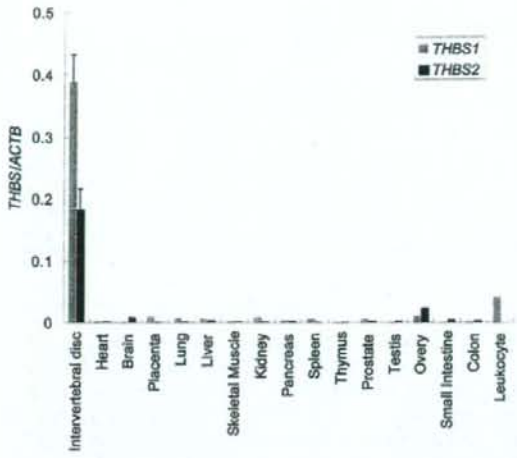

Figure 1. Real-Time PCR Analysis of *THBS1* and *THBS2* mRNAs in Various Human Tissues

THBS1 and *THBS2* mRNAs are abundantly expressed in the intervertebral disc. Data represent the ratio of *THBS* mRNAs to beta-actin (*ACTB*) mRNA. Error bars represent ± standard error of the mean (SEM) (n = 7).

human tissues by using real-time RT-PCR. We detected specific and high expression levels of both genes in intervertebral disc tissue (Figure 1), which further supported the candidacy of these genes for the LDH-susceptibility gene.

On the basis of information in the HapMap phase II database (International HapMap Consortium), we selected SNPs with minor allele frequencies of more than 10% that covered most haplotypes in the *THBS1* and *THBS2* genes. We selected 11 tag SNPs, two in *THBS1* and nine in *THBS2*. Together, these SNPs covered more than 80% of the alleles in each gene that had an r^2 value of 0.8 or greater. We first genotyped the tag SNPs in 188 individuals with LDH and 188 control individuals. We performed χ^2 tests for genotype, dominant, recessive, and allele-frequency models and identified three SNPs in *THBS2* showing nominal p values of less than 0.05 in any of the four models (data available from authors). We found no association in *THBS1* tag SNPs. Next, we genotyped the nine *THBS2* tag SNPs in additional 337 LDH cases and 376 controls. In a total of 525 cases and 564 controls (Japanese A population), we found one SNP (rs6422747) that was significantly associated with LDH (Table 1).

To locate the disease-causing sequence variation in *THBS2*, we resequenced in and around all exons of the gene and its promoter sequence (approximately 1 kb) in 24 LDH individuals and identified a total of 15 sequence variations other than two tag SNPs. We selected five of these by excluding SNPs that were in linkage disequilibrium (LD) with the landmark SNP (rs6422747), having an r^2 value of less than 0.1. We genotyped them for the Japanese A population and identified one SNP (IVS10-8C → T, rs9406328) that had the most significant association with LDH (χ^2 = 16.85, p = 0.000040; comparison of allele frequency, Table 1). The association was significant even after Bonferroni's correction of multiple testing (corrected p = 0.00064).

We estimated LD across the *THBS2* region by using genotyping data from control samples (n = 564, data available from authors). We then examined haplotypes based on the seven SNPs containing the SNP IVS10-8C → T. Six haplotypes with frequencies of more than 0.01 represented more than 95% of both the case and control populations. The haplotype associations were far less significant than that of the SNP IVS10-8C → T alone (Table 2). Therefore,

Table 1. Association of SNPs in *THBS2* with Lumbar-Disc Herniation

			LDH				Control				Test for Allele Frequency	
dbSNP ID	Assay[a]	Location	11	12	22	Sum	11	12	22	Sum	p Value[b]	Odds Ratio[c] (95% CI)
rs7382358	T	IVS4	367	142	16	525	342	201	21	564	0.0032	1.38 (1.11–1.72)
rs9379341	T	IVS5	266	212	47	525	251	246	67	564	0.023	1.24 (1.03–1.48)
rs13192849	D	IVS6	398	114	13	525	377	167	19	563	0.0019	1.45 (1.15–1.83)
rs9283850	T	IVS9	396	124	5	525	437	121	5	563	0.42	0.90 (0.70–1.16)
rs9406328	D	IVS10	203	243	79	525	164	271	129	564	0.000040	1.43 (1.20–1.70)
rs6940420	T	IVS14	360	146	19	525	362	181	19	562	0.24	1.14 (0.92–1.41)
rs6422748	D	IVS14	130	266	128	524	195	261	107	563	0.00036	0.74 (0.62–0.87)
rs9505891	D	IVS14	275	209	41	525	236	255	73	564	0.000087	1.44 (1.20–1.73)
rs6422747	T	IVS14	130	266	128	524	195	261	108	564	0.00043	0.74 (0.62–0.87)
rs11966235	T	IVS16	367	140	17	524	390	157	17	564	0.84	1.02 (0.82–1.28)
rs12665573	T	IVS17	422	99	4	525	437	117	10	564	0.15	1.22 (0.93–1.59)
rs10945405	T	IVS21	162	267	96	525	148	286	128	562	0.035	1.20 (1.01–1.42)
rs9393165	D	IVS22	161	267	97	525	148	286	128	562	0.044	1.19 (1.00–1.41)
rs8089	T	3′-UTR	424	98	3	525	453	105	5	563	0.81	1.03 (0.78–1.37)

LDH, lumbar-disc herniation; CI, confidence interval. Allele 1 and allele 2 indicate the major and minor allele, respectively.

[a] T, Tag SNPs; D, SNPs discovered by resequencing.

[b] Not corrected for multiple testing.

[c] Odds ratio for allele 1 versus allele 2.

Table 2. Association of Haplotypes of the *THBS2* Region with Lumbar-Disc Herniation

SNP							Haplotype Frequency		
rs13192849	rs9283850	rs9406328	rs6940420	rs6422748	rs9505891	rs6422747	LDH	Control	p Value[a]
T	T	T	C	C	C	C	0.489	0.409	0.00017
G	T	C	C	G	G	T	0.130	0.181	0.0010
T	T	C	T	G	G	T	0.139	0.156	0.25
T	C	T	C	G	C	T	0.115	0.106	0.50
T	T	C	C	G	C	T	0.076	0.084	0.50
T	T	C	T	G	C	T	0.022	0.020	0.66

LDH, lumbar-disc herniation.
[a] Not corrected for multiple testing.

we considered the presence of a hidden causal SNP to be unlikely.

We analyzed potential confounding factors such as age, BMI, and sex to evaluate for pseudopositive associations with LDH. There was no significant difference in mean age, BMI, or sex distribution among genotypes of the SNP IVS10-8C → T, indicating that the SNP IVS10-8C → T in *THBS2* is an independent risk factor of LDH. By using genomic control methods,[20] we also assessed the stratification of the Japanese A population by genotyping 24 randomly selected SNPs. The significance of stratification (p) and the inflation factor (λ) were 0.98 and 1.00 (95^{th} percentile upper bound 1.53), respectively. Because the maximum factor (the highest χ^2 statistic/threshold χ^2 statistic) in this study is 1.56, it is unlikely that population stratification is the cause of our case-control association result.

To confirm the association, we genotyped the SNP IVS10-8C → T by using an independent Japanese case-control population consisting of 322 cases and 332 controls (Japanese B population). Significant association was replicated in this population (Table 3). Again, we saw no significant difference in mean age, BMI, and sex distribution among genotypes of the SNP IVS10-8C → T. In total, the SNP IVS10-8C → T was genotyped in 847 individuals with LDH and 896 control individuals, and our results clearly demonstrated a strong association with LDH in the Japanese population ($\chi^2 = 21.79$, $p = 0.0000028$; comparison of allele frequency, Table 3). The association was significant after Bonferroni's correction (corrected $p = 0.000045$).

To investigate potential effects of the SNP IVS10-8C → T on *THBS2* transcription, we performed RT-PCR with CS-OKB and OUMS-27 cells. The genotypes of the SNP IVS10-8C → T in these cell lines were C/C and T/T, respectively. Both cell lines contained a 418 bp PCR product in addition to the expected 592 bp product (Figure 2A). Sequence analysis revealed that the 418 bp product lacked exon 11 but retained the original reading frame. Further, we observed a greater amount of the 418 bp product in OUMS-27 than in CS-OKB, suggesting potential allelic differences in the rate of exon 11 skipping.

We followed up this initial observation in vivo by performing RT-PCR and allele-specific real-time PCR in 29 normal human fibroblast cell samples. Fourteen of the samples had the genotype T/T at the SNP IVS10-8C → T locus, 11 had T/C, and the remaining four had C/C. Our analysis revealed significant association between the genotype of the SNP IVS10-8C → T and the rate of exon 11 skipping in vivo (Figures 2B and 2C). The rate of exon 11 skipping for the T/T genotype was approximately 3.8-fold higher than that of C/C, confirming allelic differences in *THBS2* transcription.

