selenocysteine transfer RNA, which mediates the incorporation of selenocysteine into selenoproteins, such as glutathione peroxidase and thioredoxin reductase [119].

We recently characterized one cisplatin-inducible gene, the mitochondrial ribosomal protein S11 (*MRP S11*) gene [53], which is a component of the ribosomal small subunit and binds to 12S rRNA [120]. Although there is no evidence that MRP S11 is directly related to DNA damage signals, it has been shown that the apoptosis-related protein DAP3 is a component of the small subunit of the mitochondrial ribosome (mitoribosome) [121]. This suggests that the mitoribosome, with MRP S11 as one of the constituents, might be involved in the apoptotic pathway. The Staf binding site is located in the promoter region of the *MRP S11* gene. Functional analysis of the MRP S11 promoter showed that ZNF143 regulates both basal and cisplatin-inducible promoter activities. Expression of the *ZNF143* gene was upregulated, while *ZNF76*, which is another human homologue of Staf, was downregulated by cisplatin treatment in cancer cells. We also found that γ-irradiation and anti-cancer agents, such as etoposide and adriamycin, induced the expression of *ZNF143*. Furthermore, ZNF143 preferentially recognizes cisplatin-modified DNA, as is YB-1 [53]. It is important to investigate whether elevated expression of ZNF143 induces cisplatin resistance and to identify the molecules interacting with ZNF143.

Mitochondrial Transcription Factor A (mtTFA)

Mitochondria are the major site of ROS production in eukaryotic cells. The accumulation of ROS, which causes oxidative damage to the mitochondrial DNA (mtDNA), has been implicated in aging, cancer and various degenerative diseases [122]. mtDNA is more susceptible to oxidative damage than genomic DNA because of its lack of nucleosome structures, and mainly contains an oxidized form of the guanine base 8-oxo-7, 8-dihydroguanine (8-oxo-dG) [123]. Moreover, mtDNA might easily form cisplatin-adducts similar to 8-oxo-dG induced oxidative stress.

mtTFA is a member of the HMG-box protein family [124] that stimulates the transcription of mitochondrial genes by binding to the mitochondrial D-loop region. Nuclear HMG-box proteins, including HMG1/HMG2, are ubiquitous in higher eukaryotic cells and bind preferentially to cisplatin-damaged DNA [48, 49]. mtTFA is essential not only for mitochondrial gene expression but also for mtDNA maintenance and repair [125]. Additionally, increased apoptosis has been observed in *mtTFA*-knockout animals, suggesting that mtTFA plays an important role in apoptosis [125]. We have previously demonstrated that mtTFA binds preferentially to oxidatively damaged DNA containing 8-oxoguanine in addition to cisplatin modified DNA [126], whereas HMG1/HMG2 does not bind. Furthermore, the binding affinity of mtTFA for oxidized DNA is higher than that of mtMYH, which has DNA glycosylase activity and protects mtDNA from the mutagenic effects of oxidized DNA.

We examined the number of cisplatin-targeted sequences in mtDNA from databases (NCBI; National Center for Biotechnology Information, and UCSC Genome Browser) and found that cisplatin-targeted sequences, such as G-stretch sequences, are more numerous in humans and gorillas than in rodents, frogs and flies. Interestingly, G-stretch sequences appear much more frequently in mtDNA than in nuclear DNA in humans (Table (3)). These observations suggest that mitochondria might be the main targets of cisplatin in human cancer cells. We previously showed that p53 physically interacts with mtTFA in mitochondria [127]. Binding of mtTFA to cisplatin-modified DNA was significantly enhanced by p53, similar to HMG1, whereas binding to oxidized DNA was inhibited. Thus, the interaction of p53 with mtTFA might contribute to cisplatin-induced apoptosis. The amount of mtTFA protein was also increased after cisplatin treatment [127]. Moreover, there is a Staf-binding site in the promoter region of the *mtTFA* gene, indicating that mtTFA is upregulated by the transcription factor ZNF143 during cisplatin treatment (data not shown). These findings suggest that mtTFA might act as a pivotal decision center in mechanisms of protection from cisplatin-induced apoptosis, and could become a potential target for cancer chemotherapy.

Other Transcription Factors

Activator protein-1 (AP-1) is a transcription factor that induces various genes involved in cell proliferation,

Table 3. Number of Cisplatin-Targeted DNA Sequences in Mitochondrial DNA

	GG	AG	GGG	GGGG	GGGGG	Total number of mtDNA	Accession number
Human	**2,202**	**2,233**	**703**	**250**	**85**		
(Expectation)	**2,071**	**2,071**	**518**	**129**	**32**	**16,565**	**AY255136**
(Nuclear DNA)	**1,919±319**	**2,292±101**	**501±77**	**118±25**	**35±14**		
Gorilla	2,137	2,185	667	232	77	16,364	NC_001645
Rat	1,696	2,041	440	113	36	16,300	NC_001665
Mouse	1,501	2,015	346	83	22	16,300	AJ512208
Xenopus	1,536	2,174	331	72	9	17,553	M10217
Drosophila	615	1,332	98	26	9	16,019	NC_001322

Expectation indicates the probable number of each DNA sequence in human mitochondrial DNA.

Nuclear DNA indicates the average of numbers of cisplatin-targeted DNA sequence in human nuclear DNA, which encodes each gene such as *Sp1* (NM_138473), *YB-1* (NM_004559), *ZNF143* (NM_003442) and *collagen type-I α2* (NM_000089) by way of example.

differentiation, tumor invasion and apoptosis [128]. A recent study has shown that an adenovirus expressing a dominant-negative form of AP-1, lacking DNA binding capability, is able to selectively inhibit cisplatin resistance [129]. Furthermore, downregulation of c-Jun and c-fos, which are components of the AP-1 transcription complex, by antisense oligonucleotides increases cisplatin sensitivity [130, 131]. c-Jun expression is also closely linked with cellular GSH content [130].

Nuclear factor-κB (NF-κB) is a transcription factor involved in the inflammatory and immune responses, which also functions as an anti-apoptotic molecule in TNF- and cancer therapy-induced apoptosis [132]. Moreover, it has been reported recently that NF-κB activation via inhibition of the ERK signaling pathway increases resistance to cisplatin in human cervical carcinoma cells [133].

Oct1, which is a member of the POU (Pit-Oct-Unc) homeodomain family, is ubiquitously expressed and plays a role in activating the transcription of various genes [134]. Recently, Oct1 was shown to be induced after cells are treated with UV irradiation and anti-cancer agents, including cisplatin, etoposide, camptothecine and TAS-103 [135, 72]. The V-ATPase c subunit gene, pH regulator, is overexpressed in cisplatin-resistant cell lines [70] and is induced by treatment with anti-cancer agents in an Oct1-dependent manner [72]. We previously showed that intracellular pH is significantly higher in cisplatin-resistant cell lines than in sensitive parental cell lines, and that *in vitro* cisplatin-DNA cross-link formation is markedly enhanced in low pH conditions [70]. Therefore, intracellular pH is involved in drug sensitivity and Oct1 might play a critical role in intracellular pH regulation. Furthermore, HMG2, which recognizes cisplatin-modified DNA, functionally interacts with the octamer transcription factors Oct1, Oct2 and Oct6 to enhance transcriptional activity [136]. Although there is no direct evidence to implicate Oct1 expression with resistance to cisplatin, these data indicate that Oct1 is potentially involved in drug resistance.

Sp1 is a member of the C2-H2 zinc-finger family and was one of the first transcription factors to be identified in mammalian cells. Sp1 binds to GC boxes within promoter regions and regulates various genes, including housekeeping genes and genes involved in cell growth [137]. It has been previously shown that Sp1 regulates nitric oxide (NO)-induced expression of the DNA-dependent protein-kinase catalytic subunit (DNA-PKcs), which is one of the key enzymes involved in the repair of double-stranded DNA breaks, to protect cells from DNA damaging agents such as X-ray radiation, adriamycin, bleomycin and cisplatin [138]. Interestingly, Sp1 and Oct1 also bind to the promoter of the human thioredoxin reductase 1 gene, which has antioxidant and redox regulatory functions that are involved in resistance to cisplatin [139].

Hypoxia, acidosis and a transient decrease in intracellular pH are common characteristics of solid tumors [68]. Tumor cells can survive under severely energy-deficient and acidic conditions, having developed several mechanisms to escape from cellular acidosis such as pH regulators [68]. One of the cellular pH regulators is the proton pump V-ATPase; its expression is thought to be regulated by Sp1 in addition to Oct1 [69, 72]. We have also shown that the DNA binding activity of Sp1 and its interaction with TBP are increased in low pH conditions [140]. Therefore, it is favorable for Sp1 to function well at low pH, enabling the cell to grow rapidly. A recent report from Wang *et al.* has shown that strong Sp1 expression is detectable in human gastric cancers and might be a significant predictor of survival [141]. Sp1 might thus be involved in cisplatin resistance and be a potentially promising molecular target for cancer chemotherapy.

SUMMARY

This review has focused on the transcription factors involved in the sensitivity of solid tumors to cisplatin. Transcription factors are DNA binding proteins, so it is possible that they are able to recognize DNA damage and participate in DNA repair action. Several transcription factors are activated by cisplatin treatment. We have previously reported various transcription factors—including YB-1, CTF2, ATF4, ZNF143 and mtTFA—that act as inducing genes involved in drug resistance and DNA repair. YB-1 and ZNF143 might directly participate in DNA repair reactions, because both transcription factors can preferentially recognize DNA damage. Furthermore, transcription factors with upregulated expression in cisplatin-resistant cells, such as YB-1, ATF4, ZNF143, Oct1 and Sp1, might also control the expression of the proteins that are involved in redox processes induced by cisplatin. We believe that transcription factors might represent a potential target for cancer chemotherapy. However, only nuclear hormone receptors are widely recognized as good targets for chemotherapy against hormone-dependent tumors. Molecular interactions with transcription factors induced by DNA damage are thought to be important for either survival or apoptosis, suggesting that these interactions might be also promising targets for cancer chemotherapy. Further studies to identify DNA damage-induced transcription factors and characterize functions of these factors might allow us to understand cisplatin sensitivity and to develop novel molecular-targeted drugs in future.

