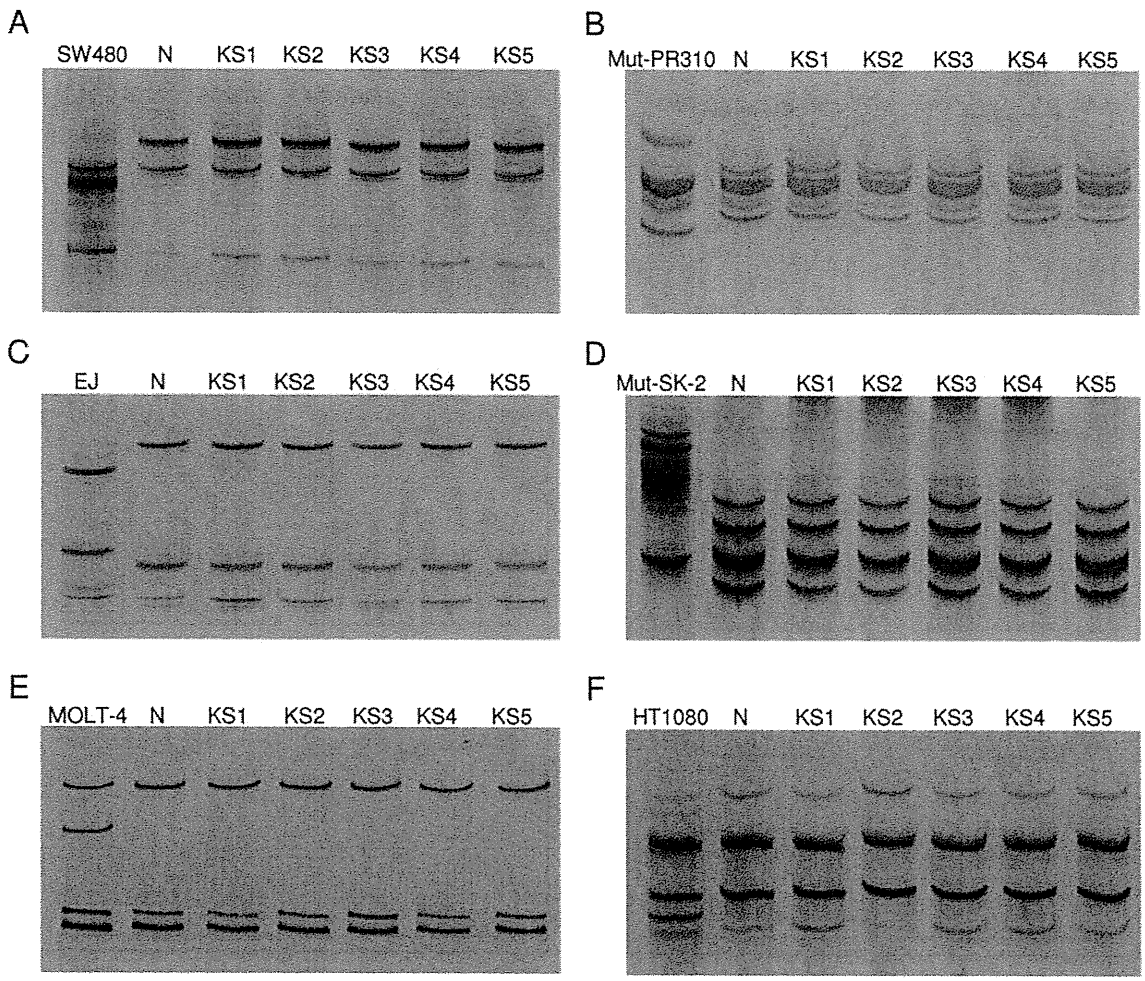

Figure 2. *PCR-SSCP analyses of STS samples. DNA extracted from five STS samples (KS1 to KS5) analyzed by PCR-SSCP along with positive and negative controls of each region are shown. A, codon 12-13 of KRAS; B, codon 61 of KRAS; C, codon 12-13 of HRAS; D, codon 61 of HRAS; E, codon 12-13 of NRAS; F, codon 61 of NRAS. N, normal skin fibroblasts.*

Table IV. *RAS mutations in soft tissue sarcomas.*

Samples			No. of samples with mutations (%)						Methods	Reference
Diagnoses	Country	No.	*KRAS*		*HRAS*		*NRAS*			
			No. of samples with mutations (%)							
STSs	USA	50	0	(0)	1	(2)	–	–	PCR Direct sequencing	14
Leiomyosarcoma	USA	51	7	(14)	–	–	–	–	DGGE	10
Embryonal RMS	UK	14	2	(14)	0	(0)	3	(21)	Probe hybridization	9
MFH	Germany	32	–	–	9	(28)	–	–	PCR Direct sequencing	10
MFH	Poland	35	–	–	0	(0)	–	–	PCR-RFLP & Direct sequencing	12
STSs	Sweden	3	–	–	0	(0)	–	–	Probe hybridization	16
STSs	Korea	45	20	(44)	7	(16)	–	–	Direct sequencing	13
Synovial sarcoma	Japan	49	–	–	3	(6)	–	–	PCR-RFLP	15
RMS	Japan	29	–	–	0	(0)	–	–	PCR-RFLP	17
STSs	Japan	100	0	(0)	0	(0)	0	(0)	PCR-SSCP & Direct sequencing	This study

STSs, Soft tissue sarcomas; RMS: rhabdomyosarcoma; MFH: malignant fibrous histiocytoma; DGGE: denaturing gradient gel electrophoresis; PCR-RFLP: PCR restriction fragment length polymorphism; –, not examined.