Because exon 11 of *THBS2* encodes one of three THBS type 1 repeat (TSR) domains that interact with MMP2 and MMP9,[11] we examined the effect of exon 11 skipping on the interaction between THBS2 and MMP2 or MMP9. We performed immunoprecipitation experiments with S-tagged, recombinant partial human THBS2 proteins produced in *E. coli*. Both the wild-type TSR domains (WT-THBS2) and the exon-11-skipped TSR domains (Skip-THBS2) that lack the third TSR domain coprecipitated with MMP2 and MMP9

Table 3. Association of rs9406328, IVS10-8C → T, in *THBS2* with Lumbar-Disc Herniation

	LDH					Control						
	Genotype					Genotype					Test for Allele Frequency	
Population	TT	TC	CC	Sum	Allele T Frequency	TT	TC	CC	Sum	Allele T Frequency	p Value[a]	Odds Ratio (95% CI)
Japanese A	203	243	79	525	0.618	164	271	129	564	0.531	0.000040	1.43 (1.20–1.70)
Japanese B	106	173	43	322	0.598	92	170	70	332	0.533	0.018	1.30 (1.05–1.62)
Combined[b]	309	416	122	847	0.610	256	441	199	896	0.532	0.0000028	1.38 (1.21–1.58)

LDH, lumbar-disc herniation; CI, confidence interval.
[a] Not corrected for multiple testing.
[b] The sum of the two Japanese populations.

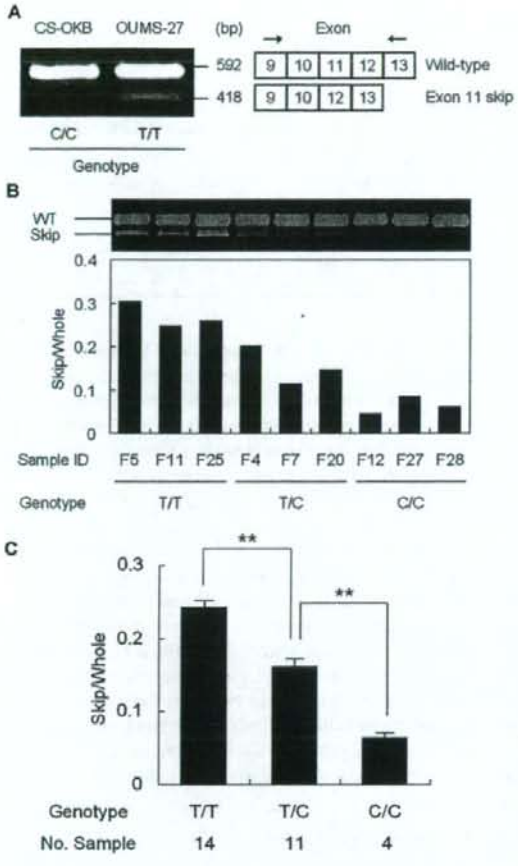

Figure 2. Effects of the LDH-Associated SNP, IVS10-8C → T, on *THBS2* Exon 11 Splicing In Vivo

(A) Left: RT-PCR products from CS-OKB and OUMS-27 cells representing the wild-type and exon-11-skipped transcripts. Right: patterns of exon skipping. Arrows indicate the positions of PCR primers.

(B) Rates of exon 11 skipping for each genotype, detected by RT-PCR (top) and allele-specific real-time PCR (bottom). Of the 29 normal human fibroblast cell samples examined (F1-F29), three representatives for each genotype are shown.

(C) Allelic differences in the rate of exon 11 skipping in 29 normal human fibroblast cells, detected by allele-specific real-time PCR. Data represent the mean ± SEM. ** indicates $p < 0.01$ (Student's t test).

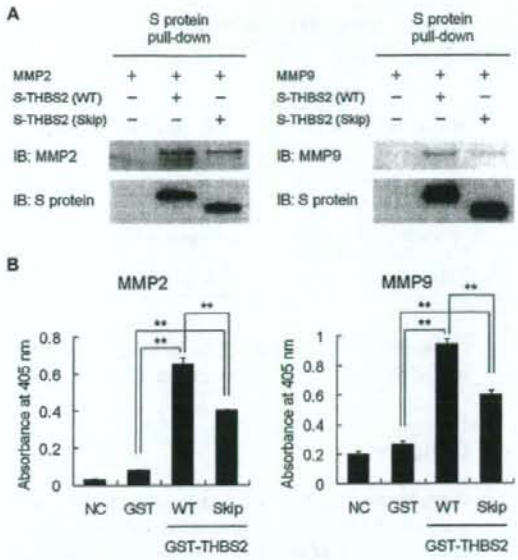

Figure 3. Effects of Exon 11 Skipping on the Interaction of THBS2 with MMP2 and MMP9 In Vitro

(A) S protein pull-down assay. S-tagged recombinant human THBS2 was incubated with purified human MMP2 (left) or MMP9 (right). MMP2 and MMP9 were detected with antibodies, and S-tagged THBS2 was detected with S protein HRP conjugate.

(B) Solid-phase binding assay. Microplate wells coated with GST or GST-tagged recombinant human THBS2 were incubated with or without purified human MMP2 (left) or MMP9 (right). Binding of GST-tagged THBS2 to MMP2 or MMP9 was quantified by colorimetric assay at $A_{405\ nm}$. Data represent the mean ± SEM in quadruplicate assays. ** indicates $p < 0.01$ (Student's t test). NC indicates noncoated.

(Figure 3A). Solid-phase binding assays confirmed that WT-THBS2 bound to MMP2 or MMP9 more strongly than did Skip-THBS2 (Figure 3B).

Binding of THBS2 with MMP2 and MMP9 implies a causal link of MMPs with LDH. Therefore, we evaluated whether *MMP2* and *MMP9* also are associated with LDH. We genotyped 15 tag SNPs in *MMP2* and *MMP9*, selected with the same criteria as for the *THBS* genes, in the Japanese A population (525 individuals with LDH and 564 controls). One missense SNP (Q279R, rs17576) in *MMP9* was strongly associated with LDH (Table 4). *MMP2* tag SNPs did not show any significant association. We resequenced in and around all exons of *MMP9* and its promoter sequence (approximately 1 kb) in 24 LDH individuals and identified a total of nine sequence variations. We genotyped the landmark SNP (rs17576) and other two SNPs that were in LD with the landmark SNP in 847 LDH cases and 896 controls (Japanese A and Japanese B populations). We found the most significant association in the landmark SNP (combined $p = 0.00049$; comparison for allele frequency, Table 4), which was significant even after Bonferroni's correction (corrected $p = 0.0083$).

The functional relationship between *THBS2* and *MMP9* raises the possibility of a combinatorial effect of these genes in LDH. We examined this possibility by combining genotype data on a pair of loci in 847 cases and in 896 controls. Significant combinatorial association was observed for *THBS2* (rs9406328) and *MMP9* (rs17576) with LDH. The genotype that was homozygous with respect to

Table 4. Association of rs17576, Q279R, in *MMP9* with Lumbar-Disc Herniation

	LDH					Control						
	Genotype					Genotype					Test for Allele Frequency	
Population	GG	GA	AA	Sum	Allele G Frequency	GG	GA	AA	Sum	Allele G Frequency	p Value[a]	Odds Ratio (95% CI)
Japanese A	265	203	57	525	0.698	234	250	80	564	0.637	0.0023	1.32 (1.10–1.58)
Japanese B	154	133	35	322	0.685	142	140	50	332	0.639	0.077	1.23 (0.98–1.55)
Combined[b]	419	336	92	847	0.693	376	390	130	896	0.637	0.00049	1.29 (1.12–1.48)

LDH, lumbar-disc herniation; CI, confidence interval.
[a] Not corrected for multiple testing.
[b] The sum of the two Japanese populations.

susceptible alleles of both SNPs showed the highest odds ratio (3.03; 95% confidence interval = 1.58-5.77, Table 5).

Discussion

Our genetic and functional data indicate that THBS2 is involved in LDH via the regulation of MMPs in the intervertebral disc ECM. MMP2 and MMP9 activities are mainly regulated by endocytosis, a general clearance mechanism for ECM. Endocytic clearance of MMPs is mediated by the scavenger receptor, low-density lipoprotein receptor-related protein (LRP).[25,26] THBS2 promotes LRP-mediated endocytosis of MMP2 and its subsequent lysosomal degradation.[25] Thus, THBS2 may play a major role in intervertebral disc ECM homeostasis as a modulator of MMP2 and MMP9 endocytosis.