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ABBREVIATIONS

ASK1	=	Apoptosis signal-regulating kinase 1
ATF4	=	Activating transcription factor 4
ATR	=	Ataxia telangiectasia mutated and Rad3-related protein
CTF/NF-I	=	CTF/nuclear factor I
CTF2	=	CCAAT-binding transcription factor 2
ER	=	Endoplasmic reticulum
ERK	=	Extracellular signal-regulated kinase
GPX	=	Glutathione peroxidase
GSH	=	Glutathione
GST	=	Glutathione *S*-transferase

HMG = High-mobility group
JNK = c-Jun N-terminal kinase
MAPK = Mitogen-activated protein kinase
MDR1 = Multidrug resistance 1
MMR = Mismatch repair
MRP2 = Multidrug resistance-associated protein 2
mtDNA = Mitochondrial DNA
mtTFA = Mitochondrial transcription factor A
NER = Nucleotide-excision repair
NF-κB = Nuclear factor-κB
Nrf1 = Nuclear-factor erythroid 1
ROS = Reactive oxygen species
Staf = Selenocysteine tRNA gene-transcription activating factor
TBP = TATA-binding protein
TrxR = Thioredoxin reductase
UV = Ultraviolet
V-ATPase = Vacuolar H^+-ATPase
YB-1 = Y-box binding protein-1
ZNF143 = Zinc-finger factor 143

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PKCδ and MAPK mediate G_1 arrest induced by PMA in SKBR-3 breast cancer cells

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Abstract

The effects of activating endogenous protein kinase C (PKC) on cell proliferation and the cell cycle were investigated by treating the breast cancer cell line SKBR-3 with phorbol 12-myristate 13 acetate (PMA). This inhibited cell growth in a concentration-dependent manner, causing a marked arrest of cells in G_1. Pre-treatment with GF109203X completely blocked the antiproliferative effect of PMA, and pre-treatment with the PKCδ inhibitor rottlerin partially blocked it. Infecting SKBR-3 cells with an adenovirus vector containing wild-type PKCδ, WTPKCδAdV, had similar effects on PMA. Infecting the cells with a dominant-negative PKCδAdV construct blocked the growth inhibition induced by PMA. Downstream of PKC, PMA treatment inhibited extracellular signal-regulated kinase mitogen-activated protein kinase phosphorylation, up-regulated c-jun NH_2-terminal kinase phosphorylation, and inhibited retinoblastoma (Rb) phosphorylation. These results strongly implicated PKC (mainly PKCδ) in the G_1 arrest induced by PMA and suggested PKC as a target for breast cancer treatment.

Keywords: SKBR-3 breast cancer cells; G_1 arrest; Phorbol ester; PKC; MAPK

Phorbol esters, such as phorbol 12-myristate 13 acetate (PMA), cause proliferation, differentiation, malignant transformation, and death in many cells [1–3]. These effects are exerted through receptors, which include several protein kinase C (PKC) isozymes and novel non-kinase receptors (α- and β-chimaerins, and Ras-GRP). PKCs are phospholipid-dependent serine/threonine kinases [4] that are involved in regulating basic cell functions, such as proliferation and differentiation. To date, at least 11 isozymes of PKC have been identified [2,5–7] and have been divided into three subgroups. PCKα, -βI, -βII, and -γ are considered to be classical PKC (cPKC) isozymes, which are activated by calcium, diacylglycerol (DAG), and PMA. PKCδ, -ε, -η, and -θ are classified as novel PKC (nPKC) isozymes, which are also activated by DAG and PMA but are not calcium dependent. PKCζ and -λ/ι are atypical PKC (aPKC) isozymes, which are neither calcium dependent nor activated by DAG or PMA.

Some stimuli that activate cPKCs and nPKCs function by activating a G protein that then activates phospholipase C to release DAG from phosphatidylinositol-4, 5-bisphosphate (PIP_2), which is a trace component of the cell membrane; DAG then activates PKCs [7–9]. By contrast, phorbol esters are promoters of carcinogenesis that pass through the cell membrane and activate almost all PKC isozymes by directly binding them. As DAG is metabolized rapidly, it activates PKC

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PKCδ and MAPK mediate G_1 arrest induced by PMA in SKBR-3 breast cancer cells

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transiently. By contrast, phorbol esters have long half-lives, and so cause prolonged PKC activation. Several reports have described the biological effects of phorbol esters on cancer cells [10–15]. In the breast cancer cell line, MDA-MB-231, PMA induced the translocation of PKCα from the cytoplasm to the cell membrane, where it inhibited cell spreading and motility by negatively regulating the signaling pathway downstream of EGFR [16]. PMA has been reported to inhibit the growth of MCF7 cells by up-regulating PKCδ and p21 [17]. Another phorbol ester, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), not only acts as a tumor promoter, but also induces apoptosis in breast cancer cell lines by a mechanism that is thought to involve the up-regulation of p21 and Bax, but to be independent of p53 [18].

The distinct functions of different PKC isozymes are gradually being clarified [19–25]. For example, in MCF7 breast cancer cells, PKCη inhibited TNF-α-induced cell death by blocking caspase activation [8], and tamoxifen induced PKCε translocation to the cell membrane and inhibited growth [26]. The atypical PKCζ blocked the phosphorylation of Akt in breast cancer cells [27], while the over-expression of PKCα inhibited the estrogen receptor (ER) [28]. In breast cancer patients, changes in the expression and localization of PKCη [29] and down-regulation of PKCα [30] have been reported. Therefore, the effects of different PKC isozymes on the proliferation, differentiation, and death of cancer cells might also depend on the type of cell. By clarifying the role of PKCs in breast cancer cells, we expected these experiments to contribute to the treatment of breast cancer patients.

Members of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinase MAPK (ERK MAPK), c-jun NH_2-terminal kinase (JNK), and p38, are crucial regulators of the protein kinase cascades that transfer extracellular signals to the nucleus. ERK MAPK is activated by many growth factors, and this involvement in cell proliferation and differentiation has made it a focus for carcinogenesis research [31,32]. JNK and p38 are thought to play a role in stress responses, inflammatory reactions, and apoptosis [33,34].

Many reports suggest that PKC plays a role in MAPK activation. In PMA-treated lung cancer cells, two PKC isozymes, PKCα and PKCε, were shown to be involved in the activation of JNK [35]. In breast cancer cells, HER2 and PKCδ were shown to be involved in the activation of ERK MAPK by estrogen [36], and PKCα, -β, and -δ translocation preceded the acceleration of ERK MAPK phosphorylation and cell proliferation, induced by angiotensin II [37]. Thus, there appears to be a close relationship between PKC and MAPK; if this could be clarified in breast cancer cells, PKC could be established as a new target for breast cancer therapy.

This study aimed to investigate the effects of activating endogenous PKC with PMA on cell proliferation and the cell cycle in SKBR-3 breast cancer cells. We aimed to identify the PKC isozymes involved in cell proliferation using PKC inhibitors and recombinant adenovirus vectors expressing wild-type and dominant-negative PKCα and -δ genes. The signaling pathways downstream of PKC were investigated by looking at changes in ERK MAPK and JNK phosphorylation.

Materials and methods

Materials. PMA, GF109203X (bisindolylmaleimide I), Gö6976, and rottlerin were purchased from Alexis (San Diego, CA).

Cell culture. The SKBR-3 human breast cancer cell line was purchased from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in 5% CO_2.

Treatment with phorbol esters. SKBR-3 cells were treated with 1, 3 or 10 nM PMA in DMEM with 10% FBS for 1 h and then the medium was changed. This short treatment with low PMA concentrations would not down-regulate PKC. Cells were counted at 24, 48, 72, and 96 h after 0.05% trypsin treatment with a hemocytometer and cell-proliferation curves were prepared.

Flow cytometry. Cells were fixed with 70% ethanol, stained with 1 mg/ml propidium iodide, and the cell-cycle distribution was analyzed by flow cytometry (FACScan; Becton–Dickinson, CA).

Western blot analysis. SKBR-3 cells in sample buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.000125% bromophenol blue, and 5% β-mercaptoethanol) (10 μg protein) were separated by sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) and transferred to nitrocellulose membranes. Membranes were blocked for 24 h with phosphate-buffered saline (PBS) containing 5% milk and 0.1% Tween 20, and then incubated for 24 h with one of the following primary antibodies: anti-PKCα and anti-PKCδ (1:1,000; Transduction Laboratories, Lexington, KY); anti-phospho ERK MAPK, and anti-phospho JNK (1:1,000; Cell Signaling Technology, Beverly, MA); anti-p21 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA); anti-cyclin A (1:1000; Upstate Biotechnology, Lake Placid, NY); anti-cyclin D1 and anti-cyclin E (1:1000; PharMingen, San Diego, CA); anti-β-actin (1:1000, Sigma, Saint Louis, MO); or anti-Rb and anti-phospho Rb (Ser807/811, Ser780, and Ser795) (1:1000; Cell Signaling Technology, Beverly, MA). After washing three times with PBS containing 0.1% Tween 20, the membrane was incubated for 60 min with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1:3000; Bio-Rad, CA) and bands were detected by enhanced chemiluminescence (ECL; Western blotting detection system; Amersham Biosciences, Buckinghamshire, UK).

Wild-type PKC and dominant-negative PKC adenovirus. Wild-type PKCα adenovirus (WTPKCαAdV), dominant-negative PKCα adenovirus (DNPKCαAdV), wild-type PKCδ adenovirus (WTPKCδAdV), and dominant-negative PKCδ adenovirus (DNPKCδAdV) were obtained from Dr. M. Oba (Showa University, Tokyo) and Dr. T. Kuroki (Gifu University, Gifu), and have been described elsewhere [38,39]. AdVs were amplified in HEK 293 cells using standard techniques [40]. A LacZ-expressing adenovirus (LacZAdV) was used as a control [10,41]. For infection experiments, SKBR-3 cells were seeded into six-well plates, grown to a density of 60–70%, and infected for 14 h at multiplicities of infection (MOI) from 1 to 100 plaque-forming units/cell (pfu/cell). The medium was then changed and the cells were cultured for a further 24 h.