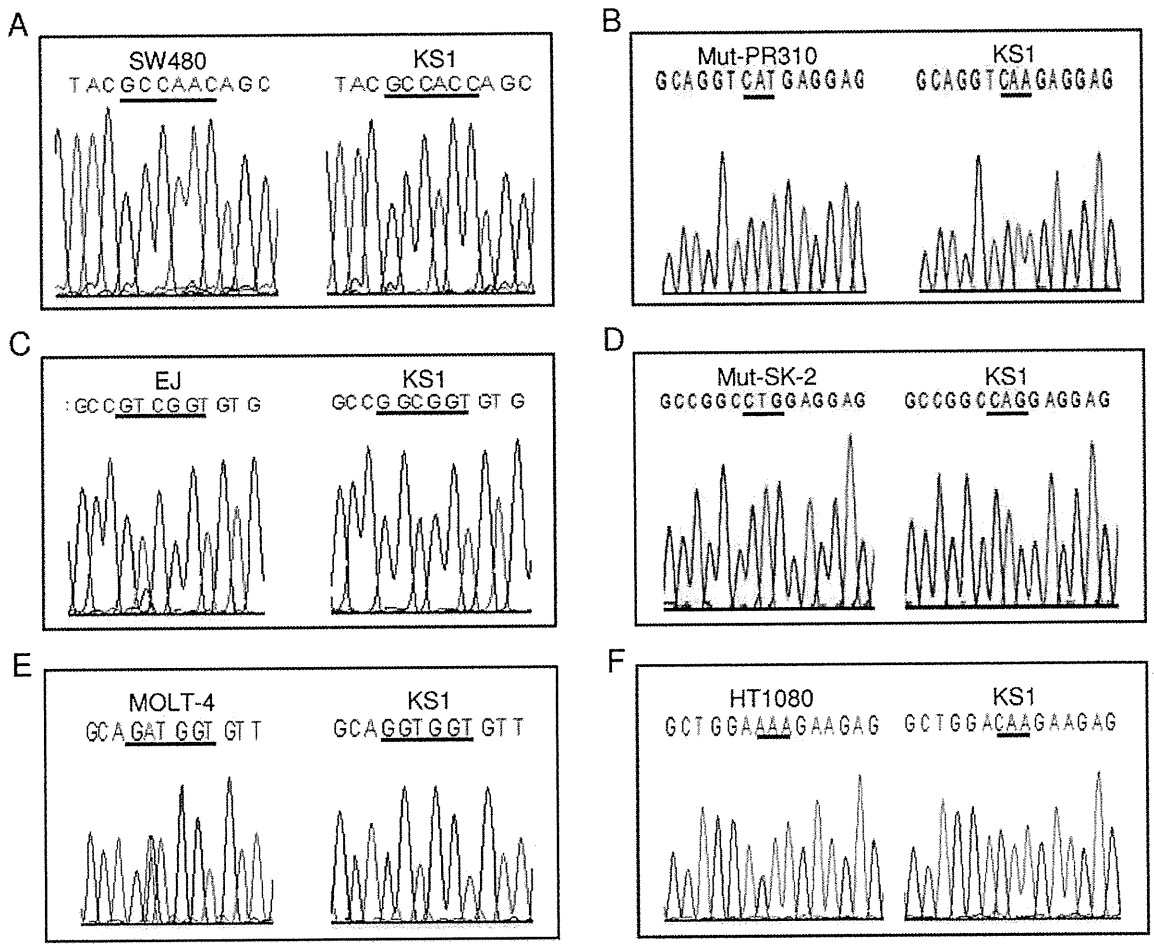

Figure 3. *Direct sequencing analyses of RAS genes. Sequencing data for the positive control and one STS sample (KS1) are shown. Sequences of target regions are underlined. A, codon 12-13 of KRAS; B, codon 61 of KRAS; C, codon 12-13 of HRAS; D, codon 61 of HRAS; E, codon 12-13 of NRAS; F, codon 61 of NRAS. All sequence data were obtained with forward primers except A, which was obtained with a reverse primer.*

and detected *KRAS* and *HRAS* mutations in 44% and 16%, respectively (13). Interestingly, they detected only 1 (2%) mutation at codon 12 of *HRAS* in 50 STS samples from American patients using the same analytical procedures (14), suggesting that genetic and/or environmental factors may affect the occurrence of *RAS* mutations. This might be one of the reasons for our failure to detect *RAS* mutations in the STS samples from Japanese patients. Evidence supporting the above assumption can be found in different types of carcinomas (35, 36). The low frequency of STSs with *RAS* gene mutations may raise concern about the use of HT1080 as a representative cell line of STS.

In conclusion, activated *RAS* mutations are uncommon in STSs samples from Japanese patients and considering the important oncogenic function of Ras-mediated signals, molecular mechanisms compensating for the role of mutated *RAS* may exist in STSs, which could be candidates for targeting of molecular therapy.

Conflict of Interest

None to be declared.

Acknowledgements

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Mesenchymal stem cells cultured under hypoxia escape from senescence *via* down-regulation of p16 and extracellular signal regulated kinase

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ABSTRACT

Hypoxia has been considered to affect the properties of tissue stem cells including mesenchymal stem cells (MSCs). Effects of long periods of exposure to hypoxia on human MSCs, however, have not been clearly demonstrated. MSCs cultured under normoxic conditions (20% pO_2) ceased to proliferate after 15–25 population doublings, while MSCs cultured under hypoxic conditions (1% pO_2) retained the ability to proliferate with an additional 8–20 population doublings. Most of the MSCs cultured under normoxic conditions were in a senescent state after 100 days, while few senescent cells were found in the hypoxic culture, which was associated with a down-regulation of *p16* gene expression. MSCs cultured for 100 days under hypoxic conditions were superior to those cultured under normoxic conditions in the ability to differentiate into the chondro- and adipogenic, but not osteogenic, lineage. Among the molecules related to mitogen-activated protein kinase (MAPK) signaling pathways, extracellular signal regulated kinase (ERK) was significantly down-regulated by hypoxia, which helped to inhibit the up-regulation of *p16* gene expression. Therefore, the hypoxic culture retained MSCs in an undifferentiated and senescence-free state through the down-regulation of p16 and ERK.

Introduction

Mesenchymal stem cells (MSCs) are tissue stem cells with multi-directional differentiation potential, though molecular and cellular definitions remain controversial. At present, MSCs are defined as mononuclear adherent cells capable of differentiating into the osteo-, chondro-, and adipogenic lineages [1]. Despite the equivocal definition, the clinical application of MSCs to tissue regeneration and engineering has already been launched [2,3]. Like other tissue stem cells, MSCs have limited growth potential and cease to proliferate due to cellular senescence [4]. Cellular senescence is induced by both intrinsic and extrinsic factors [5]. The shortening of telomeres is the most important intrinsic factor, and mitogenic stimuli and DNA damage are main extrinsic factors. Oxidative stress caused by reactive oxygen species (ROS) is one of the factors inducing DNA damage [6]. Although cells with the properties of MSCs have been isolated from adipose tissue [7], synovial tissue [8], and umbilical cord [9], bone marrow is the most frequent source of MSCs [10]. The oxygen concentration (pO_2) in bone marrow varies by the distance from the sinus ranging from 1% to 7% [11]. Therefore, although the precise location of MSCs in bone marrow is not known, the physiological oxygen concentration surrounding MSCs is much lower than the ambient oxygen concentration in cultures (20% pO_2). The high concentration of oxygen in standard culturing systems may produce excess oxidative stress, driving MSCs into a senescent state. Based on this concept, a number of studies have cultured MSCs under hypoxic conditions [12–17], and demonstrated that hypoxia is beneficial to the growth and also differentiation of MSCs, although the precise molecular mechanisms responsible for these phenotypic changes are not clear. The expression of the *p16* tumor suppressor gene is also important to cellular senescence [18,19]. We have shown that *p16* gene expression was gradually up-regulated during the life of MSCs *in vitro*, and tightly associated with the induction of cellular senescence [20]. Inhibition of *p16* gene expression by short interfering RNA for *p16* endowed senescent MSCs with the ability to re-proliferate and rescued them from senescence, indicating

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the important role of p16 in the growth of MSCs [20]. A number of factors are involved in the up-regulation of *p16* gene expression *in vitro* including ROS [21].