MMP activity is also regulated at different levels with activation of the latent enzyme and inhibition by tissue inhibitor of metalloproteinase (TIMP). MMP2 and MMP9 mostly exist in tissues as latent enzymes that make complexes to their specific inhibitors, TIMP2 (MIM 188825) and TIMP1 (MIM 305370), respectively.[27,28] These MMP inhibitors, as well as other MMP regulators, could also be a candidate for LDH-susceptibility genes, although the clearance of MMP2-TIMP2 complex is THBS2-independent process.[29]

Our findings suggest that the third TSR in THBS2 plays an important role in its interaction with MMPs and in LDH pathogenesis overall. TSRs are small cystein-knot modules of approximately 60 amino acids, containing six conserved cystein residues as well as conserved tryptophan, serine, and arginine residues.[30] The TSR domain of THBS2 consists of three repeats and interacts with MMP2 and MMP9.[11] These three repeats share conserved cystein and tryptophan residues but differ in other amino acid positions, suggesting that each repeat functions differently. Several short motifs within TSRs have been reported to support protein-protein interactions.[31,32]

The susceptibility SNP in *MMP9* (Q279R) resides within the highly conserved gelatinase-specific fibronectin type II domains, which presumably enhance substrate binding.[33,34] This SNP has previously been reported to associate with metastasis of lung cancer[35] and trachoma;[36] however, its function is unknown. We can hypothesize that this nonconservative amino acid change affects enzyme activity by modifying its substrate binding capacity. Further characterization of the interaction between THBS2 and MMPs, as well as the identification of other genes functioning within this metabolic pathway, should reveal promising targets for treatment and novel therapeutic strategies for LDH.

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Table 5. Combinatorial Effect between *THBS2* and *MMP9*

	Genotype of *THBS2* (rs9406328)								
	LDH (n = 847)			Control (n = 896)			Odds Ratio (95% CI)		
Genotype of *MMP9* (rs17576)	TT	TC	CC	TT	TC	CC	TT	TC	CC
GG	154	213	52	105	193	78	3.03 (1.58–5.77)	2.28 (1.21–4.27)	1.38 (0.69–2.75)
GA	123	159	54	116	186	88	2.19 (1.14–4.18)	1.76 (0.94–3.32)	1.27 (0.64–2.51)
AA	32	44	16	35	62	33	1.89 (0.88–4.06)	1.46 (0.72–2.98)	1

LDH, lumbar-disc herniation; CI, confidence interval.

Web Resources

The URLs for data presented herein are as follows:

Applied Biosystems, http://www.appliedbiosystems.com/index.cfm

International HapMap Project, http://hapmap.org/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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Growth suppression and apoptosis induction in synovial sarcoma cell lines by a novel NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ)

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Abstract

Synovial sarcoma is a relatively common soft tissue sarcoma with an aggressive clinical course. Although surgery is currently the first treatment modality, improvement of adjuvant chemotherapy is deemed essential to improve the clinical outcome. Nuclear factor-κB (NF-κB) is constitutively activated in various cancer cells and has emerged as a potential therapeutic molecular target; however, the possible involvement of NF-κB in the pathology of sarcomas remains to be clarified. Herein we examined the effects of a novel NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ) on two synovial sarcoma-derived cell lines, HS-SY-II and SYO-1. The growth of both cell lines was completely inhibited by DHMEQ and apoptosis was induced at 10 μg/ml. Additionally, we found that DHMEQ showed additive effects when used in combination with other cytotoxic agents. These observations indicate that inhibition of NF-κB activity may serve as a potential therapeutic target for synovial sarcoma.

Keywords: Synovial sarcoma; NF-κB inhibitor; Dehydroxymethylepoxyquinomicin (DHMEQ); Chemotherapy

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1. Introduction

Synovial sarcoma is the fourth most common soft tissue sarcoma, which accounts for approximately 5–10% of the malignancy arising in soft tissue [1]. Although the name of the tumor suggests synovial cell origin, the exact histogenesis or origin of the tumor remains controversial. Histologically, synovial sarcoma can be subdivided into two major forms; biphasic and monophasic, based on the presence or absence of areas of glandular epithelial differentiation. Cytogenetic studies in the past decade have revealed the presence of a characteristic chromosomal translocation, t(X;18)(p11.2;q11.2), which gives rise to a chimeric protein, SYT-SSX, in the majority of these tumors [2,3]. Although synovial sarcoma is relatively slow-growing, it is a clinically aggressive tumor and patients have a poor prognosis, with the 5-year survival rate being approximately 50% [1]. Hence, establishment of more efficient therapeutic strategies is mandatory to improve the prognosis of synovial sarcoma.

In recent years, nuclear factor-κB (NF-κB) has emerged as a potential molecular target for the treatment of several malignancies [4–6]. Active NF-κB complexes are dimers of various combinations of the Rel family of polypeptides consisting of p50 (NF-κB1), p52 (NF-κB2), c-Rel, v-Rel, Rel A (p65) and Rel B. NF-κB is activated by a wide variety of stimuli and cytokines, including UV radiation, chemical carcinogens, tumor necrosis factor α and IL-1β, which cause dissociation of the binding of inhibitory IκB proteins and consequently lead to the relocation of NF-κB complex into the nucleus [7,8]. Activated NF-κB promotes over 150 target transcripts [9], which include various genes involved in cell proliferation, angiogenesis, metastasis and suppression of apoptosis, indicating NF-κB as a valid therapeutic target for cancer.

DHMEQ (dehydroxymethylepoxyquinomicin) is a novel NF-κB inhibitor derived from the structure of the antibiotic epoxyquinomicin [10,11]. It has been shown that DHMEQ inhibits NF-κB transcriptional activity via blocking its translocation into the nucleus [10,12]. Previous studies have shown that this agent suppresses the growth of various tumors, such as hormone-refractory prostate cancer, multiple myeloma, thyroid cancer cell, breast cancer and squamous cell carcinoma *in vitro* and also in tumor xenograft models in mice without any apparent side effects (see [13] for review); however, the potential efficacy of DHMEQ against sarcomas remains to be clarified.

In the current study, we examined the effects of DHMEQ on the growth inhibition and induction of apoptosis in synovial sarcoma cell lines, HS-SY-II and SYO-1. We herein show that DHMEQ suppresses growth and induces apoptosis, presumably via the activation of caspase-3 and downregulation of cIAP-1 in synovial sarcoma-derived cell lines, and that a combination of cytotoxic drugs and DHMEQ exhibits additive effects against these cells. These observations indicate that targeting the NF-κB pathway may serve as a novel adjuvant therapy for the treatment of synovial sarcoma.

2. Materials and methods

2.1. Reagents

DHMEQ was synthesized as previously described [10]. Anti-p65 NF-κB subunit antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cIAP-1 antibody and anti-cleaved caspase-3 antibody were from R&D Systems (Minneapolis, MN) and Cell Signaling Technology (Danvers, MA), respectively. TOTO3 was from Molecular Probes (Camarillo, CA). pNFκB-Luc vector, which contains the firefly luciferase gene from *Photinus pyralis* and multiple copies of the NF-κB consensus sequence, was from Clontech. Fluorescence-conjugated secondary antibody was from Jackson Labs (Bar Harbor, ME). Vincristine sulfate (VCR) and doxorubicin hydrochloride (DXR) were from Shionogi (Osaka, Japan) and Kyowa Hakko Kogyo (Tokyo, Japan), respectively. All other reagents were from Sigma-Aldrich unless otherwise described.

2.2. Cell lines

Detailed descriptions on the establishment of the synovial sarcoma-derived cell lines, SYO-1 and HS-SY-II, have previously been described [14,15]. Both cell lines were maintained in DMEM supplemented with 5% fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin.

2.3. Cell growth assay

Cells plated on a 96-well plate were incubated overnight and then treated with DHMEQ for the designated time period. After incubation, the cells were fixed with 4% paraformaldehyde (PFA)/PBS

and stained with 0.1% crystal violet solution. The stained cells were washed several times with tap water and the dye was dissolved with 1% SDS solution. The absorbance value of each well was determined at 550 nm using a microplate reader (Bio-Rad, Hercules, CA).

2.4. Immunostaining and confocal microscopy

Cells were fixed with 4% PFA/PBS and permeabilized with 0.1% Triton X-100/PBS. The cells were then blocked with 1% BSA/PBS, incubated with the primary antibody at 4 °C for 1 h, washed three times with PBS and incubated with the secondary antibody for another hour. Cells mounted in FluoroGuard (Bio-Rad) were viewed and photographed with an Olympus FV1000 confocal microscope and Olympus Fluoview software.