Statistical analysis. Statistical analysis was performed using Student's t test, with p values <0.05 considered to be statistically significant.

Results

Effect of PMA on SKBR-3 cell growth and cell cycle

When the PKC activator, PMA, was added to SKBR-3 cell cultures at 1, 3, and 10 nM for 1 h, it inhibited cell growth in a concentration-dependent manner (Fig. 1A). After 96 h, cell numbers were reduced to 42% at 3 nM PMA and 60% at 10 nM PMA of the control.

The effect of PMA on the cell cycle was analyzed by flow cytometry. While 56% of the cells in the control group were in the G_0/G_1 phase, 75% of the cells treated with 3 nM PMA, and 78% of those treated with 10 nM PMA, were in G_0/G_1 after 72 h (Fig. 1B). This marked increase in the number of cells in G_0/G_1 suggested that the role of PKC in growth arrest should be investigated further.

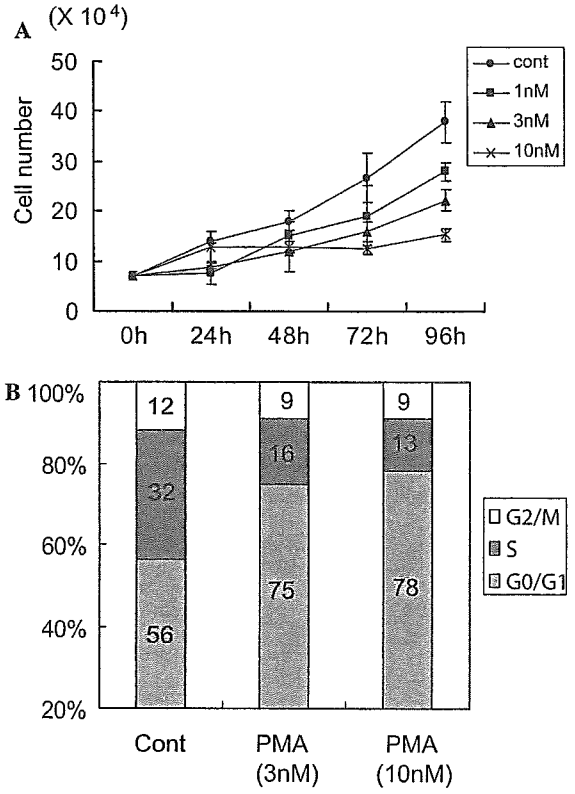

Fig. 1. Effect of PMA on SKBR-3 cell growth and cell cycle. (A) Different concentrations of PMA (1–10 nM) were added to SKBR-3 cells for 1 h in six-well plates, then washed with PBS and cultured with 10% FBS–DMEM. Cell number was counted after 24, 48, 72, and 96 h. Data are expressed as means ± standard error (SE) of three independent experiments. (B) PMA (3 or 10 nM) was added to cell cultures for 1 h, then washed with PBS and cultured with 10% FBS–DMEM. After 72 h the cells were harvested, fixed, stained with propidium iodide, and analyzed by flow cytometry. The results of a representative experiment are shown. Similar results were obtained in two additional experiments.

Effect of PMA on cell-cycle regulators

We focused our studies on the cell-cycle regulators that are essential for G_0/G_1 progression, looking at changes in the phosphorylation of Rb protein, and in the expression of p21 and cyclin proteins. SKBR-3 cells were treated with 3 nM PMA for 1 h to activate endogenous PKC and subjected to Western blotting after 0, 24, and 48 h. After 24 h, phosphorylation of Rb protein at Ser 807/811 and Ser 795 was inhibited, but there was no change in the phosphorylation at Ser 780 (Fig. 2). This provided clear evidence that phosphorylation of Rb protein, which has an important role in G_0/G_1 progression, was inhibited by PMA. By contrast, no p21 protein expression was detected, and no changes were seen in the expression of cyclins A, D1, and E (data not shown).

Effect of PKC inhibitors

We next used a PKC inhibitor (GF109203X), a specific PKCδ inhibitor (rottlerin), and a specific classical PKC inhibitor (Gö6976) to confirm that PKC mediated the effect of PMA on SKBR-3 cells. As SKBR-3 cells do not express PKCβ or PKCγ [42], Gö6976 acted as a specific PKCα inhibitor in these cells. Cells were pre-treated with the PKC inhibitors, 1 h before the addition of PMA (3 nM for 1 h), and the effects on cell proliferation were assessed after 24 h. GF109203X (5 μM) almost completely blocked the inhibition of proliferation induced by PMA alone, with a final cell count similar to that of the control without PMA. Rottlerin (0.5 μM) partially blocked the effect of PMA, with a significant difference ($p < 0.05$) between

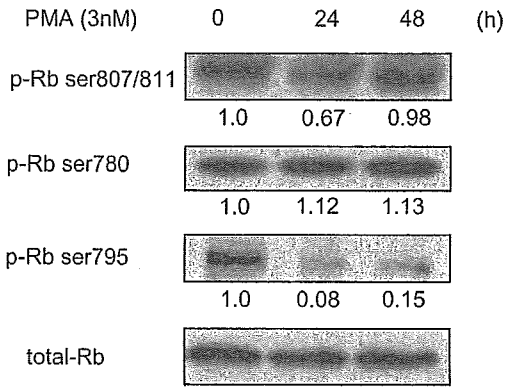

Fig. 2. PMA-induced changes in phosphorylation of Rb protein. Three nanomolar PMA was added to SKBR-3 cells for 1 h and Rb phosphorylation was assessed by Western blot analysis. Values indicate density of the band.

the cell numbers after PMA treatment alone, and after treatment with the PKCδ-specific inhibitor plus PMA. However, Gö6976 (0.2 μM) had no effect on the PMA-induced inhibition of proliferation, with no significant difference observed from the PMA treatment alone, demonstrating that PKCα did not have a role in regulating SKBR-3 cell proliferation. By contrast, the effects of the other PKC inhibitors showed that the inhibition of SKBR-3 cell proliferation by PMA did involve PKC activation, with PKCδ implicated as the isozyme involved (Fig. 3).

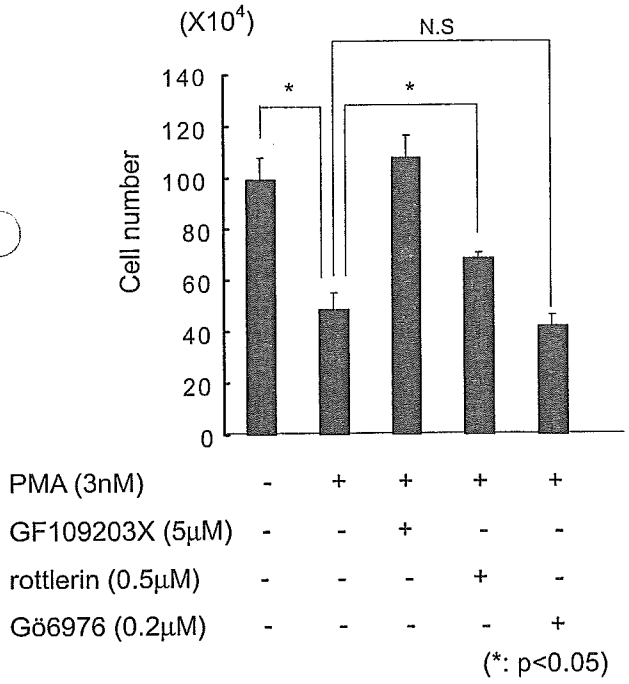

Fig. 3. Effects of PKC inhibitors. SKBR-3 cells were incubated with 5 μM GF109203X, 0.5 μM rottlerin or 0.2 μM Gö6976 1 h before the addition of PMA (3 nM for 1 h). Cell numbers were determined after 24 h. Data are expressed as means ± SE of three independent experiments (NS, not significant).

Effects of over-expressing wild-type PKCα and PKCδ in SKBR-3 cells using adenovirus vectors

To confirm that PKCδ, and not PKCα, has a role in the inhibition of SKBR-3 cell proliferation, we looked at the effects of over-expressing these proteins using adenoviral vectors. When SKBR-3 cells were infected with either WTPKCαAdV or WTPKCδAdV at MOIs between 1 and 100 pfu/cell, dose-dependent increases in the levels of PKCα and PKCδ were observed at 24 h after infection (data not shown). SKBR-3 cells were infected with 3 pfu/cell WTPKCαAdV, WTPKCδAdV or LacZAdV and the cells were counted after 24, 48, and 72 h. No differences were seen between the control and LacZAdV groups. By contrast, in the WTPKCδAdV-infected group, cell proliferation was strongly inhibited (Fig. 4B), but no inhibition was observed in the WTPKCαAdV-infected group (Fig. 4A).

The cell-cycle distribution of SKBR-3 cells infected with 3 pfu/cell WTPKCαAdV or WTPKCδAdV was analyzed by flow cytometry. After infection with WTPKCδAdV, the percentage of cells in G_0/G_1 increased to 84%, compared to 66% in the control cells, and 74% in the PMA-treated cells. By contrast, the percentage of cells in G_0/G_1 after infection with WTPKCαAdV was as low as 56%, which was lower than that in the control group (Fig. 4C). This increase in the percentage of SKBR-3 cells in the G_0/G_1 phase when PKCδ was over-expressed strongly supported the involvement of PKCδ in the induction of G_1 arrest by PMA. The results of over-expressing PKCαAdV excluded the involvement of PKCα.