Here we cultured MSCs long term (more than 200 days) under normoxic (20% pO_2) or hypoxic (1% pO_2) conditions, and compared growth profiles and the potential to differentiate into three lineages. We found that hypoxia increased the life span of MSCs by down-regulating *p16* gene expression and endowed superior properties for differentiation into the chondro-, and adipogenic lineages, which were associated with the down-regulation of extracellular signal regulated kinase (ERK).

Materials and methods

Primary cultured cells

The isolation of bone marrow-derived MSCs from donors was performed as described previously [22]. The Ethics Committee of the Faculty of Medicine, Kyoto University, approved the procedure and informed consent was obtained from each donor. Mononuclear cells containing MSCs were suspended in α-minimal essential medium with GlutaMAX (Invitrogen Co., Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, South Logan, UT), 100 U/ml penicillin, and 100 mg/ml streptomycin, and separately cultured at a density of 2.5×10^5 cells/cm^2 under normoxic (20% pO_2 and 5% pCO_2) and hypoxic (1% pO_2 and 5% pCO_2) conditions at 37 °C. At 80% confluence, cells were collected, counted, and reseeded at a density of 3000 cells/cm^2. From this point, the number of population doublings (PD) was calculated based on the total cell number at each passage.

Senescence-associated-β-galactosidase assay

Cells were cultured on four-well chamber slides. The senescence-associated-β-galactosidase (SA-β-gal) assay was performed with a Senescence Detection Kit (BioVison, Mountain View, CA).

Induction of differentiation and quantitative evaluation

Differentiation was induced using standard methods [23], and evaluated quantitatively as follows.

Osteogenic differentiation. After 14 days, calcium deposits were visualized by alizarin red staining, and calcium content was quan-

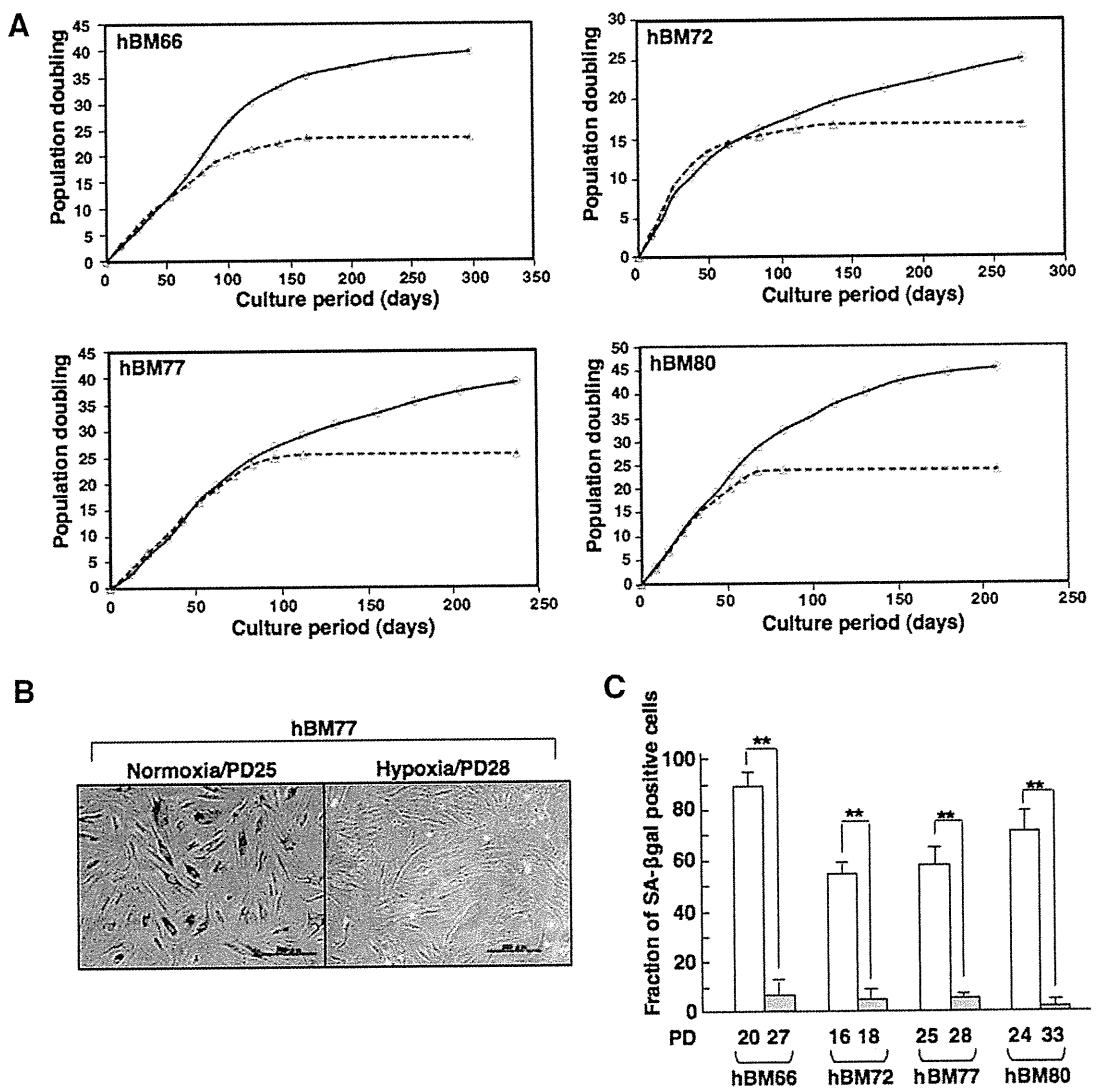

Fig. 1. Hypoxia extended the life span of MSCs *in vitro*. (A) Growth profiles of MSCs under normoxic (blue triangle) and hypoxic (red triangle) conditions. (B) Expression of SA-β-gal. hBM77 cells cultured for about 100 days under normoxic (PD25) or hypoxic (PD28) conditions were stained. (C) Quantitative analyses of SA-β-gal-positive cells among four hBM cell preparations at the indicated PD. White and gray box indicate normoxic and hypoxic conditions, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

tified based on the OCPC method with a Calcium C-Test Wako Kit (Wako Pure Chemical Industries, Osaka, Japan).