2.5. Luciferase assay

The activity of NF-κB was monitored using a luciferase plasmid containing a specific binding sequence for NF-κB (pNFκB-Luc). Cells plated on a 24-well plate were transfected with the plasmid vector using Fugene HD (Roche) as instructed by the manufacturer and were incubated overnight. Fresh medium containing various concentrations of DHMEQ was added to the cells and incubated for 3 h. After incubation, the cells were washed with PBS and lysed with the lysis buffer provided in the Luciferase Assay kit (Promega, Madison WI). Luciferase activity in the lysate was measured by a luminometer (TD 20/20, Tuner Designs, Sunnyvale, CA).

2.6. Detection of apoptosis

Apoptosis was detected using a TUNEL-based apoptosis detection kit (ApopTag, Chemicon). In short, cells plated on a cover slip were incubated with DHMEQ (10 μg/ml) for 48 h. After incubation, the cells were fixed with 1% PFA/PBS, permeabilized with a 1:2 mixture of acetic acid and methanol, and the free 3′-OH DNA termini were detected as instructed by the manufacturer. Nuclei were counterstained with TOTO3 and the cells were photographed as described above.

2.7. Western Blot

The cells were washed with ice-cold PBS twice and removed mechanically. Whole cell lysate was collected by 1% Triton X/PBS supplemented with Protease inhibitor Cocktail (Sigma-Aldrich). Nuclear protein was extracted as previously described [16]. Samples separated by SDS–PAGE were blotted onto nitrocellulose membranes and detected by the designated primary antibody.

2.8. Statistical analysis

Student's *t-test* for two samples assuming equal variances was used to calculate *P*-values. *P*-values smaller than 0.05 were considered significant.

3. Results

3.1. DHMEQ suppresses the growth of synovial sarcoma cell lines

To evaluate the effects of DHMEQ on the growth of HS-SY-II and SYO-1, cells plated on 96-well plates at low density (4000 cells/well) were treated with DHMEQ at different concentrations (0–20 μg/ml) for 48 h. The cells were subsequently PFA-fixed and the number of viable cells was evaluated by crystal violet staining. As shown in Fig. 1A, DHMEQ showed significant growth inhibitory effects in a concentration-dependent manner. Statistically significant inhibitory effects were observed at 2.5 μg/ml and higher, and 50% cell growth inhibition was observed at approximately 5 μg/ml in both cell lines. Next, we incubated the cells in the presence of DHMEQ (0, 5 and 10 μg/ml) for a different time course (0, 24 and 48 h). DHMEQ completely inhibited and even reduced the number of viable cells at 5 μg/ml in HS-SY-II cells, whereas SYO-1 cells were less sensitive to DHMEQ and complete growth suppression was observed at approximately 10 μg/ml (Fig. 1B). We then examined the effects of DHMEQ on the induction of apoptosis in HS-SY-II and SYO-1 cells. Cells grown to confluence were treated with DHMEQ for 48 h and apoptosis in these cells was detected by a TUNEL-based method, as described in Section 2. As shown in Fig. 1C, DHMEQ strongly induced apoptosis in both HS-SY-II and SYO-1 cells. Since NF-κB regulates the expression of several anti-apoptotic genes [6], we examined if this was also the case for apoptosis induced by DHMEQ in HS-SY-II and SYO-1 cells. Western blotting analysis revealed a significant decrease in the expression level of cIAP-1, a member of the inhibitor of apoptosis protein (IAP) family genes, which coincided with the appearance of cleaved caspase-3 in both cell lines (Fig. 1D). There was no apparent difference in the expression levels of Bcl-xl (data not shown). These observations indicate the possible involvement of the caspase-3 pathway in apoptosis induced by DHMEQ. To confirm that the effects of DHMEQ were derived from the downregulation of NF-κB transcriptional activity, we next performed a

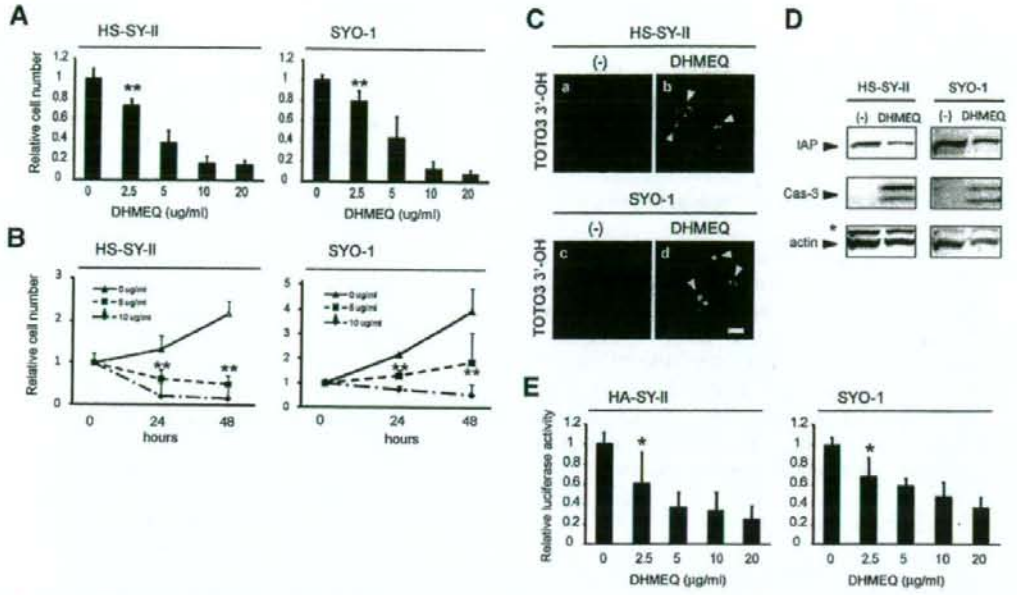

Fig. 1. Effects of DHMEQ on growth and apoptosis in HS-SY-II and SYO-1 cells. (A) HS-SY-II and SYO-1 were treated with designated concentrations of DHMEQ for 48 h. The number of viable cells is presented as a relative value to the non-treated control (0 μg/ml). (B) HS-SY-II and SYO-1 were treated without (0 μg/ml) or with DHMEQ (5 or 10 μg/ml) for the designated time period. The number of surviving cells was evaluated by crystal violet assay and presented as a relative value to the control (0 μg/ml, 0 h). Each value represents the mean derived from at least three individual experiments; error bars, SD. $^{**}P < 0.005$. (C) HS-SY-II and SYO-1 cells were incubated with or without DHMEQ (10 μg/ml) for 48 h and the cells underwent apoptosis (b and d, arrowheads) were detected by a TUNEL-based assay. The panels show merged images of nuclei stained with TOTO3 (shown here in red) and 3′-OH DNA termini stained with fluorescein (in green). Bar, 20 μm. (D) Immunoblot analysis of cIAP-1 (IAP), cleaved caspase-3 (Cas-3) and actin. *, non-specific band. (E) HS-SY-II and SYO were transfected with NF-κB luciferase reporter vector and incubated with various concentrations of DHMEQ for 3 h. Each represents the mean derived from at least three individual experiments; error bars, SD.

luciferase assay using an NF-κB luciferase reporter gene. Cells grown to approximately 80% confluency were transfected with a vector harboring the NF-κB binding site upstream of luciferase and incubated for 18–24 h. The cells were then washed with fresh medium and further incubated with DHMEQ at various concentrations (0–20 μg/ml). Significant suppression of NF-κB activity by DHMEQ was observed in both HS-SY-II and SYO-1 at a concentration of 2.5 μg/ml and higher (Fig. 1E). These data indicate that the suppression of constitutive NF-κB activity leads to apoptosis in these synovial sarcoma-derived cell lines.