Effects of dominant-negative PKC adenoviral vectors in SKBR-3 cells

SKBR-3 cells were infected with 3 pfu/cell of DNPKCαAdV, DNPKCδAdV or LacZAdV, and then treated with 3 nM PMA, to look at the effect of blocking

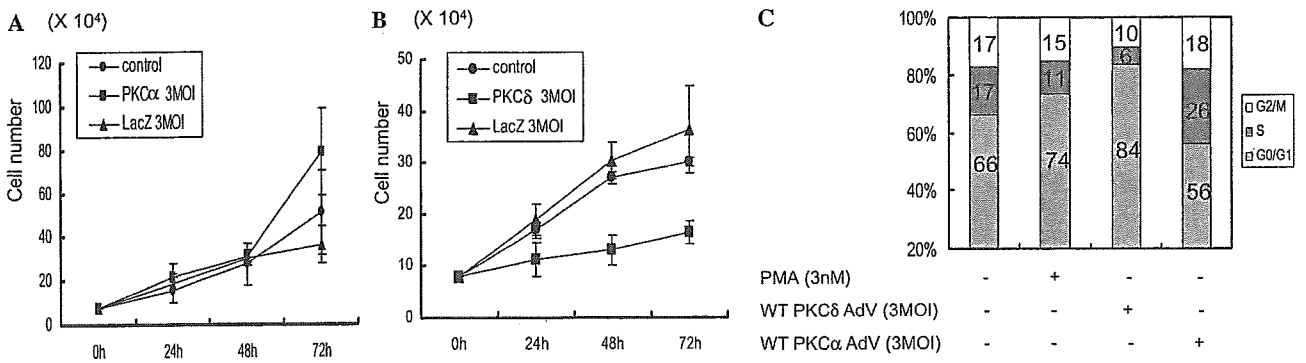

Fig. 4. The effects of WTPKCαAdV and WTPKCδAdV on cell numbers and cell-cycle distribution. (A) Cells were infected with WTPKCαAdV or LacZAdV, or (B) WTPKCδAdV or LacZAdV, at 3 pfu/cell for 14 h. Cell numbers were determined 24, 48, and 72 h later. Data are expressed as means ± SE of three independent experiments. (C) Cells were infected with PKCαAdV or PKCδAdV (MOI = 3 pfu/cell for 14 h), or treated with 3 nM PMA, harvested 72 h later, fixed, stained with propidium iodide, and analyzed by flow cytometry. The results of a representative experiment are shown. Similar results were obtained in two additional experiments.

PKC expression on the inhibition of proliferation by PMA. Cells infected with the control LacZAdV vector showed no difference from PMA-treated uninfected SKBR-3 cells. The proliferation of DNPKCδAdV-infected cells was significantly different ($p < 0.05$) from the cells infected with LacZAdV after PMA treatment, showing that the inhibition of cell proliferation by PMA was blocked by DNPKCδ. However, there was no significant difference in the proliferation of cells infected with DNPKCαAdV or LacZAdV after PMA treatment (Fig. 5). These results provided further evidence that PKCδ, rather than PKCα was involved in the PMA-induced G_1 arrest of SKBR-3 cells.

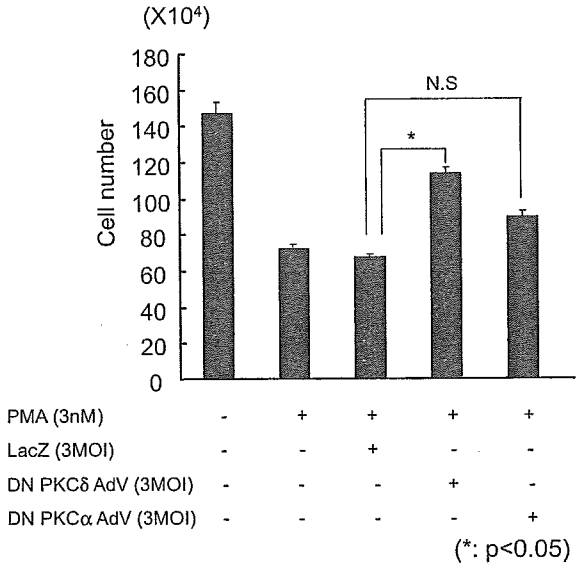

Fig. 5. The effects of DNPKCαAdV and DNPKCδAdV on cell numbers. Cells were infected with DNPKCαAdV, DNPKCδAdV or LacZAdV at 3 pfu/cell for 14 h, and then 3 nM PMA was added to cell cultures for 1 h. Cell numbers were determined 24 h later. Data are expressed as means ± SE of three independent experiments (NS, not significant).

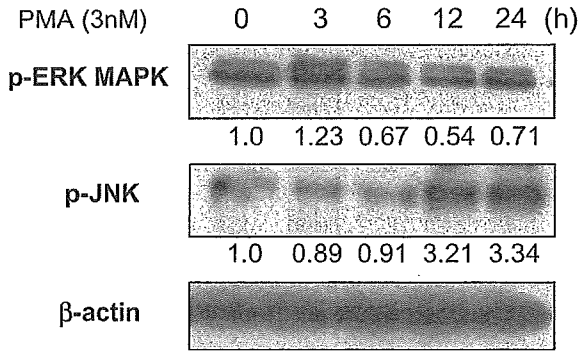

Fig. 6. The effect of PMA on ERK MAPK and JNK phosphorylation. SKBR-3 cells were treated with 3 nM PMA for 1 h and then used in Western blot analysis for phospho-ERK MAPK, phospho-JNK, and β-actin. Similar results were obtained in two additional experiments. Values indicate density of the band.

Effect of PMA on MAPK activation

In the next series of experiments, we used Western blot analysis to investigate the downstream signaling from PKC, looking at changes in the phosphorylation of ERK MAPK and JNK after PMA treatment. Phosphorylation of ERK MAPK was inhibited 6 h after exposure to PMA and remained low for at least 24 h. By contrast, JNK phosphorylation was increased by 12 h after PMA treatment (Fig. 6).

Discussion

The results presented here have clarified the role of PKC in the inhibition of SKBR-3 cell proliferation by PMA. The effects of PKC inhibitors demonstrated that the activation of PKC by PMA was essential to the induction of G_1 arrest, and that the PKC isozyme involved was PKCδ and not PKCα. Experiments that infected SKBR-3 cells with recombinant adenovirus vectors containing wild-type and dominant-negative PKCα and PKCδ supported these conclusions. The over-expression of PKCδ using WTPKCδAdV inhibited cell proliferation and induced G_1 arrest in a similar way to PMA treatment, whereas infection with WTPKCαAdV had no effect. Similarly, when SKBR-3 cells were infected with dominant-negative adenoviral vectors, the blockade of the PMA-induced inhibition of cell proliferation was significant with DNPKCδAdV but not with DNPKCαAdV. Although these results clearly demonstrated the involvement of PKCδ, the PKCδ-specific inhibitor, rottlerin, only partially inhibited the PMA-induced effects, suggesting that other isozymes could also be involved. We have recently shown that over-expression of other novel PKCs, PKCη, and PKCε, inhibited proliferation, although more weakly than PKCδ (unpublished data), suggesting that the effects of PMA were mediated by the activation of several nPKC isozymes.

There have been other reports that PKC regulated the cell cycle. Fima et al. [43] reported that PKCη promoted the proliferation of MCF7 cells, and that expression of PKCη up-regulated the G_1-associated cyclins D and E. Inhibition of PKCα and PKCθ has also been shown to arrest cells in G_1, and to be accompanied by the induction of p21 expression and the inhibition of Rb phosphorylation [44]. These reports, along with our data, suggest that PKC might be an important cell-cycle regulator. However, in this study, PMA did not induce changes in cyclin expression, suggesting that G_1 arrest in these cells might be caused by the reduction of cyclin dependent kinase (cdk) activity or some other unidentified factor.

Other studies with SKBR-3 cells have shown that 10 nM TPA treatment for 48–72 h induced apoptosis by

a mechanism involving the up-regulation of p21 and Bax [18], and that TPA-induced growth arrest, increased the expression of p21, and decreased the expression of c-Myc [45]. Both reports identified the importance of changes in p21 expression, but neither mentioned the role of PKC. We found that PMA induced G_1 arrest when used at a low concentration (3 nM) for a short period (1 h), but we saw no evidence of apoptosis (data not shown). We suggest that when cells are exposed to phorbol esters for long periods, activated PKC is gradually metabolized and disappears, and its activation is down-regulated. Therefore, it appears that phorbol esters have different biological effects under different experimental conditions, even when the same cell line is used, and that p21 is not involved in the inhibition of proliferation under our experimental conditions.

We also looked at the signaling pathways downstream of PKC after PMA treatment, and found that ERK MAPK phosphorylation was inhibited and JNK phosphorylation was increased. We therefore propose that the inhibition of SKBR-3 cell proliferation induced by PMA involves a pathway initiated by the activation of PKC, followed by the inhibition of ERK MAPK phosphorylation for 6 h and the promotion of JNK phosphorylation for 12 h, which then inhibits the phosphorylation of Rb proteins and causes G_1 arrest. Others have reported similar signaling relationships between PKC and JNK [46], and in MCF7 cells that were induced to secrete MMP9 by TPA, which promoted the activation of Ras, the phosphorylation of c-Raf, MEK1/2, and ERK1/2, and also involved the PKCδ Ras/ERK signaling pathway [47].

In conclusion, PKC (mainly PKCδ) causes G_1 arrest in the SKBR-3 breast cancer cell line by a mechanism involving a PKC-ERK MAPK-JNK-Rb protein signaling pathway, suggesting that PKC could be a target for breast cancer treatment.