Adipogenic differentiation. After 21 days, lipid-vacuoles were detected using Oil-Red-O staining, and the amount of triglyceride (TG) was quantified with a serum triglyceride kit (Sigma–Aldrich, St. Louis, MO). Protein content was quantified using BCA protein assay reagent (Pierce Biotechnology, Rockford, IL).

Chondrogenic differentiation. After 14 days, cartilage matrix was evaluated by alcian blue staining of cryosections, and the glycosaminoglycan (GAG) content in pellets was quantified with BLYSCAN Dye and Dissociation reagents (BIOCOLOR, Belfast, UK). DNA content was quantified using a PicoGreen dsDNA Quantitation kit (Invitrogen, Carlsbad, CA).

Western blotting

Western blotting was performed as described [22]. The primary antibodies used were as follows: MAB1536 for HIF-1α, purchased from R&D Systems (Minneapolis, MN), and 551153 for p16, M12320 for ERK1, E23920 for phosphorylated ERK1/2, P19820 for p38, P39520 for phosphorylated p38, M54920 for pan-JNK, and S37220 for phosphorylated JNK, purchased from BD Biosciences Pharmingen (San Diego, CA). Blots were probed with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized using a chemiluminescence reagent ECL Plus Detection Kit (GE Healthcare UK Ltd., Little Chalfont, UK). The intensity of each band was calculated with Quantity One software, and evaluated as a ratio to that of control (β-actin).

Cell treatment

MAPK/ERK kinase (MEK) inhibitor treatment. Cells (2×10^5) were treated with U0126 (10 μM) (Promega, Madison, WI) for 96 h, and proteins were extracted every 24 h for Western blotting.

n-Propyl gallate (nPG) treatment. Cells (8×10^4) were treated with nPG (Sigma–Aldrich) at a concentration of 25–200 μM for 4 h, and proteins were extracted for Western blotting.

Statistical analyses

Statistical analyses were performed using Statcel software. Data were assessed using the Pearson product-moment correlation coefficient and Student's *t*-test.

Results

Hypoxia elongated growth of MSCs

MSCs were isolated from bone marrow of four donors, designated hBM66, hBM72, hBM77, and hBM80, respectively, and cultured under normoxic or hypoxic conditions as described in Materials and methods. In the case of hBM66, there was no significant difference between normoxic and hypoxic cultured-cells in terms of growth profile until PD15 (Fig. 1). After this stage, normoxic cultured-cells ceased to proliferate, whereas hypoxic cultured-cells kept on growing, and the number of PD at the last observation under normoxic and hypoxic conditions was 24 and 40, respectively. The theoretically accumulated cell number was 65,000-fold

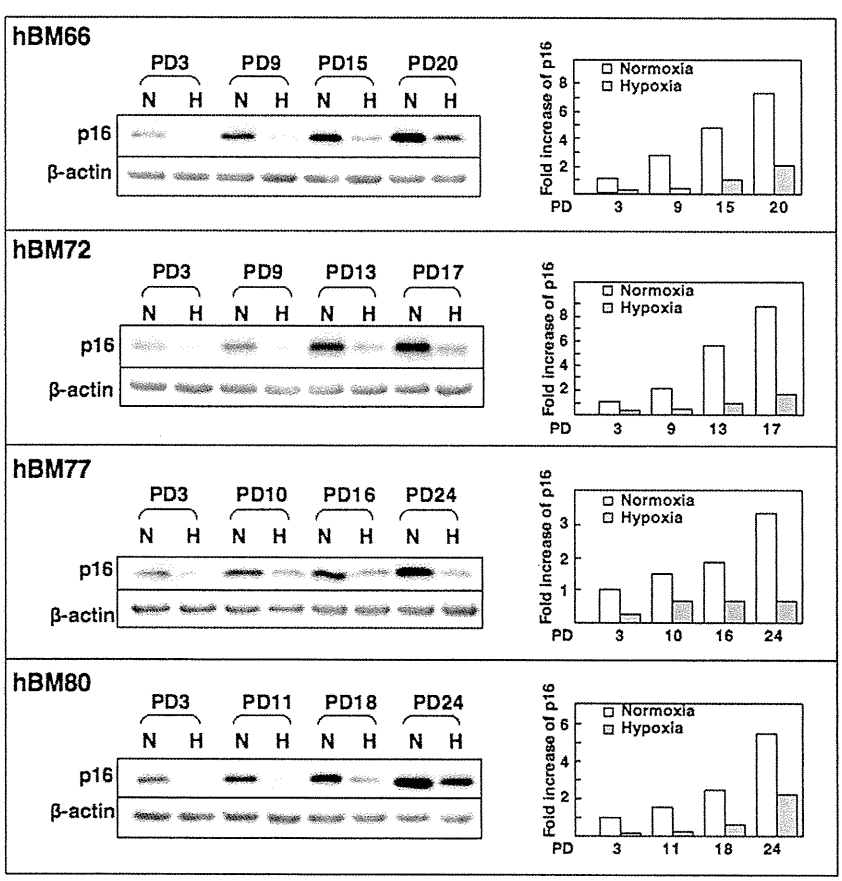

Fig. 2. Hypoxia down-regulated the expression of p16 in MSCs. Expression of p16 was evaluated by Western blotting using antibody for p16 protein. The relative expression level was determined using the value at PD3 of normoxic cultured-cells as a standard. White and gray box indicate normoxic and hypoxic conditions, respectively.

higher in the hypoxic culture. Similar results were obtained with the other three hBM cells (Fig. 1), and the final number of PD in normoxic and hypoxic cultured-cells was 17 and 25 in hBM72, 25 and 39 in hBM77, and 24 and 45 in hBM80.

Hypoxia rescued MSCs from cellular senescence

In the case of hBM77, most of the normoxic cultured-cells at PD25 were positive for SA-β-gal, whereas few were stained in the hypoxic culture at PD28 (Fig. 1B), and the difference was highly significant (Fig. 1C). Similar results were obtained with the other three hBM cells (Fig. 1C), indicating that the hypoxic culture protected MSCs from cellular senescence, which may be the cause of the increase in life span *in vitro*.