3.2. DHMEQ inhibits nuclear translocation of NF-κB in synovial sarcoma cell lines

It has been previously shown that DHMEQ suppresses NF-κB activity by inhibiting the translocation of NF-κB complex into the nucleus [11]. We next examined if a similar inhibitory mechanism was also present in synovial sarcoma by monitoring the subcellular localization of p65 NF-κB subunit. Cells plated on glass coverslips were incubated with TNFα and/or DHMEQ alone or in combination for 3 h. After incubation, the cells were fixed with PFA and the localization of p65 NF-κB subunit was detected by immunofluorescence. In a non-stimulated state (i.e., without DHMEQ or TNFα) high amounts of the p65 NF-κB subunit were found in the cytoplasm of both HS-SY-II and SYO-1 cells (Fig. 2A, C, panels a and e). Since fluorescent staining was not sensitive enough to detect the further decrease of p65 in the nucleus as shown by the comparable staining pattern between the control and that of DHMEQ-treated cells (Fig. 2A, B, panels b and f), we next used TNFα to upregulate NF-κB nuclear translocation. Incubation with TNFα highly induced the accumulation of p65 NF-κB subunit in the nucleus (Fig. 2A, C, panels c and g), while addition of DHMEQ inhibited the nuclear translocation of NF-κB to a comparable level to that of untreated cells (Fig. 2A, C, panels d and h). Consistently, DHMEQ completely attenuated NF-κB transcriptional activity driven by

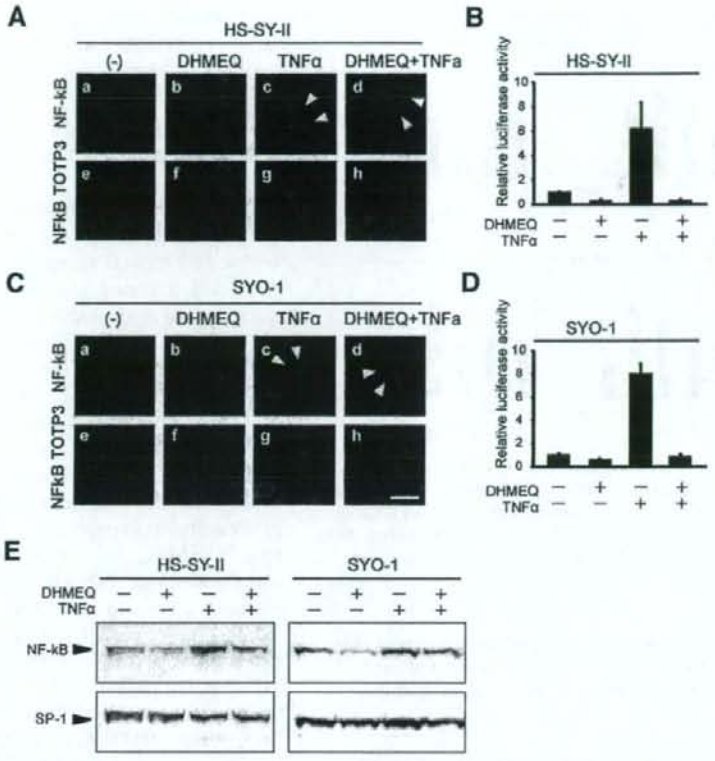

Fig. 2. DHMEQ downregulates NF-κB activity by suppressing nuclear translocation of NF-κB. (A) and (C) Immunostaining of p65. HS-SY-II (A) and SYO-1 (C) were treated with DHMEQ (10 μg/ml) or TNFα (10 ng/ml) alone or in combination for 3 h. After incubation, cells were immunostained with anti-p65 antibody and nuclei were counterstained with TOTO3 (e and f, merged images). Nuclear accumulation of p65 (c, arrowheads) induced by TNFα was significantly blocked by co-incubation of DHMEQ (d, arrowheads). Bar, 20 μm. HS-SY-II (B) or SYO-1 (D) transiently expressing NF-κB luciferase reporter vector were incubated with DHMEQ (D, 10 μg/ml) and/or TNFα (T, 10 ng/ml) for 3 h. Luciferase activity was measured as described in Section 2. Each value represents the mean derived from at least three individual experiments; error bars, SD. (E) Immunoblot analysis of NF-κB and SP-1. HS-SY-II (left panels) and SYO-1 (right panels) were treated with DHMEQ (10 μg/ml) or TNFα (10 ng/ml) alone or in combination for 3 h. Nuclear extracts from cells were subjected to analysis. Immunoblot of a transcription factor SP-1 serves as an internal control.

TNFα (Fig. 2B and D). These results suggest that DHMEQ suppressed NF-κB activity by inhibiting the nuclear translocation of NF-κB also in synovial sarcoma cells. Western blotting analysis using the nuclear extract from HS-SY-II and SYO-1 cells revealed essentially the same results (Fig. 2E). DHMEQ reduced both basal p65 levels and TNFα-induced accumulation of p65 in the nucleus in both tumor cell lines.

3.3. Cytotoxic agents, VCR and DXR, upregulate NF-κB activity

It has previously been shown that various chemotherapy agents, such as VCR, DXR, cisplatin and etoposide, activate the NF-κB signaling pathway in tumor cell lines, and thereby make the tumor cells less sensitive to chemotherapy [4]. We next examined if the activation of NF-κB can also be observed in synovial sarcoma cell lines using DXR and VCR. DXR is an anthracycline antibiotic which disrupts DNA replication and transcription, and is one of the most potent chemotherapeutic agents used in the treatment of synovial sarcoma [17,18]. VCR is a mitotic inhibitor that is often used in the treatment of various malignancies, including leukemia, malignant lymphoma, neuroblastoma and soft tissue sarcomas [19]. Cell lines transfected with NF-κB reporter vector were incubated in the presence of various concentrations (0–10 μM) of VCR or DXR for 3 h and luciferase activity at the end of incubation was measured by a luminometer. As shown in Fig. 3A and B, both VCR and DXR upreg-

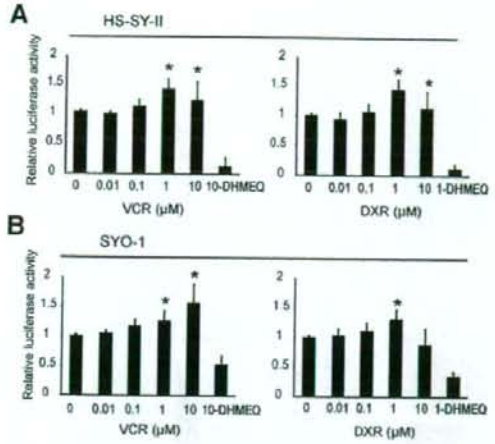

Fig. 3. DXR and VCR upregulate NF-κB activity in HS-SY-II and SYO. HS-SY-II (A) and SYO-1 (B) transfected with NF-κB luciferase reporter vector were incubated with or without DXR or VCR at various concentrations for 3 h. Each value represents the mean derived from at least three individual experiments; error bars, SD. $^{*}P < 0.05$.

ulated NF-κB activity in a dose-dependent manner in HS-SY-II and SYO-1 cell lines. Moreover, the upregulation of NF-κB activity with these agents was completely inhibited by DHMEQ.

3.4. DHMEQ sensitizes the growth inhibitory effects of DXR and VCR

Since the above results suggest that the upregulation of NF-κB activity by DXR and VCR could render the cells less prone to apoptosis, we next examined if a combination DHMEQ with these agents has additive or synergistic effects on the induction of apoptosis. HS-SY-II and SYO-1 cells grown to confluence were incubated with DXR or VCR in the presence or absence of DHMEQ for 24 h and the number of variable cells at the end of incubation was evaluated by crystal violet staining. As shown in Fig. 4A and B, in all cases, DHMEQ showed additive effects when used in combination with DXR or VCR, suggesting that DHMEQ sensitized these cell lines to DXR and VCR. We next asked whether the downregulation of IAP levels was also involved in this effect. The cells were incubated with DXR or VCR in the presence or absence of DHMEQ for 24 h and cell lysates were subjected to Western blotting analysis. Both DXR and VCR had reduced expression levels of cIAP-1 compared to the non-treated control (data not shown). As shown in Fig. 4C, the addition of DHMEQ to VCR- or DXR-treated cells further reduced the expression levels of cIAP-1. The degree of reduction in cIAP-1 levels was nearly in reverse proportion to that of the increase in the cytotoxic effect induced by DHMEQ. These results indicate that the addictive effect of DHMEQ and chemotherapeutic agents has, at least in part, derived from downregulation of the expression levels of cIAP-1.

4. Discussion

Despite extensive studies on the pathogenesis of synovial sarcoma in the past decade, the clinical course of synovial sarcoma remains unsatisfactory, providing a strong incentive to improve the treatment regime against this tumor. In the current study we explored the possible involvement of NF-κB in the pathoetiology of synovial sarcoma by using a novel NF-κB inhibitor, DHMEQ. Currently, various NF-κB inhibitors target the NF-κB pathway at different points in the signaling cascade, including anti-oxidants, proteosome inhibitors, peptides, small molecules and dominant-negative or constitutively active polypeptides [20]. Among these inhibitors, DHMEQ is unique in that it inhibits the translocation of NF-κB proteins into the nucleus without changing the amount of IκB proteins [12,21]. It was initially used in an *in vivo* model of rheumatoid arthritis where activation of the NF-κB signaling pathway by TNFα plays a pivotal role in its pathogenesis [22]. Subsequently, it has been shown that DHMEQ could attenuate renal inflammation in an *in vivo* model [23], inhibit osteoclastogenesis induced by RANKL and suppress the growth of various cancer and leukemia cells in both in vitro and *in vivo* models [13].