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ATP-binding cassette superfamily transporter gene expression in human soft tissue sarcomas

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The phenomenon of multidrug resistance (MDR) in various malignant neoplasms has been reported as being caused by one or multiple expressions of ATP-binding cassette (ABC) superfamily protein, including P-glycoprotein/multidrug resistance (MDR) 1 and the MDR protein (MRP) family. However, their expression levels and distribution within soft tissue sarcomas remain controversial. In 86 cases of surgically resected soft tissue sarcoma, intrinsic mRNA levels of *MDR1*, *MRP1*, *MRP2* and *MRP3* were assessed using a quantitative reverse transcriptase-PCR (RT-PCR) method. Moreover, immunohistochemical protein expressions of P-glycoprotein (P-gp), MRP1, MRP2, MRP3 and p53 protein were evaluated in concordant paraffin-embedded material. The mRNA expression and immunohistochemical expression of ABC superfamily transporters were compared to clinicopathologic parameters and proliferative activities as evaluated by the MIB-1-labeling index (LI). Among the various histologic types, malignant peripheral nerve sheath tumor (MPNST) showed significantly high levels of *MDR1* ($p=0.017$) and *MRP3* ($p=0.0384$) mRNA expression, compared to the other tumor types. When the immunohistochemical method was compared to the RT-PCR technique to assess ABC transported expression at the protein and mRNA levels, a significantly close relationship was found between the 2 methods ($p<0.05$). P-gp expression was significantly correlated with large tumor size (≥5 cm, $p=0.041$) and high AJCC stage (stages III and IV) ($p=0.0365$). Furthermore, cases with nuclear expression of p53 revealed significantly higher levels of *MDR1* mRNA expression, compared to those with negative immunoreaction for p53 ($p=0.0328$). Our results suggest that MDR1/P-gp expression may have an important role to play in tumor progression in the cases of soft tissue sarcoma, and p53 may be one of the active regulators of the *MDR1* transcript. In addition, the high levels of both *MDR1* and *MRP3* mRNA expression in MPNST may help to explain the poor response of this tumor to anticancer-drugs.

Key words: Soft tissue sarcoma; quantitative RT-PCR; immunohistochemistry; ABC transporter; p53

"Soft tissue sarcomas" define a group of histologically and genetically diverse cancers that are relatively rare, accounting for less than 1% of all cancers.[1] Recent progress in the treatment of soft tissue sarcomas is due to improvements in pathological definition, staging, the use of radiotherapy as an adjunct to other modalities, the definition of doxorubicin and ifosfamide as active drugs and surgical advances in functional preservation. As for chemotherapy in cases of soft tissue sarcoma, the beneficial role of adjunctive chemotherapy remains controversial. An Italian group[2] suggested that high-dose epiribicin and ifosfamide are of benefit in the treatment of high-grade extremity sarcomas. Moreover, anthracyclines and ifosfamide have been established as the most active chemotherapeutic agents for metastatic adult soft tissue sarcomas, with a single-agent response rate of 16–36%.[3]

Multidrug resistance (MDR) to anticancer agents is a major problem in the chemotherapy treatment of cancer patients. One mechanism of MDR is the overexpression of ATP-dependent membrane proteins that function as a drug efflux pump. The classical drug efflux pump is P-glycoprotein (P-gp), which is coded by MDR1.[4] The expression of P-gp has been extensively investigated in various types of carcinoma and sarcoma. The family of multidrug resistance-associated protein (MRP) also belongs to the ATP-binding cassette superfamily of transporters and the MRP family is comprised of 9 related ABC transporters that are able to transport structurally diverse lipophilic anions and function as a drug efflux pump.[5] Overexpression of P-gp and MRP1 in human cancer cells leads to drug resistance against anthracyclines vinca alkaloids, and epipodophyllotoxins,[6,7] which are involved in the regimen of chemotherapy for soft tissue sarcoma.

Recent studies have demonstrated that the expression of ABC transporters is highly regulated, particularly at the level of transcription, by several molecular mechanisms.[8] A tumor suppressor protein could also influence the expression of a drug-resistant gene. Several authors have observed that wild-type p53 repressed the transcription of the *MDR1* gene,[9–11] while P-gp expression showed a positive correlation with p53 nuclear expression in breast cancer,[12] gastric cancer[13] and oral squamous cell carcinoma.[14] Wild-type p53 has also been shown to repress the transcription of the human MRP1 promoter,[15–17] whereas aberrant p53 expression is correlated with increased MRP1 expression in nonsmall cell lung cancer[18] and gastrointestinal tract cancer.[19,20] Recently, we evaluated the expression of ABC transporter proteins at mRNA and protein levels in synovial sarcoma and found a significant correlation between immunohistochemical P-gp expression and biphasic type tumors.[21] However, we failed to demonstrate their correlation with any clinicopathological parameters. The aims of the current study were to find out whether the mRNA of any of the specific ABC transporter proteins (MDR1, MRP1, MRP2 and MRP3) is expressed in a specific histologic type of soft tissue sarcomas and whether these transporters are associated with clinicopathologic parameters. Furthermore, we searched for a correlation between p53 protein expression and the transcription levels of ABC transporter protein.

Material and methods

Tissue samples

Eighty-six specimens of soft tissue sarcoma were obtained from 83 patients who had undergone surgery at Kyushu University Hospital, the National Kyushu Cancer Center and the National Kyushu Medical Center, Fukuoka, Japan, between 1988 and 2001. The samples were snap-frozen in liquid nitrogen immediately after

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TABLE I - CLINICAL AND PATHOLOGICAL CHARACTERISTICS OF THE EXAMINED 83 CASES OF SOFT TISSUE SARCOMA AND THEIR CORRELATION WITH ABC TRANSPORTER mRNA EXPRESSION

Parameters	*MDR1* mean A.U.	*MRP1* mean A.U.	*MRP2* mean A.U.	*MRP3* mean A.U.
Age (n=83)				
< 20 years (n=11)	159.365	40.665	0.478	129.333
≥ 20 years (n=72)	68.287	58.747	0.776	159.093
Sex (n=83)				
Male (n=44)	77.495	44.845	0.971	180.940
Female (n=39)	83.588	69.331	0.472	126.052
Location (n=83)				
EX and TR (n=63)	70.877	58.745	0.843	160.883
AC or TC, RP or Vis (n=20)	110.222	48.807	0.401	137.088
Size (n=83)				
< 5 cm (n=22)	17.860	73.480	0.222	139.164
≥ 5 cm (n=61)	102.898	50.172	0.922	160.914
Depth (n=83)				
Superficial (n=5)	25.265	26.027	0.319	190.948
Deep (n=78)	83.889	58.294	0.763	152.854
Mitosis (n=86 tumors)				
0–19/10 HPFs (n=67)	78.147	51.486	0.677	161.37
≥ 20/10 HPFs (n=19)	75.465	73.841	0.916	128.639
Necrosis (n=86 tumors)				
0–50% (n=78)	79.627	52.644	0.446[1]	166.895
> 50% (n=8)	57.354	93.283	3.502[1]	29.762
AJCC stage (n=83)				
Low (Stage I, II) (n=32)	24.572	65.783	0.346	122.164
High (Stage III, IV) (n=51)	115.361	50.432	0.982	175.846

[1]Statistically significant difference in mRNA expression between the 2 groups of clinicopathologic parameters, EX: Extremities, TR: Trunk, AC: Abdominal cavity, TC: Thoracic cavity, RP: Retroperitoneum, Vis: Viscera, AJCC: American Joint Committee on Cancer, HPFs: High-power fields.

surgical resection and stored at −80°C until use. Their clinicopathological characteristics are summarized in Table I.

Tumor stage was evaluated according to the American Joint Committee on Cancer (AJCC) staging system.[22] The patients comprised 44 males and 39 females. Seventy-two patients were aged 20 years or older. Sixty-three tumors had occurred in the extremities or trunk, and 20 had arisen in the abdominal or thoracic cavity, retroperitoneum or viscera. Sixty-one tumors were 5 cm or larger. Only 5 tumors were superficially situated, and the remaining 78 tumors were deeply situated. Fifty-four tumors had less than 9 mitoses per 10 high-power fields (HPFs), whereas 13 had between 10 and 19 mitoses per 10 HPFs. Nineteen tumors had more than 20 mitoses per 10 HPFs. In 54 cases, necrosis was absent. Extensive tumor necrosis covering more than 50% of the examined slides was recognized in 8 cases, whereas 24 cases showed tumor necrosis covering less than 50%. Eighteen cases of the primary tumors were considered to be AJCC stage I, 14 cases stage II, 45 cases stage III and 6 cases stage IV.

Histologic subtypes are summarized in Table II. As for 5 "others" tumors are comprised of 1 case each of angiosarcoma, unclassified sarcoma, alveolar rhabdomyosarcoma, dermatofibrosarcoma protuberans and epithelioid hemangioendothelioma (Table II). The ABC transporter expression in 22 out of the 23 synovial sarcomas has been published previously.[21] Three tumors were obtained from recurrent or metastatic sites as well as the primary site in the same patients, 1 from a metastatic lung lesion of DFSP with FS area, and 2 from recurrent lesions of pleomorphic MFH. To avoid contaminating normal tissue, the tumor samples were evaluated in conjunction with histologic sections. Furthermore, 6 frozen samples of normal skeletal muscles from patients with other diseases were used for a nontumorous control. Histological diagnosis was confirmed by corresponding paraffin-embedded materials and when necessary, panels of immunohistochemical study were carried out carefully, according to the most up-to-date diagnostic criteria.[23] The number of evaluated histologic slides ranged from 1 to 32, with a mean of 4 slides per case. As for the diagnosis of all synovial sarcoma cases, the presence of SYT-SSX fusion transcripts was confirmed by RT-PCR. All materials reported here, even in the recurrent or metastatic lesions, were obtained prior to chemotherapy so that only intrinsic ABC transporter expression was detected.