Hypoxia inhibited the up-regulation of p16 expression

We have demonstrated that the induction of cellular senescence in MSCs is tightly associated with the up-regulation of *p16* gene expression [20]. Consistent with previous findings, the level of p16 increased with the life span of normoxic cultured-hBM66, which was seven-times higher at PD20 than at PD3 (Fig. 2, upper panel). In contrast, hypoxic cultured-hBM66 retained a lower level of the p16 expression, which was only two-times higher at PD20 than at PD3 (Fig. 2, upper panel). Similar results were obtained with the other three hBM cells (Fig. 2), suggesting that hypoxia inhibited the induction of *p16* gene expression, which then protected cells from cellular senescence.

Hypoxia affected the ability to differentiate into chondro- and adipogenic, but not osteogenic, lineage

The ability to differentiate into osteo-, chondro-, and adipogenic lineages in the early phase was confirmed in all four hBM cells (data not shown). After long-term culture (about 100 days), no significant difference in osteogenic differentiation, measured based on Ca content, was observed between normoxic and hypoxic cultured-MSCs (Fig. 3A). In contrast, the ability to differentiate into the chondrogenic (Fig. 3B) and adipogenic (Fig. 3C) lineages, measured from GAG and TG content, respectively, was significantly more superior in hypoxic than normoxic conditions.

Hypoxia inhibited the activation of ERK

In the case of hBM66, the expression of molecules related to MAPK signals showed no significant difference between normoxic

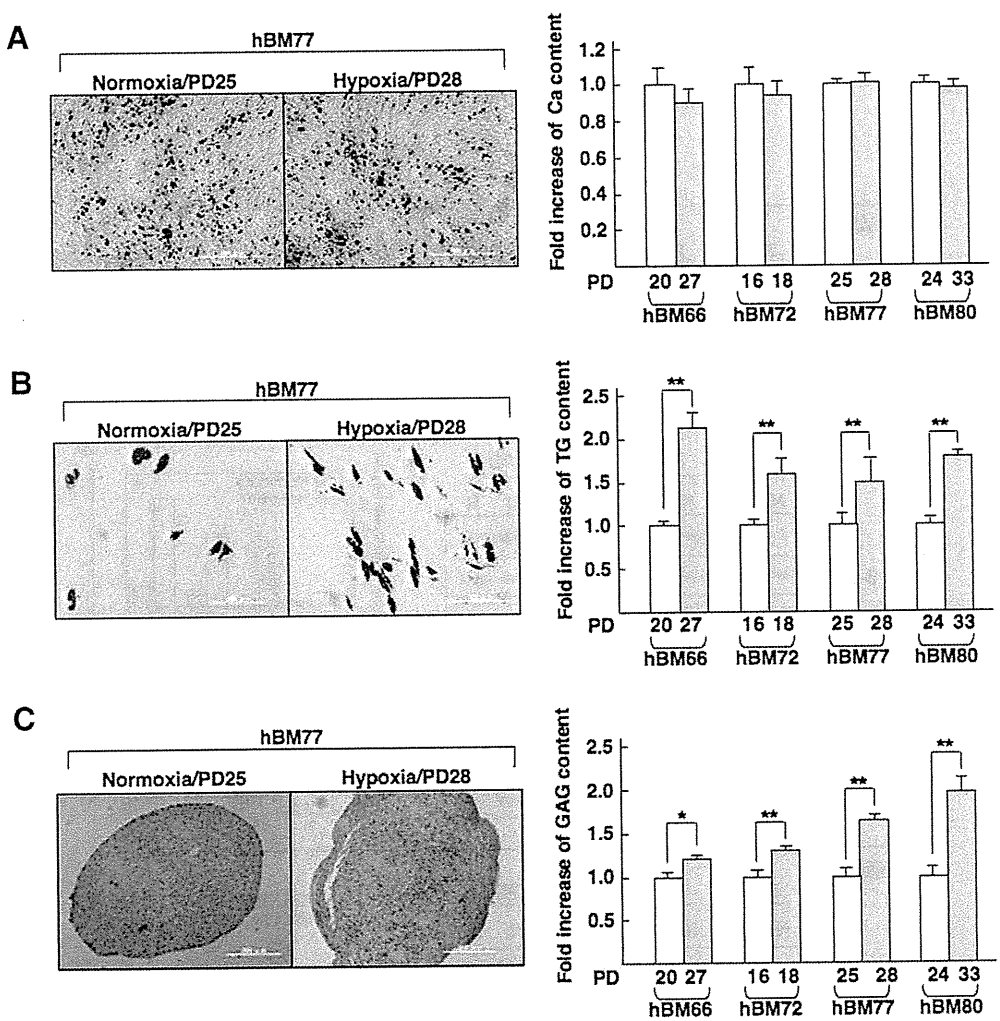

Fig. 3. Hypoxia enhanced differentiation properties of MSCs. Each preparations of MSCs was cultured for approximately 100 days under either normoxic or hypoxic conditions and then induced to undergo (A) osteogenic, (B) chondrogenic, or (C) adipogenic differentiation under normoxic conditions. The results were quantified based on the content of Ca, GAG, or TG, respectively. PD numbers of the MSCs used for these experiments are indicated. White and gray box indicate normoxic and hypoxic conditions, respectively.

and hypoxic cultured-cells except that of ERK (Fig. 4A). Levels of both phospho-ERK1 and phospho-ERK2 were much lower in hypoxic cultured-cells than in normoxic cultured-cells throughout the culture period, and similar results were obtained with the other three hBM cells (Fig. 4B). nPG is an antioxidant, which induces the production HIF-1α protein [24]. Treatment of hBM72 cells (PD3) cultured under normoxic conditions with nPG successfully induced the expression of HIF-1α, but failed to reduce the level of phospho-ERK1/2 (Fig. 4C), indicating that the reduction in phospho-ERK is not a direct consequence of HIF-1α's activation. To investigate the relationship between ERK and p16, the activity of MEK, by which ERK is phosphorylated, was inhibited by U0126. When hBM72 cells (PD3) cultured under normoxic conditions were treated with U0126 for 4 days, activation of ERK was inhibited throughout the culture period (Fig. 4D). During this period, the level of *p16* increased two-fold in control cells, but only slightly in U0126 treated-cells (Fig. 4D), suggesting that the ERK signal is one of the factors inducing the expression of the *p16* gene.