NF-κB is known to inhibit apoptosis through the induction of anti-apoptotic proteins and suppression of pro-apoptotic genes. Constitutive activation of NF-κB is often found in various malignancies, including breast cancer, prostate cancer, multiple myeloma, and Hodgkin lymphoma. Consistent with these observations, previous studies have shown that inhibition of NF-κB activity led to growth arrest and apoptosis in the above tumor cells [13]. The effects of DHMEQ on the expression levels of anti-apoptotic genes have not been clarified yet. A couple of reports have shown that DHMEQ reduced the expression levels of Bcl family genes [24,25], while others have suggested the possible involvement of IAP, but not of Bcl, family genes [21,26–28]. The current study also indicates cIAP-1 as one of the target genes for DHMEQ; however, the effects of DHMEQ on these anti-apoptotic molecules may differ among tumors. Since the inhibition of growth and induction of apoptosis by DHMEQ

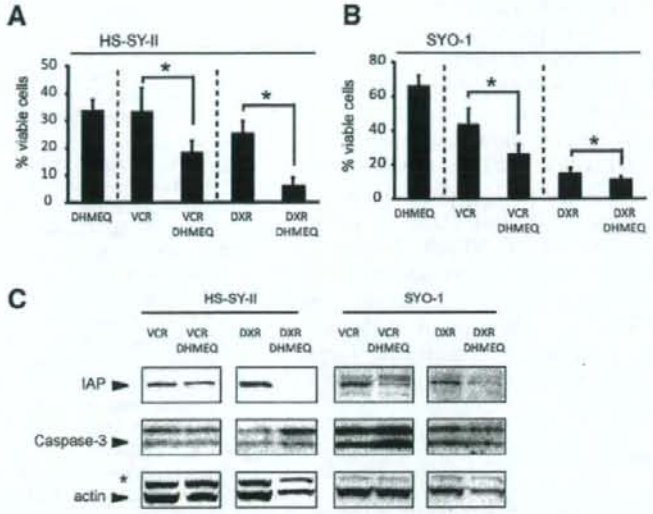

Fig. 4. Additive effects of the combination of DHMEQ and DXR/VCR on growth inhibition. HS-SY-II (A) or SYO-1 (B) grown to confluence was incubated in the presence of a cytotoxic drug (DXR, 1 μM or VCR, 1 μM) alone or with DHMEQ (10 μg/ml) for 48 h. After incubation, the number of viable cells was evaluated by crystal violet assay. Each value represents the mean derived from at least three individual experiments; error bars, SD. $^{*}P < 0.05$. (C) Immunoblot analysis of cIAP-1 (IAP), cleaved caspase-3 and actin. Synovial sarcoma cells were treated with chemotherapeutic agents alone or in combination with DHMEQ for 24 h. Whole cell extracts were collected and subjected to analysis.

was seen in both HS-SY-II and SYO-1 cells at comparable or even lower levels (5–10 μg/ml) than in previous studies, we assumed that NF-κB signaling would also be constitutively activated in synovial sarcoma cell lines. Western blot analysis revealed that a certain amount of NF-κB was present in the nucleus of both HS-SY-II and SYO-1 cells in an unstimulated state, and that DHMEQ reduced the accumulation of NF-κB in the nucleus, indicating that NF-κB is constitutively activated to a certain level in these cell lines. On the other hand, accumulation of the p65 subunit in the nucleus, when visualized by fluorescence staining, was relatively low at the unstimulated basal level, and there was little difference in the staining pattern between non-treated cells and cells treated with DHMEQ. This discrepancy may be due to differences in the sensitivity of fluorescence and immunoblotting, or it is also possible that DHMEQ inhibits NF-κB activity by other mechanisms. In fact, it was recently shown that DHMEQ inhibited NF-κB activity in human Burkitt lymphoma, HS0-Sultan and Daudi cells, without changing the localization of NF-κB proteins [27].

It is also noteworthy that NF-κB in HS-SY-II and SYO-1 cells was upregulated in response to chemotherapeutic agents, such as DXR and VCR, as has been previously described in other tumor cells [4]. Since the activation of NF-κB by anti-cancer drugs in tumor cells could make them less prone to apoptosis and more resistant to chemotherapy, inhibition of NF-κB activation could potentially lead to the reversal of chemoresistance [4,5,13]. This would further validate NF-κB as a rational molecular target for the treatment of synovial sarcoma. Possible mechanisms behind NF-κB activation by chemotherapeutic agents have been extensively studied. In the case of DXR and VCR, it has been shown that the phosphorylation and degradation of IκB through the increased activity of protein kinase C is involved in NF-κB activation [29]. The observation that DXR- or VCR-induced NF-κB activity can be inhibited by DHMEQ may also have significant clinical implications. Since successful chemotherapy is crucial to increase the survival rate of patients with synovial sarcoma, the reversal of chemoresistance has potential to significantly improve the clinical outcome; however, given the wide range of functions of NF-κB, it is crucial to clarify whether the inhibition of NF-κB activity suppresses immunity against tumors and infections since patients are often treated with drugs which

have myelosuppressive side effects. Moreover, since NF-κB activation can be pro-apoptic under certain situations depending on the stimulus, it will be mandatory to evaluate the possible adverse effects and specificity against tumor cells.

In summary, our results show that a novel NF-κB inhibitor, DHMEQ, inhibits growth and induces apoptosis in synovial sarcoma cells. To our knowledge, this is the first study to report the possible involvement of NF-κB activity in the pathology of synovial sarcoma and to show the efficacy of DHMEQ against synovial sarcoma cells. The observations in the current study suggest that NF-κB is an attractive molecular target in developing a more potent therapeutic regime against synovial sarcoma.

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Association of *STAT4* With Susceptibility to Rheumatoid Arthritis and Systemic Lupus Erythematosus in the Japanese Population

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Objective. ***STAT4*** **encodes a transcriptional factor that transmits signals induced by several key cytokines, and it might be a key molecule in the development of autoimmune diseases. Recently, a *STAT4* haplotype was reported to be associated with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) in Caucasian populations. This was replicated in a Korean RA population. Interestingly, the degree of risk of RA susceptibility with the *STAT4* haplotype was similar in the Caucasian and Korean populations. The present study was undertaken to investigate the effect of *STAT4* on susceptibility to RA and SLE in the Japanese.**

Methods. **We performed an association study using 3 independent Japanese RA case–control populations (total 3,567 cases and 2,199 controls) and 3 independent Japanese SLE populations (total 591 cases). All samples were genotyped using the TaqMan fluorogenic 5′ nuclease assay for single-nucleotide polymorphism (SNP) rs7574865, which tags the susceptibility haplotype. The association of the SNP with disease susceptibility in each case–control study was calculated using Fisher's exact test, and the results were combined, using the Mantel-Haenszel method, to obtain combined odds ratios (ORs).**

Results. **We observed a significant association of the *STAT4* polymorphism with susceptibility to both RA and SLE. The combined ORs for RA and SLE, respectively, were 1.27 ($P = 8.4 \times 10^{-9}$) and 1.61 ($P = 2.1 \times 10^{-11}$) for allele frequency distribution; these ORs were quite similar to those previously observed in the Caucasian population.**

Conclusion. **We conclude that *STAT4* is associated with RA and SLE in the Japanese. Our results indicate that *STAT4* is a common genetic risk factor for autoimmune diseases, with similar strength across major racial groups.**

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Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are chronic inflammatory autoimmune diseases characterized by pathologic infiltration of lymphocytes in target organs. Although the pathogenesis of these diseases remains unclear, dysregulated lymphocyte activation via the breakdown of self tolerance is believed to be implicated in their pathogenesis, and multiple genetic and environmental factors are important in the development of these diseases.

Recently, Amos et al conducted a genome-wide linkage scan using >5,700 single-nucleotide polymorphisms (SNPs) in 642 Caucasian families with affected sibling pairs; they found the best evidence of linkage at chromosomes 2q33 (1). Following the linkage analysis, Remmers et al performed a large case–control study of 13 selected candidate genes within the linkage region and found an association between a common haplotype located in the third intron of *STAT4* and susceptibility to RA and SLE (2). The association was replicated in several independent Caucasian RA and SLE populations, and also in a Korean RA population (2,3).

STAT4 encodes signal transducer and activator of transcription 4, the STAT protein family member that is uniquely activated by interleukin-12 (IL-12) through its receptor, which has an essential downstream role in Th1 cell differentiation and proliferation (4). In addition, it has been reported that STAT-4 is necessary for the development of Th17 cells (IL-17–producing CD4+ T cells) (5). Since Th1 cells and Th17 cells play an important role in chronic inflammatory disorders and since STAT-4 is considered to be a key molecule in both the Th1 and Th17 lineages, STAT-4 may play a crucial role in the development of autoimmune diseases such as RA and SLE.