TABLE II - HISTOLOGIC TYPE OF EXAMINED TUMORS

Histologic type	Numbers
Synovial sarcoma	23
Monophasic fibrous	15
Biphasic	8
Pleomorphic malignant fibrous histiocytoma/ Undifferentiated high grade pleomorphic sarcoma	16
Leiomyosarcoma	12
Malignant peripheral nerve sheath tumor	10
Myxoid/round cell liposarcoma	4
Ewing's sarcoma/Primitive neuroectodermal tumor	3
Desmoplastic small round cell tumor	3
Alveolar soft part sarcoma	2
Clear cell sarcoma	2
Solitary fibrous tumor	2
Inflammatory myofibroblastic tumor	2
Dermatofibrosarcoma protuberans with fibrosarcomatous area	2
Others	5

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed tissue sections in concordance with frozen material. Sections were cut at widths of 4 μm from paraffin-embedded material and then dewaxed with xylene and rehydrated through a graded series of ethanol. After inhibition of endogenous peroxidase, sections were exposed to the primary antibodies at 4°C overnight, followed by staining with a streptavidin-biotin-peroxidase kit (Nichirei). The sections were then finally reacted in 3,3′-diaminobenzidine, counterstained with hematoxylin and mounted. The following monoclonal antibodies were used as the primary antibody: anti-P-glycoprotein [JSB-1, 1:20 (Sanbio, Uden, the Netherlands)], anti-MRP1 [MRPr1, 1:50 (Nichirei, Tokyo, Japan)], anti-MRP2/cMOAT [M2 III-6, 1:20 (Sanbio)], anti-MRP3[M3 II-9, 1:80

(Kamiya Biomedical Company, Seattle, WA)], anti-p53 [PAb1801, 1:100 (Oncogene Research Products, Boston, MA)] and anti-Ki-67 [MIB-1, 1:100 (Immunotech, Marseille, France)]. For staining with all the antibodies, sections were pretreated with microwave irradiation for the purpose of antigen retrieval. Tissue from a normal kidney or MDR human osteosarcoma cell line MNNG/HOS/DXR 1000, which expresses high level *MDR1* mRNA,[24] served as a control for JSB-1, while tissue from the adrenal gland served as a control for MRPr1. Tissue from a normal liver and colon was used as a control for MRP2/cMOAT and MRP3, respectively. For each procedure, a negative control was also obtained by staining the samples with secondary antibody only.[25]

ABC transporter proteins positive tumor samples were graded from 1 to 3 according to the distribution of positivity and the degree of immunostaining of the plasma membrane or cytoplasmic Golgi region,[25,26] as follows: Score 1, positive cells are less than 10% and weak immunostaining; Score 2, more than 10% of the tumor cells are positive and weak immunostaining and Score 3, more than 10% of the tumor cells are positive and strong immunoreactivity. The highest degree of positivity found in any area of the section was recorded and grade 2 or 3 was judged as positive. As for p53 protein, when more than 10% of the tumor cells showed a positive reaction in nuclei, we judged the case to be positive. The MIB-1-labeling index (LI) was estimated by counting the number of positive cells per 1,000 tumor cells.

Real-time quantitative RT-PCR

Total RNA was extracted using Trizol Reagent (Invitrogen Corp., Carlsbad, CA) and reverse transcription was performed with Superscript II reverse transcriptase (Invitrogen Corp.) according to the manufacturer's instructions. Real-time quantitative PCR (TaqMan PCR) using an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) was performed according to the manufacturer's protocol. The sequences of oligonucleotide primer pairs and TaqMan probes for MDR1, MRP1, MRP2 and MRP3 are as follows:[27] (a) *MDR1*, sense primer 5′-TGCTCAGACAGGATGTGAGTTG-3′, antisense primer 5′-TAGCCCCTTTAAC-TTGAGCAGC-3′ and probe 5′-AAA-ACACCACTGGAGCATTGACTACCAGGC-3′; (b) *MRP1*, sense primer 5′-TACCTCCTGTGGCTGAATCTGG-3′, antisense primer 5′-CCGATTGTCTTTGCTCTTCATG-3′ and probe 5′-ATGGCG-ATGAAGA-CCAAGACGTATCAGGTG-3′: (c)*MRP2*, sense primer 5′-CAAACTCTAT-CTTGCTAAGCAGG-3′, antisense primer 5′-TGAGTACAAGGGCCAGCTCTA-3′ and probe 5′-TTCGTTGGTTTTCTTCTTATTCTAGCAGCC-3′: (d) *MRP3*, sense primer 5′-CTTAAGACTTCCCCTCAACATGC-3′, antisense primer 5′-GGTCA-AGTTCCTCTTGGCTC-3′ and probe 5′-AGTGTGTCTCTGAAACGGATC-CAGCAATTC-3′. Primers and probes for GAPDH were purchased from Perkin-Elmer Applied Biosystems (TaqMan GAPDH control reagent kit). Serial 1:10 dilutions of plasmid DNA containing each of the target cDNAs were analyzed and served as standard curves from which we determined the rate of change of threshold cycle values. Copy numbers of the target cDNA were estimated by standard curves. We confirmed the sensitivity of the quantitation by demonstrating MDR1 amplification curves using low-level and high-level positive control cells comprising MNNG/HOS cells, MG63/DXR 10 cells and MNNG/HOS/DXR 1000 cells.[24] All the reactions for standard samples and samples of patients were performed in triplicate (Fig. 1). The data were averaged from the values obtained in each reaction. To determine the mRNA levels of 4 ABC transporters, we used an mRNA expression index, which is an mRNA expression level standardized by GAPDH. The mRNA expression index was calculated as follows in arbitrary units (AU):

$$\text{mRNA expression index} = \frac{\text{copy numbers of ABC transporter mRNA}}{\text{copy numbers of GAPDH mRNA}} \times 1{,}000 \text{ AU}$$

Statistical analysis

The difference in mRNA expression between tumor tissue and skeletal muscle as a control was evaluated by the Mann Whitney U test. The difference in ABC transporter mRNA expression among the various histologic types was estimated by the Kruskal-Wallis test. The difference in mRNA expression between the 2 groups including clinicopathologic parameters and p53 expression was estimated by an unpooled 2-sample *t*-test, whereas the correlation between each of the clinicopathologic parameters and immunohistochemical protein expressions was evaluated by a 2-sided chi-square test with Yates continuity correction. Correlation between mRNA expression and MIB-1-LI was estimated by the Pearson relative correlation coefficient analysis. A p value <0.05 was considered to be significant.

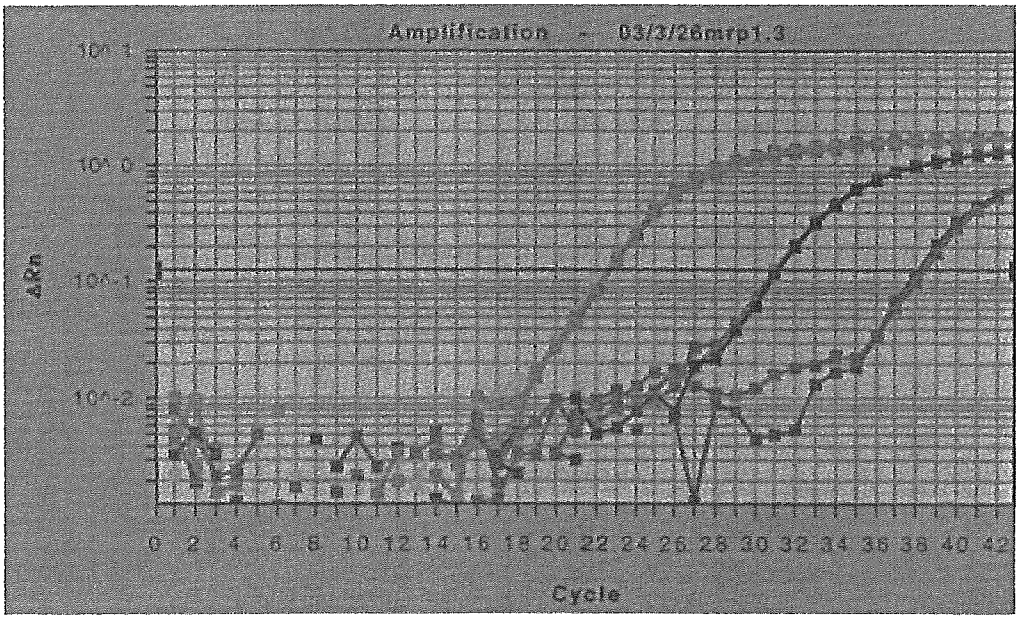

FIGURE 1 – Amplification plot for *MRP3* cDNA obtained from a pleomorphic malignant fibrous histiocytoma arising in the thigh of a 62-year-old male (middle curves). Upper and lower curves are plots for *MRP3* cDNA standards (10^7 copies for upper and 10^3 copies for lower curves, respectively). All the reactions were performed in triplicate.

TABLE III – EXPRESSION OF ABC TRANSPORTER mRNA AND HISTOLOGIC TYPE

Histologic type[1]	*MDR1* mean SD	*MRP1* mean SD	*MRP2* mean SD	*MRP3* mean SD
Synovial sarcoma (n=23)	90.471	54.110	1.429	69.818
	141.422	59.715	4.027	109.424
MPNST (n=10)	303.227*	59.105	0.387	214.693**
	538.690	113.011	0.760	286.149
MFH (n=16)	4.191	64.125	0.688	127.319
	16.114	121.481	1.113	221.591
Liposarcoma (n=4)	123.163	32.502	0.692	172.228
	142.672	35.396	1.004	186.574
Leiomyosarcoma (n=12)	23.909	74.310	0.225	149.699
	78.788	130.337	0.343	204.677
EWS/PNET (n=3)	12.754	9.889	0.020	6.454
	8.157	4.200	0.014	1.694
DSRCT (n=3)	58.408	11.314	0.096	67.794
	95.691	7.787	0.023	9.402
Skeletal muscle (n=6)	0	5.402	0.290	3.982
	0	4.190	0.416	5.950

[1]MPNST: malignant peripheral nerve sheath tumor, MFH: pleomorphic malignant fibrous histiocytoma, EWS/PNET: extraskeletal Ewing's sarcoma/primitive neuroectodermal tumor, DSRCT: desmoplastic small round cell tumor.–*p=0.0017.–**p=0.0384 by Kruskal-Wallis test.