Discussion

A number of studies have been published regarding the effects of hypoxia on the growth and differentiation of MSCs [12–17], but the results differed considerably. The discrepancies may be at least in part due to differences in species, the concentration of oxygen, and/or the length of culture periods, and the discussion hereafter focuses on the data for human MSCs. As for short-term effects, some studies showed that hypoxia increased the proliferation of MSCs by promoting progression of the cell cycle [16], but others showed no or even the opposite effect [14], consistent with the results of the current study. Two studies have analyzed the long-term effects of hypoxia on human MSCs. Grayson et al. showed that human MSCs displayed enhanced proliferation under hypoxic conditions (2% pO_2) for seven passages over 6 weeks, resulting in a 30-fold increase in cell number compared with that under normoxic conditions [15]. Fehrer et al. showed that MSCs cultured under hypoxic conditions (3% pO_2) for up to 100 days had a higher number of final PD than those cultured under normoxic conditions by ten [17], which agreed with the results of the current study. Because the PD time showed no difference during the early phase of growth (Fig. 1A), our data indicate that hypoxia did not affect the growth of cells, but extended their life span, and the marked difference in the number of SA-β-gal-positive cells between hypoxic and normoxic conditions clearly indicates that the lengthening of life span by hypoxia is due to the escape from cellular senescence. We have shown that the up-regulation of *p16* gene expression is key to inducing cellular senescence in human MSCs [20]. In the current study, we showed that hypoxia inhibited the up-regulation of *p16* gene expression. ROS induces *p16* gene expression [21,22], and the p16-Rb pathway then induces the production of ROS, which leads to cellular senescence [25].

Regarding the effects of hypoxia on the differentiation of MSCs, published results vary. To evaluate the ability of MSCs to differentiate, osteo-, chondro-, or adipogenic differentiation has been analyzed in most studies [12–17], but no studies have examined all three lineages in MSCs cultured under hypoxic conditions long term. Martin-Rendon et al. analyzed the short-term effects (24 h) of hypoxia (1% pO_2) on the differentiation into three lineages and found that only chondrogenic differentiation was improved [16]. Our data indicated that chondrogenic, as well as adipogenic, but not osteogenic, differentiation was improved in MSCs cultured under hypoxic conditions for long term (about 100 days). Although

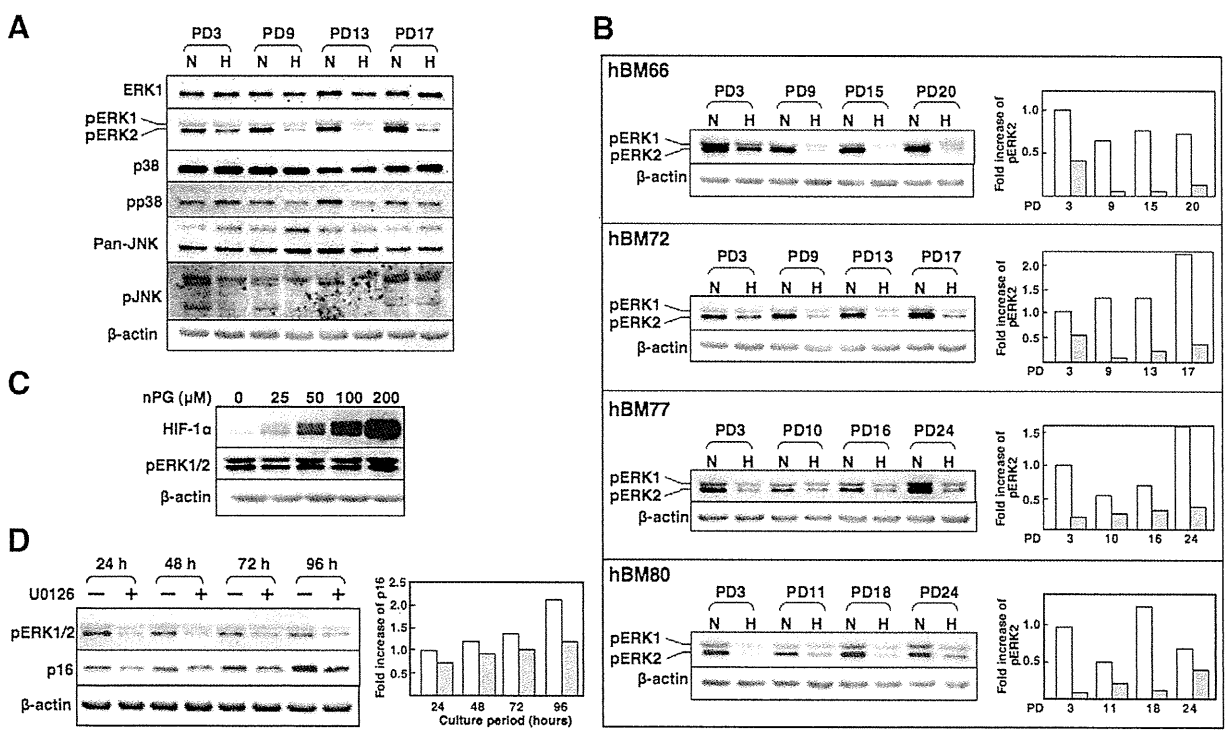

Fig. 4. Hypoxia down-regulated the activation of ERK in MSCs. (A) Expression of molecules related to MAPK signaling pathways of huBM72 at the indicated PD. (B) Expression of pERK1/2 during the life span of MSCs *in vitro*. The relative level of pERK2 was determined using the value at PD3 of normoxic cells as a standard. White and gray box indicate normoxic and hypoxic conditions, respectively. (C) Effect of nPG treatment on the expression of HIF-1α and pERK1/2. huBM72 cells cultured under normoxic conditions at PD3 were treated with nPG at the indicated concentration for 4 h, and proteins were extracted for Western blotting. (D) Effect of a MEK inhibitor on the expression of pERK1/2 and p16. huBM72 cells cultured under normoxic conditions at PD3 were treated with U0126 (10 μM) for up to 96 h, and proteins were extracted for Western blotting. The relative expression level of p16 was determined using the value of normoxic cultured-cells at 24 h as a standard. White and gray box indicate normoxic and hypoxic conditions, respectively.

the molecular mechanisms underlying this change in the behavior of MSCs are not known, the down-regulation of phospho-ERK expression caused by a reduction in oxygen is an intriguing new finding of the current study. MAPK signaling pathways have profound effects on the growth and differentiation of MSCs [26,27], and the signaling through ERK has been investigated intensively. Activation of the ERK signal triggers osteogenic differentiation [28,29]. The up-regulation of MAPK signals promoted chondrogenesis by inducing the expression of the *Sox9* gene [30]. The inhibition of ERK signals reduced the adipogenic differentiation [31]. Therefore, the ERK signal is essential to the differentiation of MSCs. In other words, inhibition of the ERK signal may restrict the "spontaneous" differentiation which maintains MSC in an undifferentiated state. The ERK signal also plays a role as a mitogenic stimulus, which promotes growth, but at the same time induces cellular senescence of MSCs. Inhibition of the ERK signal by a MEK inhibitor reduced the up-regulation of *p16* gene expression. Therefore, down-regulation of phospho-ERK expression may also help cells to escape from cellular senescence during propagation *in vitro*. Further study of mechanisms by which hypoxia down-regulates the ERK signal may provide a new method of culturing MSCs.