Genetic association between HLA–DRB1 and RA susceptibility has been well established, and several other risk genes for RA outside the HLA region have been identified. However, while DRB1 has been repeatedly shown to be an RA risk locus in Caucasian and Asian populations, the other reported RA risk genes, such as *PTPN22*, *PADI4*, and *FCRL3*, have been difficult to replicate in other ethnic populations aside from the original populations first reported (6). These conflicting results suggest that the genetic background of the disease may vary among ethnic groups.

Interestingly, the degree of risk of RA susceptibility observed with the *STAT4* haplotype was found to be similar in the Caucasian and Korean populations (2,3). This finding indicates that the risk haplotype for RA susceptibility might be common across major racial groups. In the present study, we investigated the association of *STAT4* with RA susceptibility using large series of Japanese RA cohorts. We also tried to evaluate whether the gene is associated with RA outcome measures in a Japanese RA cohort. In addition, we tested the association between the gene and susceptibility to SLE in the Japanese population. This study is the first to investigate the association of *STAT4* with SLE in an Asian population.

PATIENTS AND METHODS

Subjects and disease criteria. All patients with RA fulfilled the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 revised criteria for the disease (7). All patients with SLE met the ACR 1982 revised criteria for the disease (8).

DNA samples were obtained from subjects in 3 RA case–control series (Table 1). DNA from the case subjects in the Tokyo Women's Medical University (TWMU) Institute of Rheumatology RA cohort (IORRA) case–control series was obtained from the IORRA DNA collection. The IORRA is an observational RA cohort with an enrollment of nearly 5,000 Japanese RA patients, and DNA samples were collected from 1,504 of these patients (mean age 59.3 years, 84% female, 88% rheumatoid factor [RF] positive) (9). This DNA collection was also used to analyze the effect of the single-nucleotide polymorphism (SNP) (see below) on RA outcome measures. Demographic, clinical, and treatment information on IORRA patients as of the spring of 2003 was obtained from the IORRA database, which includes the Disease Activity Score in 28 joints (DAS28) (10) and the Japanese version of the Health Assessment Questionnaire (J-HAQ) (11). Radiographs of the hands and feet of the IORRA patients, obtained when the duration of disease was 5 years, were reviewed retrospectively, and radiographic joint damage was assessed by a single skilled reader, using the modified Sharp/van der Heijde score (SHS) (12). The SHS includes a count of erosions and joint space narrowing in the hands and feet and has a range of 0 (no damage) to 448 (highest damage). DNA samples from popu-

Table 1. Case–control series for the studies of rheumatoid arthritis and systemic lupus erythematosus

Series	No. of patients	No. of controls
Rheumatoid arthritis		
IORRA	1,504	752
RIKEN	1,113	940
Tokushima	950	507
Systemic lupus erythematosus		
TWMU	238	752*
RIKEN	188	940†
Tokushima/Fukuoka	165	212

* Genotype information was obtained from the controls in the Tokyo Women's Medical University (TWMU) Institute of Rheumatology Rheumatoid Arthritis cohort (IORRA) rheumatoid arthritis series.
† Genotype information was obtained from the controls in the Institute of Physical and Chemical Research (RIKEN) series.

lation controls were obtained from the Pharma SNP consortium (http://www.jpma.or.jp/psc/index.html).

DNA from the case subjects in the Institute of Physical and Chemical Research (RIKEN) RA case–control series (mean age 60.4 years, 82% female, 70% RF positive) was obtained from the BioBank Japan Project DNA collection. As part of the BioBank Japan Project, DNA and serum samples along with clinical data have been collected from 300,000 patients with 47 diseases, including RA (13). Sixty-six hospitals affiliated with 12 institutions are participating in the project. Population-based control subjects were recruited through the Rotary Club of Osaka-Midosuji District 2660 Rotary International in Japan.

Patients and controls in the Tokushima RA case–control series were recruited through the orthopedics clinic at University of Tokushima Hospital, its community affiliates, and the rheumatology clinic at Tokushima Kensei Hospital (Tokushima, Japan) (14). The mean age of the patients was 61.8 years, and 79% were female.

Cases with SLE were also obtained from 3 sources (Table 1). TWMU patients were recruited from Institute of Rheumatology and Aoyama Hospital, TWMU. RIKEN patients were recruited through the Specified Disease Treatment Research Program of the Japanese Ministry of Health, Labor, and Welfare. Several medical institutions nationwide are participating in the program. These 2 series included only cases; control genotype information was obtained from the RA case–control series in the IORRA and RIKEN, respectively. Patients and control subjects in the Tokushima/Fukuoka lupus case–control series were recruited from Kyushu University Hospital (Fukuoka, Japan) (15).

The ethics committee of each institution (TWMU, RIKEN, and University of Tokushima) granted approval for the study, and each individual subject signed an informed consent form after receiving a verbal explanation of the study.

SNP genotyping. A polymorphism located within intron 3 of *STAT4*, rs7574865, which tags the susceptibility haplotype, was selected for this study because it exhibited the best evidence for association in the primary study (2) and was one of the SNPs most significantly associated with RA susceptibility in the Korean replication study (3). This SNP has been considered to be in strong linkage equilibrium with a putative functional variant. Genotyping was performed using the TaqMan fluorogenic 5′ nuclease assay, according to the instructions of the manufacturer (Applied Biosystems, Tokyo, Japan). All polymerase chain reactions were performed using GeneAmp PCR System 9700 (Applied Biosystems), and end point fluorescence readings were performed with an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems).

Statistical analysis. Allele frequencies of SNP rs7574865 in each case–control series were estimated by the allele counting method. Chi-square testing was used to identify significant departure from Hardy-Weinberg equilibrium.

Association of the SNP with susceptibility to RA or SLE in each study was estimated by Fisher's exact test; we compared the allelic effect of T (suspected risk allele) with G (common allele), and the genotypic effect of the homozygous genotypes with other genotypes. After assessing heterogeneity among the studies as determined based on Woolf's method, the Mantel-Haenszel test was used to evaluate combined odds ratios (ORs) and 95% confidence intervals (95% CIs), demonstrating the population-wide impact of the polymorphism on disease susceptibility.

Differences in patient characteristics among IORRA subjects with different rs7574865 genotypes were assessed by Kruskal-Wallis test or Fisher's exact test. The allelic effect of rs7574865 on the SHS was analyzed by linear regression analysis.

All statistical tests were implemented using the R software package, version 2.6.0 (http://www.r-project.org/).

RESULTS

On average, we achieved a genotyping success rate of 98.9%, with call rates of >98.2% for each case–control series. The genotype concordance rate was 100% as assessed by random retyping across different plates. Genotype distributions for SNP rs7574865 were in Hardy-Weinberg equilibrium in each case–control series.

Association of *STAT4* polymorphism with RA. The data summarized in Table 2 show the allele fre-

Table 2. Association of single-nucleotide polymorphism rs7574865 with RA in Japanese subjects*

Series, subjects	Genotype					Allele		GG vs. others		TT vs. others	
	GG	GT	TT	Total	MAF	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
IORRA											
RA	588	694	199	1,481	0.37	1.29 (1.13–1.48)	1.7×10^{-4}	1.38 (1.15–1.66)	3.9×10^{-4}	1.41 (1.05–1.89)	0.020
Controls	355	316	74	745	0.31						
RIKEN											
RA	447	502	160	1,109	0.37	1.31 (1.15–1.50)	4.8×10^{-5}	1.38 (1.16–1.66)	3.0×10^{-4}	1.48 (1.12–1.96)	0.0048
Controls	453	389	96	938	0.31						
Tokushima											
RA	365	448	128	941	0.37	1.17 (0.99–1.38)	0.056	1.20 (0.96–1.50)	0.11	1.30 (0.92–1.86)	0.13
Controls	216	230	54	500	0.34						
Combined						1.27 (1.17–1.37)	8.4×10^{-9}	1.34 (1.20–1.49)	1.9×10^{-7}	1.41 (1.19–1.67)	8.5×10^{-5}

* RA = rheumatoid arthritis; MAF = minor allele frequency; OR = odds ratio; 95% CI = 95% confidence interval (see Table 1 for other definitions).