Results

mRNA expression of ABC transporter protein

As for clinicopathologic parameters, extensive tumor necrosis (>50%) was found to be significantly correlated with a high level of *MRP2* mRNA (p=0.0001, Table I). Although large tumor size (≥5 cm) and high AJCC stage (stages III and IV) tended to reveal a high level of *MDR1* mRNA, the difference was not statistically significant (tumor size, p=0.1207/AJCC stage, p=0.0669, Table I). There was no relationship between ABC transporter mRNA expression and other clinicopathologic parameters. With regard to histologic type, MPNST showed a significantly higher level of mRNA expression of *MDR1* and *MRP3,* compared to other tumor types (p=0.017 in *MDR1* and p=0.0384 in *MRP3* by Kruskal-Wallis test, Table III and Fig. 2). No significant difference was observed between the various histologic types with regard to *MRP1* or *MRP2* mRNA expression.

Immunohistochemical expression of abc transporter protein

For all ABC transporters, immunohistochemical staining for positive and negative controls were relevant (Fig. 3*a,b*). P-glycoprotein (P-gp) expression was observed in 32 out of 86 cases (37.2%) (Fig. 3*c*). MRP1 protein expression was recognized in 37 out of 86 cases (43%) (Fig. 3*d*), whereas MRP2/cMOAT expression was found in 27 cases (31.4%) (Fig. 3*e*). Positive immunoreactivity for MRP3 was observed in 31 out of 86 cases (36%) (Fig. 3*f*).

When comparing the immunohistochemistry and real-time quantitative RT-PCR techniques, we observed a significant correlation between the 2 detection methods for all the examined ABC transporter proteins and mRNA (p<0.05, Table IV).

The correlation between ABC transporter protein expression and clinicopathological parameters are summarized in Table V. P-gp expression was significantly correlated with large tumor size (≥5 cm) (p=0.041) and high AJCC stage (stages III and IV) (p=0.0365). MRP1 expression demonstrated a significant relationship with increased age of the patient (≥20 years old) (p=0.037). There was also a significant correlation between MRP2 expression and low mitotic figures (0–19/10 HPFs) (p=0.0231). No relationship was observed between ABC transporter protein expression and any of the other clinicopathologic parameters.

Nuclear accumulation of p53 protein was observed in 22 out of 86 cases (25.6%) (Fig. 4). There was no relationship between p53 immunoreaction and clinicopathologic parameters. However, cases with nuclear expression of p53 protein showed significantly higher levels of *MDR1* mRNA than cases with negative immunoreaction for p53 (p=0.0328) (Table VI).

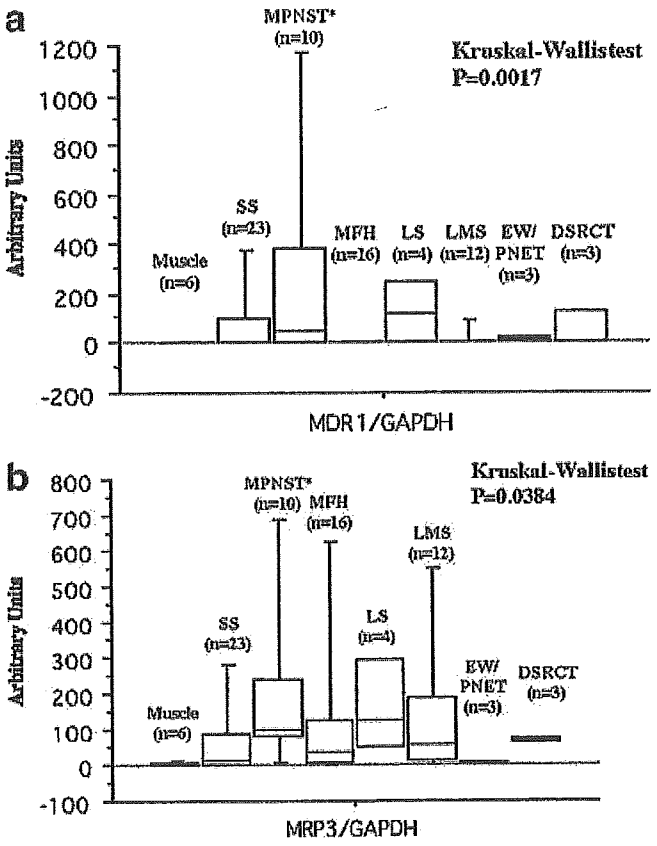

FIGURE 2 – Comparison of relative mRNA levels of (*a*) *MDR1* and (*b*) *MRP3* between various types of soft tissue sarcoma. Data for control skeletal muscle tissue are also shown. The levels of *MDR1* and *MRP3* mRNA in MPNST are significantly higher than those of other types of soft tissue sarcoma (MDR1; p=0.0017, MRP3; p=0.0384). Muscle, Skeletal muscle tissue; MPNST, Malignant peripheral nerve sheath tumor; MFH, Pleomorphic malignant fibrous histiocytoma; LS, Liposarcoma; LMS, Leiomyosarcoma; EW/PNET, Ewing's sarcoma/Primitive neuroectodermal tumor; DSRCT. Desmoplastic small round cell tumor.

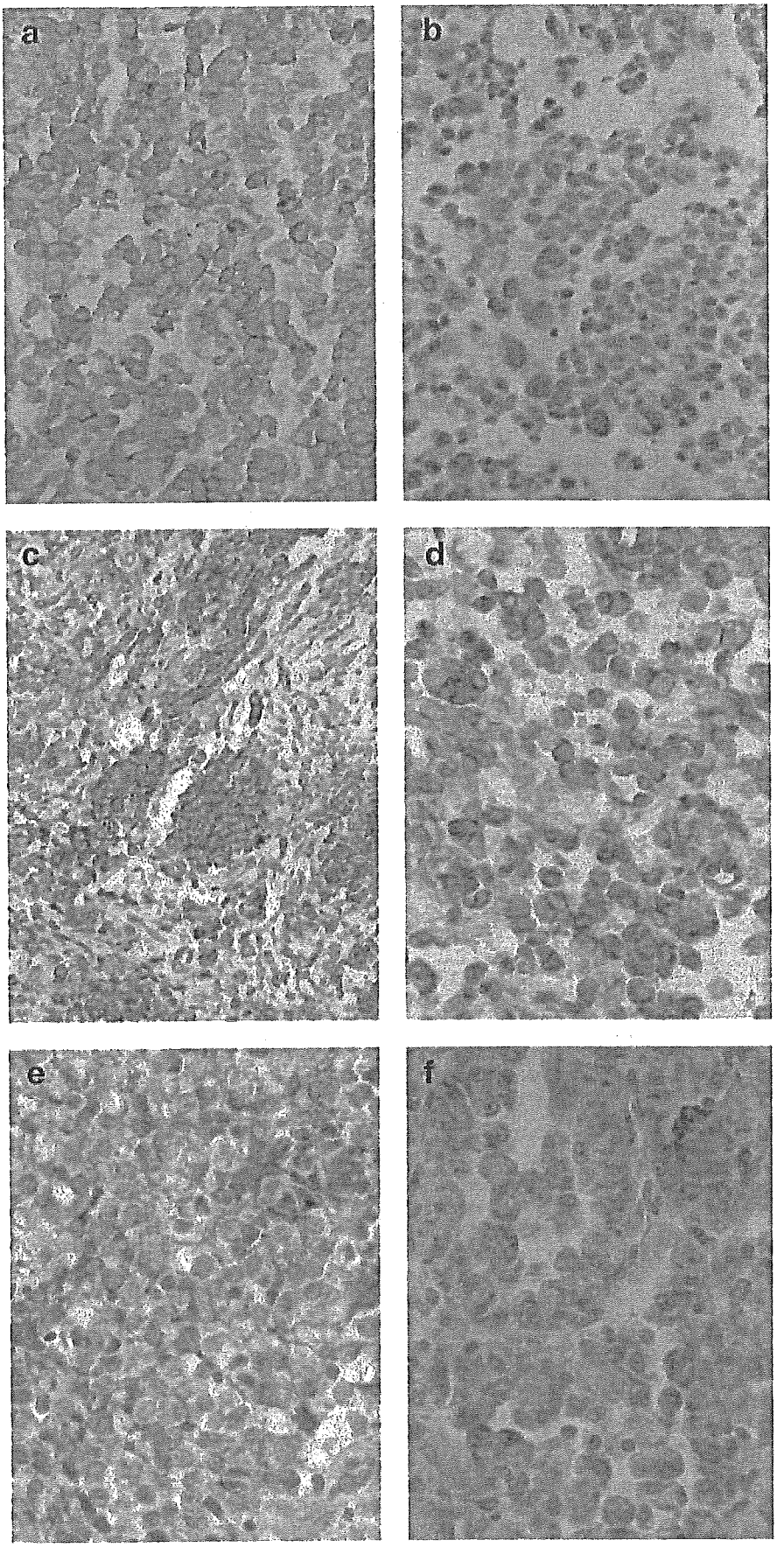

FIGURE 3 – Immunohistochemical expression of ABC transporter protein in control sections and various types of soft tissue sarcoma. (*a*) MNNG/HOS/DXR 1000 cell line for positive control shows strong membranous immunoreaction for P-glycoprotein. (*b*) Its negative control reveals no immunoreactivity. (*c*) Immunohistochemical staining of P-glycoprotein in monophasic fibrous synovial sarcoma arising in the thigh of a 40-year-old male. Tumor cells show membranous positive immunoreactivity. (*d*) Membranous immunostaining for MRP1 in MPNST arising in the spinal canal of a 2-year-old girl. (*e*) MRP2 is localized on the plasma membrane of a clear cell sarcoma in the lower leg of a 19-year-old female. (*f*) MRP3 is localized in the cytoplasm of an alveolar soft part sarcoma in the axillary region of a 16-year-old male.