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Trail-Walking Exercise and Fall Risk Factors in Community-Dwelling Older Adults: Preliminary Results of a Randomized Controlled Trial

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OBJECTIVES: To evaluate the effects of a trail-walking exercise (TWE) program on the rate of falls in community-dwelling older adults.

DESIGN: Pilot randomized controlled trial (RCT).

SETTING: This trial was conducted in Japan and involved community-dwelling older adults as participants.

PARTICIPANTS: Sixty participants randomized into a TWE group (n = 30) and a walking (W) group (n = 30).

INTERVENTION: Exercise class combined with multicomponent trail walking program, versus exercise class combined with simple indoor walking program.

MEASUREMENT: Measurement was based on the difference in fall rates between the TWE and W groups.

RESULTS: Six months after the intervention, the incidence rate ratio (IRR) of falls for the TWE group compared with the W group was 0.20 (95% confidence interval (CI) = 0.04–0.91); 12 months after the intervention, the IRR of falls for the TWE group compared with the W group was 0.45 (95% CI = 0.16–1.77).

CONCLUSION: The results of this pilot RCT suggest that the TWE program was more effective in improving locomotion and cognitive performance under trail-walking task conditions than walking. In addition, participants who took part in the TWE demonstrated a decrease in the incidence rate of falls 6 months after trial completion. Further confirmation is needed, but this preliminary result may promote a new understanding of accidental falls in older adults. J Am Geriatr Soc 58:1946–1951, 2010.

Key words: fall prevention; trail walking task; falls; RCT; older adults

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Falls in older adults are a major health problem. Approximately 30% of community-dwelling adults aged 65 and older fall at least once a year, and 6% of these falls result in fractures.[1,2] Recent meta-analyses of fall prevention programs have convincingly shown that exercise interventions are effective at reducing the risk of falls and fall injuries.[3,4]

Although the cause of falls is typically multifactorial, performance-based measures are often used to assess how balance and gait impairments contribute to fall risk, but in community-dwelling older adults, performance-based measures are not always predictive of fall risk, particularly in a high-functioning population.[5] Concurrent cognitive or motor tasks, such as talking or carrying objects, are crucial for mobility in daily life. Because of the increasingly recognized role of cognition in postural control and gait, many researchers have used dual-task (DT) paradigms incorporating a concurrent cognitive task to improve studies investigating fall risk.[6] For example, a concurrent cognitive task during standing postural control tasks has been used to distinguish between older adults with and without a history of falls.[7] In addition, complex walking tasks such as DT walking may be more sensitive than simple walking tasks for identifying early declines in postural control in nondisabled older adults without apparent mobility limitations.[8]

The ability to modulate attention may also play an important role in the acquisition of complex-task coordination skills. Therefore, a trail-walking test (TWT), in which a person walks from numbered flags in an ascending or descending order, was developed to evaluate cognitive and motor function simultaneously.[9]

This randomized controlled trial (RCT) aimed to evaluate whether the trail-walking exercise (TWE), a new 16-week exercise program, would be effective in reducing falls in community-dwelling older adults. It was hypothesized that complex-task walking is improved to a greater extent with TWE than regular walking, suggesting that TWE is more effective in preventing falls.

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METHODS

Participants

Participants were recruited using an advertisement in the local press. The following criteria were used to screen participants in an initial interview: aged 65 and older, community-dwelling, had visited a primary care physician within the previous 3 years, a Mini-Mental State Examination (MMSE)[10] score of 24 or greater, able to walk independently (or with a cane), willingness to participate in group exercise classes for at least 6 months, access to transportation, minimal hearing and vision impairments, and no regular exercise in the previous 12 months.

The interview was also used to exclude participants based on the following exclusion criteria: severe cardiac, pulmonary, or musculoskeletal disorders; pathologies associated with greater risk of falls (e.g., Parkinson's disease or stroke); osteoporosis; and use of psychotropic drugs. Written informed consent was obtained from each participant in the trial in accordance with the guidelines approved by the Kyoto University Graduate School of Medicine and the Declaration of Human Rights, Helsinki, 1975.

Study Design and Randomization

Participants were block randomized in blocks of four. Using this sequence, opaque envelopes bearing group names were numbered and the 60 participants were then randomly assigned to the TWE (n = 30) or walking (W) group (n = 30).

Intervention

All participants received 90 minutes of group training sessions once a week for 16 weeks. Participants were randomly assigned to one of the two training groups: standardized training with TWE group and standardized training with W group.

The exercise class was individualized for each group and supervised by a physiotherapist. Each exercise class used a standardized format that included 20 minutes of moderate-intensity aerobic exercise, 20 minutes of progressive strength training, 10 minutes of flexibility and balance exercises, and 10 minutes of cool-down activities, followed by exercises known to decrease fall risk.[11] The aerobic exercise involved movement of the legs, trunk, and arms to involve all joints and major muscle groups, such as dance. Strength training involved progressive resistive exercises using an elastic band. A sequence of progressively more-difficult exercises was also performed to improve static and dynamic balance. Although exercises could be performed in a sitting position, the importance of performing them in a standing position to improve balance was stressed. Physiotherapists evaluated the participants twice during the study period to ensure adherence with exercise protocols during classes.

Trail-Walking Exercise

In the TWE training field, flags were set randomly at each of 15 positions in a 25-m^2 area (5 m × 5 m; Figure 1A). Participants in the TWE group were asked to sequentially pass from number 1 to 15 (Figure 1B). A circle with a 30-cm diameter was drawn on the ground around each flag, and the participant was required to step in the circle to pass the flag. The height of the flag was 30 cm. The tester gave the following instructions to participants, "Please move to number 15 as quickly and correctly as possible."

The 16-week program included a progressive aspect of the TWT. The participants were asked to pass sequentially from number 1 to 15 during Weeks 1 to 8 and then were asked to pass sequentially from number 15 to 1 during Weeks 9 to 16. Flag positions were changed for each day of training.

Walking Exercise

Participants in the W group were instructed to attend a supervised indoor walking session. These sessions were designed as controls for the additional physical activity in the TWE session. The participants were instructed to walk as comfortably and for as long as possible for up to 30 minutes. They were permitted to stop and take breaks during the session. The indoor walking course consisted of a 300-foot loop.