Table 3. Association of single-nucleotide polymorphism rs7574865 with SLE in Japanese subjects*

Series, subjects	Genotype					Allele		GG vs. others		TT vs. others	
	GG	GT	TT	Total	MAF	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
TWMU											
SLE	76	103	48	227	0.44	1.73 (1.38–2.15)	8.3×10^{-7}	1.81 (1.31–2.50)	1.8×10^{-4}	2.43 (1.59–3.68)	2.9×10^{-5}
Controls	355	316	74	745	0.31						
RIKEN											
SLE	70	85	28	183	0.39	1.40 (1.10–1.77)	0.0059	1.51 (1.08–2.12)	0.015	1.58 (0.97–2.53)	0.053
Controls	453	389	96	938	0.31						
Tokushima/Fukuoka											
SLE	51	83	31	165	0.44	1.79 (1.31–2.45)	1.4×10^{-4}	2.07 (1.33–3.25)	0.0010	2.34 (1.22–4.59)	0.0059
Controls	102	91	19	212	0.30						
Combined						1.61 (1.40–1.85)	2.1×10^{-11}	1.74 (1.43–2.12)	4.9×10^{-8}	2.08 (1.59–2.72)	8.5×10^{-8}

* SLE = systemic lupus erythematosus; MAF = minor allele frequency; OR = odds ratio; 95% CI = 95% confidence interval (see Table 1 for other definitions).

quency and genotype distribution in RA patients and controls in each case–control series. We observed a significant difference in allele frequency and genotype distribution of the *STAT4* polymorphism between RA patients and controls in the IORRA and the RIKEN cohorts, while no significant difference was found in the Tokushima series. When study-specific ORs were combined using the Mantel-Haenszel method, the differences in allele frequency and genotype distributions of the SNP between patients and controls were significant (combined OR 1.27 [95% CI 1.17–1.37, $P = 8.4 \times 10^{-9}$]–1.41 [95% CI 1.19–1.67, $P = 8.5 \times 10^{-5}$]). There was no significant heterogeneity among the studies, as assessed by Woolf's method ($P > 0.05$).

Association of *STAT4* polymorphism with SLE. Table 3 shows the genotype distribution and minor allele frequency in the 3 SLE series. As with RA, we found differences in the allele frequency and genotype distributions of SNP rs7574865 between SLE patients and controls; these were significant in all 3 case–control series. No significant evidence of heterogeneity among the studies was identified by Woolf's method ($P > 0.05$), and the combined OR for the polymorphism as calculated by Mantel-Haenszel testing was 1.61 (95% CI 1.40–1.85, $P = 2.1 \times 10^{-11}$). Combined ORs for the recessive trait and the dominant trait were 2.08 (95% CI 1.59–2.72) and 1.74 (95% CI 1.43–2.12), respectively.

Stratified analyses of clinical and laboratory variables in RA patients. Among 1,504 patients with available DNA samples, 1,335 participated in the IORRA clinical data collection in the spring of 2003, and information on their demographic, clinical, and treatment details as of that time could be obtained from the IORRA database (Table 4). Consistent with previous findings by Lee et al in a Korean population (3), there was no significant genotypic association with age at disease onset or sex. We also found no significant differences among the genotypes in age, disease duration, family history of RA, RF status, DAS28 score, or J-HAQ score. There was a trend toward an association of risk allele with elevated levels of inflammation markers and patient's assessment of global health, but these were not significant. Only glucocorticoid usage and glucocorticoid dosage were found to differ significantly among the genotypes, with the difference increasing in a stepwise manner according to the number of risk alleles (median dosage 0, 1, and 2.5 mg equivalent prednisolone, respectively, among patients with the GG, GT, and TT genotypes).

The SHS after 5 years of disease could be measured in 163 patients, of whom 160 were genotyped. Although a trend toward an effect of the risk allele on the SHS was observed, it was not significant ($P = 0.22$) (median score 40, 45, and 46, respectively, among patients with the GG, GT, and TT genotypes [n = 67, 79, and 14, respectively]).

We did not perform a stratified analysis on anti–cyclic citrullinated peptide antibody (anti-CCP) positivity, since anti-CCP data were not available on most of the patients from the IORRA DNA collection. However, Lee and colleagues suggested that, at least among Asians, the risk of RA susceptibility associated with the *STAT4* variant may not be restricted to the anti-CCP positive disease subset (3).

DISCUSSION

This study is the first to investigate the association of a *STAT4* polymorphism with genetic susceptibility to lupus in any Asian population, and susceptibility to

Table 4. Genotypic differences in clinical or laboratory variables in RA patients*

	Total	Genotype			P†
		GG	GT	TT	
No. (%) of patients	1,335	521 (40)	610 (46)	183 (14)	
Age, years	60 (53–68)	61 (53–68)	60 (53–68)	60 (52–66)	0.14
Female, no. (%)	1,125 (84)	441 (85)	507 (83)	157 (86)	0.63
Disease duration, years	10 (5–16)	10 (5–17)	10 (5–16)	10 (5–17)	0.71
Age at RA onset, years	48 (39–57)	49 (40–57)	49 (40–57)	48 (38–55)	0.18
Family history of RA, no. (%)	415 (32)	157 (31)	187 (31)	63 (35)	0.54
RF positive, no. (%)‡	1,195 (90)	468 (90)	543 (89)	164 (90)	0.92
RF titer, IU/ml‡	116 (48–282)	115.5 (49–296)	116 (49–278)	122 (46–283)	0.97
Treatment§					
NSAID, no. (%)	980 (73)	378 (73)	446 (73)	143 (78)	0.32
DMARD, no. (%)	1,228 (92)	478 (92)	560 (92)	171 (93)	0.78
Glucocorticoid, no. (%)	719 (54)	266 (51)	325 (53)	117 (64)	0.01
Prednisolone, mg	1 (0–4.9)	0 (0–4)	1 (0–5)	2.5 (0–5)	0.01
DAS28	3.6 (2.7–4.5)	3.5 (2.7–4.4)	3.6 (2.8–4.5)	3.8 (2.8–4.6)	0.39
TJC	1 (0–3)	1 (0–3)	1 (0–3)	1 (0–3)	1.00
SJC	1 (0–3)	1 (0–3)	1 (0–3.75)	1 (0–4)	0.25
Patient's global assessment by VAS, mm	27 (10–54)	24 (9–54)	27 (11–55)	33 (14–53)	0.12
ESR, mm/hour	28 (16–48)	27 (16–46)	29 (16–48)	31 (16–54)	0.51
CRP, mg/dl	0.7 (0.2–1.6)	0.6 (0.2–1.6)	0.7 (0.2–1.6)	0.95 (0.3–1.9)	0.08
J-HAQ	0.625 (0.125–1.375)	0.625 (0.125–1.375)	0.625 (0.125–1.25)	0.625 (0.125–1.375)	0.96

* Data on some variables were missing for a small number of patients (maximum 3.1%). Data on genotype were missing for 21 patients (1.6%); therefore, values in the individual columns under Genotype are for 1,314 patients (521 for GG, 610 for GT, 183 for TT). Except where indicated otherwise, values are the median (interquartile range). RA = rheumatoid arthritis; NSAID = nonsteroidal antiinflammatory drug; DMARD = disease-modifying antirheumatic drug; DAS28 = Disease Activity Score in 28 joints; TJC = tender joint count (28 joints); SJC = swollen joint count (28 joints); VAS = visual analog scale; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; J-HAQ = Japanese version of the Health Assessment Questionnaire.

† By Kruskal-Wallis test or Fisher's exact test.

‡ The highest rheumatoid factor (RF) value measured in the cohort project during 2000–2006 for each individual was used. Cutoff for positivity = 15.0 IU/ml.

§ Biologic agents were not available in Japan at this time (spring 2003). Glucocorticoid dosage was calculated as the prednisolone equivalent dosage in milligrams.

RA in a Japanese population. Although replication studies using other ethnic populations are essential for establishing any genetic association, results are often reported as negative in the other populations. One of the reasons for this is that the degree of genetic risk differs among ethnic groups.

Concerning RA genetics, many study groups worldwide have made great efforts to newly identify susceptibility genes and to replicate findings of other groups, particularly using Caucasian or Asian populations. However, findings for most susceptibility genes identified outside the HLA region have not been replicated in the populations different from the population used in the primary study. A typical example of this is an association between *PTPN22* and susceptibility to RA. A missense SNP in *PTPN22* known as R620W was discovered as a common genetic risk factor for several autoimmune diseases including RA in a Caucasian population, and the finding has been replicated in many Caucasian RA cohorts (16). However, the risk allele is extremely rare in Asians, and attempts to validate the association in Asian populations have been unsuccessful (17). In contrast, the association between *PADI4* and RA susceptibility is thought to be strong among Asian populations, and indeed, most replication studies in Asian populations have succeeded in validating this association. However, a meta-analysis of studies using Caucasian populations revealed the combined OR for the association to be as low as 1.1 (18), and as a result, replication studies in Caucasian populations have seldom validated the association.

Failure to replicate a genetic association in a different ethnic population from the population used in the primary study is often due to low statistical power. To avoid this problem, it is important to make the sample size as large as possible, as we did in the present multicenter Japanese case–control study. We collected 3,567 RA cases and 591 SLE cases to validate the association between *STAT4* and susceptibility to RA and lupus in the Japanese. Consistent with previous reports (2,3), we observed a significant association of the *STAT4* polymorphism with both RA and SLE susceptibility in