TABLE IV – CORRELATION BETWEEN REAL-TIME QUANTITATIVE RT-PCR AND IMMUNOHISTOCHEMISTRY

MDR1 mRNA (A.U.)		Average	SD	
P-gp: IHC[1]	+ (n=32)	186.96	323.91	p=0.0002*
	− (n=54)	12.72	48.47	
MRP1 mRNA (A.U.)		Average	SD	
MRP1: IHC[1]	+ (n=37)	98.96	115.91	p=0.0001*
	− (n=49)	24.31	53.02	
MRP2 mRNA (A.U.)		Average	SD	
MRP2: IHC[1]	+ (n=27)	2.04	3.67	p=0.0001*
	− (n=59)	0.13	0.23	
MRP3 mRNA (A.U.)		Average	SD	
MRP3: IHC[1]	+ (n=31)	301.42	405.51	p=0.0002*
	− (n=55)	71.12	127.48	

[1]IHC: Immunohistochemistry.–*Statistically significant.

TABLE V – CORRELATION BETWEEN CLINICAL AND PATHOLOGICAL PARAMETERS AND ABC TRANSPORTER PROTEIN EXPRESSION[1]

Parameters	PGP		MRP1		MRP2		MRP3	
	+	−	+	−	+	−	+	−
Age (n=83)								
< 20 yrs. (n=11)	4	7	8	3*	4	7	5	6
≥ 20 yrs. (n=72)	28	44	28	44*	22	50	25	47
Sex (n=83)								
Male (n=44)	16	28	16	28	12	32	16	28
Female (n=39)	16	23	20	19	14	25	14	25
Location (n=83)								
EX and TR (n=63)	26	37	30	33	21	42	22	41
AC or TC, RP or Vis (n=20)	6	14	6	14	5	15	8	12
Size (n=83)								
< 5 cm (n=22)	3	19*	8	14	4	18	7	15
≥ 5 cm (n=61)	29	32*	28	33	22	39	23	38
Depth (n=83)								
Superficial (n=5)	2	3	0	5	2	3	2	3
Deep (n=78)	30	48	36	42	24	54	28	50
Mitosis (n=86)								
0–19/10 HPFs (n=67)	25	42	31	36	25	42*	27	40
≥ 20/10 HPFs (n=19)	7	12	6	13	2	17*	4	15
Necrosis (n=86)								
0–50% (n=78)	29	49	33	45	24	54	30	48
> 50% (n=8)	3	5	4	4	3	5	1	7
AJCC stage (n=83)								
Low (Stage I, II) (n=32)	8	24*	12	20	9	23	11	21
High (Stage III, IV) (n=51)	24	27*	24	27	17	34	19	32

[1]+: positive, −: negative, p: p values.–*Statistically significant difference in immunohistochemical expression between the 2 groups of clinicopathologic parameters, EX: Extremities, TR: Trunk, AC: Abdominal cavity, TC: Thoracic cavity, RP: Retroperitoneum, Vis: Viscera, AJCC: American Joint Committee on Cancer, HPFs: High-power fields.

The MIB-1 LI ranged from 1.3 to 67.6. It demonstrated no correlation with either ABC transporter mRNA expression or with ABC transporter protein.

Discussion

Except extraskeltal Ewing's sarcoma and rhabdomyosarcoma, the value of systemic chemotherapy remains controversial.[23] According to the review of van Glabbeke *et al.*,[28] the responses to chemotherapy in all soft tissue sarcomas are still low (26%). In their series, MPNSTs are not mentioned as separate groups with bad response. Furthermore, Stein *et al.*[29] reported that 7 out of 8 MPNSTs (neurofibrosarcomas) (88%) showed intermediate *MDR1* expression at high or intermediate frequency. In our study, expressions of *MDR1* and *MRP3* in MPNST were significantly higher than those in other sarcomas. Although MDR1/P-gp contributes to drug resistance in a wide spectrum of anti-cancer agents, MRP3 has been reported to confer only low levels of resistance to etoposide and teniposide *in vitro*.[30] On the other hand, Steinbach *et al.*[31] recently demonstrated that a high level of *MRP3* gene expression is correlated with failure of remission in AML and they suggested that this gene expression is involved in drug resistance in childhood AML. Moreover, they also demonstrated a positive correlation between *MRP3* mRNA expression and poor outcome in childhood ALL, especially in the case of the T-cell immunophenotype.[32] Overexpression of these 2 types of ABC transporter protein mRNA may have an important role to play in the poor chemoresponse seen in cases of MPNST. These 2 ABC transporter expressions are possible targets for the development of specific drugs to overcome multidrug resistance in MPNST.

Some investigators have reported that the tumor suppressor protein, wild-type p53, repressed the transcription of the *MDR1* gene *in vitro*,[9–11] while Li *et al.*[33] showed that the induction of wild-type p53 protein activates the expression of the *MDR1* gene. As for clinical samples, Linn *et al.*[12] demonstrated a positive correlation between P-gp and the nuclear accumulation of p53 protein in breast cancer using an immunohistochemical method.

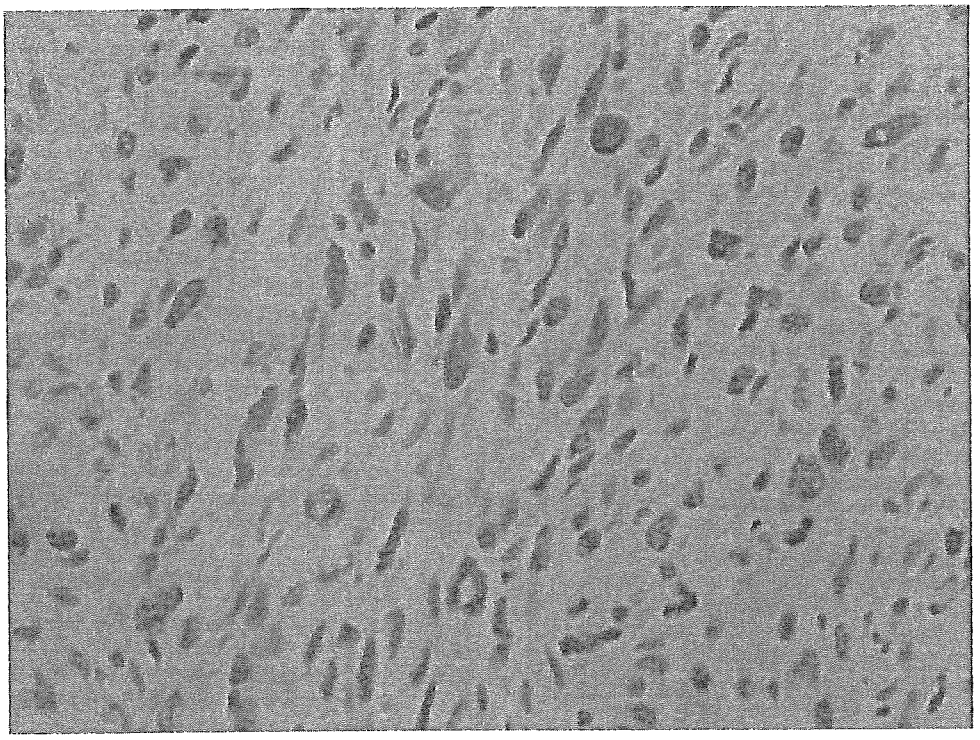

FIGURE 4 – Diffuse nuclear accumulation of p53 protein is observed in pleomorphic MFH arising in the thigh of 76-year-old female.

TABLE VI – CORRELATION BETWEEN ABC TRANSPORTER mRNA EXPRESSION AND IMMUNOHISTOCHEMICAL p53 EXPRESSION

MDR1 mRNA (A.U.)				
		Average	SD	
p53	+ (n=22)	162.17	382.86	p=0.0328*
p53	− (n=64)	48.47	105.17	
MRP1 mRNA (A.U.)				
		Average	SD	
p53	+ (n=22)	47.51	43.45	p=0.6054
p53	− (n=64)	59.49	104.96	
MRP2 mRNA (A.U.)				
		Average	SD	
p53	+ (n=22)	0.55	0.97	p=0.6557
p53	− (n=64)	0.79	2.52	
MRP3 mRNA (A.U.)				
		Average	SD	
p53	+ (n=22)	248.23	471.39	p=0.0715
p53	− (n=64)	121.79	175.21	

*Statistically significant.

Similar immunohistochemical results have been reported in gastric cancer[13] and oral squamous cell carcinoma.[14] A significant correlation between p53 mutation and *MDR1* mRNA expression has also been documented in advanced colon cancer[34] and in nonsmall cell lung cancer.[35] Paradoxically, a negative correlation between p53 expression and *MDR1* mRNA was also reported in colon cancer,[36] myelodysplastic syndrome[37] and CLL.[38] In our study, aberrant nuclear accumulation of p53 was found to be significantly correlated with a high level of *MDR1* mRNA in soft tissue sarcomas, although the clone of p53 antibody (PAb 1801) was different from that used in the study of Linn *et al.*[12] (DO1). As for the transcriptional regulation of *MRP1*, wild-type p53 has also been shown to repress the transcription of the human *MRP1* promoter.[15–17] However, the immunohistochemical nuclear accumulation of p53 protein has been found to be correlated with MRP1 expression in clinical nonsmall lung cancer,[18] gastric cancer[19] and colon cancer.[20] In this study, no significant correlation was observed between p53 expression and *MRP1* mRNA expression. Stein *et al.*[39] reported that hyperthermia causes nuclear translocation of the Y-box transcription factor, YB-1, and that it is associated with *MDR1* and *MRP1* gene activity in human colon carcinoma cells. Recently, we demonstrated that nuclear localization of YB1 protein is correlated with the overexpression of P-gp and *MDR1* mRNA in synovial sarcoma.[21]. Further studies are required to assess the correlation between nuclear YB-1 expression and the ABC transporter gene expression within several kinds of soft tissue sarcoma.

MDR1 mRNA expression in soft tissue sarcomas has been reported by some investigators using Northern blotting,[40] dot blot,[41] or RT-PCR assay.[42] No authors have analyzed several kinds of ABC transporter mRNA expressions systematically in soft tissue sarcomas. We previously failed to reveal relationship between *MDR1* expression by RNA level with simple RT-PCR method and the protein level with immunohistochemistry.[42] In our study, we showed significant relationship between the different immnohistochemistry and real-time RT-PCR technique to demonstrate all ABC transporter expression in several kinds of soft tissue sarcomas.