Falls

The primary outcome of this trial was the occurrence of falls, which were measured 6 and 12 months after trial completion. A fall was defined as an event that resulted in a

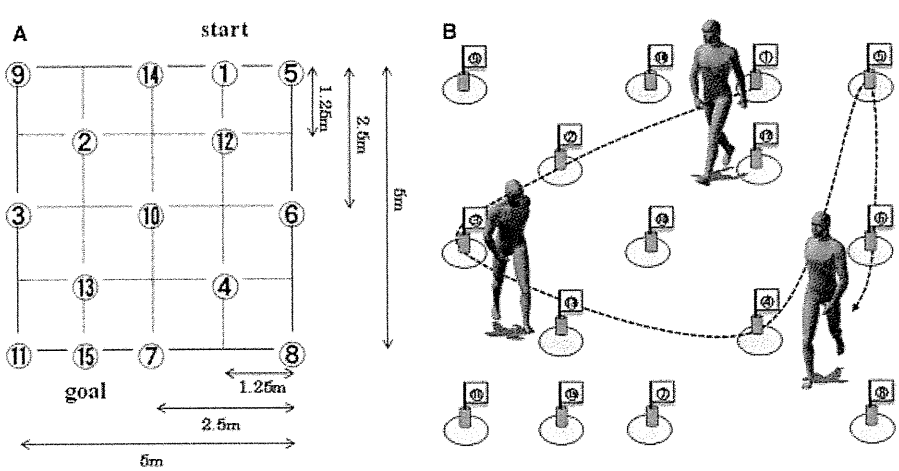

Figure 1. Schematic representation of the trail-walking test. (A) Participants are asked to pass sequentially from number 1 to 15. (B) Image scheme of a task excursion.

person unintentionally coming to rest on the ground, floor, or other lower level with or without loss of consciousness or injury.[12] Falls resulting from extraordinary environmental factors (e.g., traffic accidents or falls while riding a bicycle) were excluded. The participants were asked to record any falls in fall diaries that were mailed to the research assistants every month. If participants failed to send in the fall diaries, research assistants collected data on falls over the telephone. All participants who had fallen were interviewed during these calls using a structured questionnaire about the fall event and its consequences.

Secondary Outcome Measures

All participants underwent seven measurements: the Timed Up and Go (TUG) Test,[13] the functional reach (FR) test,[14] the one-leg stand (OLS) test, 10-m walking time under single-task conditions (ST walking),[15] 10-m walking time under DT conditions,[16] the TWT,[9] and the Trail-Making Test (TMT) Part A.[17] A physiotherapist blinded to group allocation administered these measures at baseline, upon completion of the 16-week intervention (16 weeks), and 6 and 12 months after completion of the intervention (6 months, 12 months). All baseline measures were completed before randomization. All pretest measures were completed before randomization. Before commencing the study, all staff members received training on correct protocols for administering all assessment measures included in the study from one of the authors (MY). If a walking aid was normally used at home, this aid was used during the TUG Test, ST walking, DT walking, and TWT.

In the TUG Test, participants were asked to stand up from a standard chair with a seat height of 40 cm, walk a distance of 3 m at a normal pace, turn, walk back to the chair, and sit down. The time recorded from two trials was averaged to obtain the TUG score.

In the FR test, each participant was positioned next to a wall with one arm raised at 90° and fingers extended. A meterstick was mounted on the wall at shoulder height. The distance that a participant could reach while extending forward from an initial upright posture to the maximal anterior leaning posture without moving or lifting the feet was visually measured in centimeters according to the position of the tip of the third finger against the mounted meterstick. The distances measured in two trials were averaged to obtain the FR score.

In the OLS test, participants were instructed to start from a standing position with a comfortable base as support with eyes open and arms at their sides. They were then instructed to stand unassisted on either leg. OLS was measured in seconds from the time one foot was lifted from the floor to when it touched the ground or the standing leg.

In ST walking, participants walked 15 m at a speed they felt comfortable with. A stopwatch was used to record the time required to reach the 10-m point that was marked in the middle of this walk. The time recorded in two trials was averaged as the ST walking score.

In DT walking, participants walked 15 m at a speed they felt comfortable with while counting backwards from 50 out loud as the DT. All participants were asked to walk and count simultaneously to the best of their ability without prioritizing either task. Possible counting mistakes were not corrected.[16]

The field for the TWT was the same as that used for the TWE. The test–retest reliability using the intraclass correlation coefficient was 0.945.[9] The positions in which the flags were placed are shown in Figure 1. The tester gave the following instruction to participants, "Please move to number 15 as quickly and as correctly as possible," and timed the trial using a stopwatch to the nearest 0.01 s following a standard procedure. The TWT was performed only once for each participant at each time point.

Executive function was assessed using the TMT, a well-established psychomotor test originally developed as part of the Army Individual Test Battery.[15] Part A of the TMT is a visual scanning task. The participant is required to draw lines sequentially connecting consecutively numbered circles (1–25) randomly arranged on a page as quickly as possible.

Statistical Analysis

Baseline characteristics of the TWE and W groups were compared to examine comparability of the two.

Differences in the physical function variables between the TWE and W groups were analyzed using the Student *t*-test or the chi-square test. The Kolmogorov-Smirnov test and the Mann-Whitney *U*-test were used to test the normality of distributions and differences in physical function variables between the groups, respectively.

The number of falls was calculated from the beginning of the study period to the participant's death, withdrawal from the trial, or the end of the 12-month follow-up period. Confidence intervals (CIs) for the fall rate were calculated assuming that the number of falls followed a negative binomial distribution.

Incidences of falls with their 95% CIs were calculated for participants in the TWE and W groups and compared using negative binomial regression analysis. Results were presented using incident rate ratios (IRRs) with their 95% CIs. The effect of exercise on outcome measurements was analyzed using mixed 2 × 4 (group (TWE and W groups) × time (pretraining, posttraining, after 6 months, and after 12 months)) analysis of variance. Post hoc Bonferroni tests were used to assess which group or time periods showed significant differences.

Data were entered and analyzed using the SPSS (Windows version 11.0, SPSS, Inc., Chicago, IL). $P<.05$ was considered statistically significant for all analyses.

RESULTS

Eighty-two people were screened, and 60 (73%) who met the inclusion criteria for the trial and agreed to participate were enrolled (Figure 2). Of individuals not meeting the inclusion criteria (n = 22), most were excluded because they had exercised regularly for 6 months before screening. Three people who may have been eligible for the study declined after a telephone screening. Of the 60 individuals who enrolled in the study, 58 (97%) completed the 12-month follow-up and returned for the exit interview and final tests: 29 in the TWE group (97%) and 29 in the W group (97%).