(Sigma), and 2.5% fetal calf serum (FCS) for 10 min. Cells were analyzed on a FACScalibur flow cytometer. The percentage of cells in various stages of the cell cycle was determined with the cell cycle analysis program, MultiCycle AV (Phoenix Flow Systems, San Diego, CA).

Assessment of apoptotic cell death

Apoptotic cells were assessed by flow cytometry using PC5-conjugated APO2.7 mAb (clone 2.7; Imunotech), which was raised against the 38-kDa mitochondrial membrane protein expressed by apoptotic cells (18).

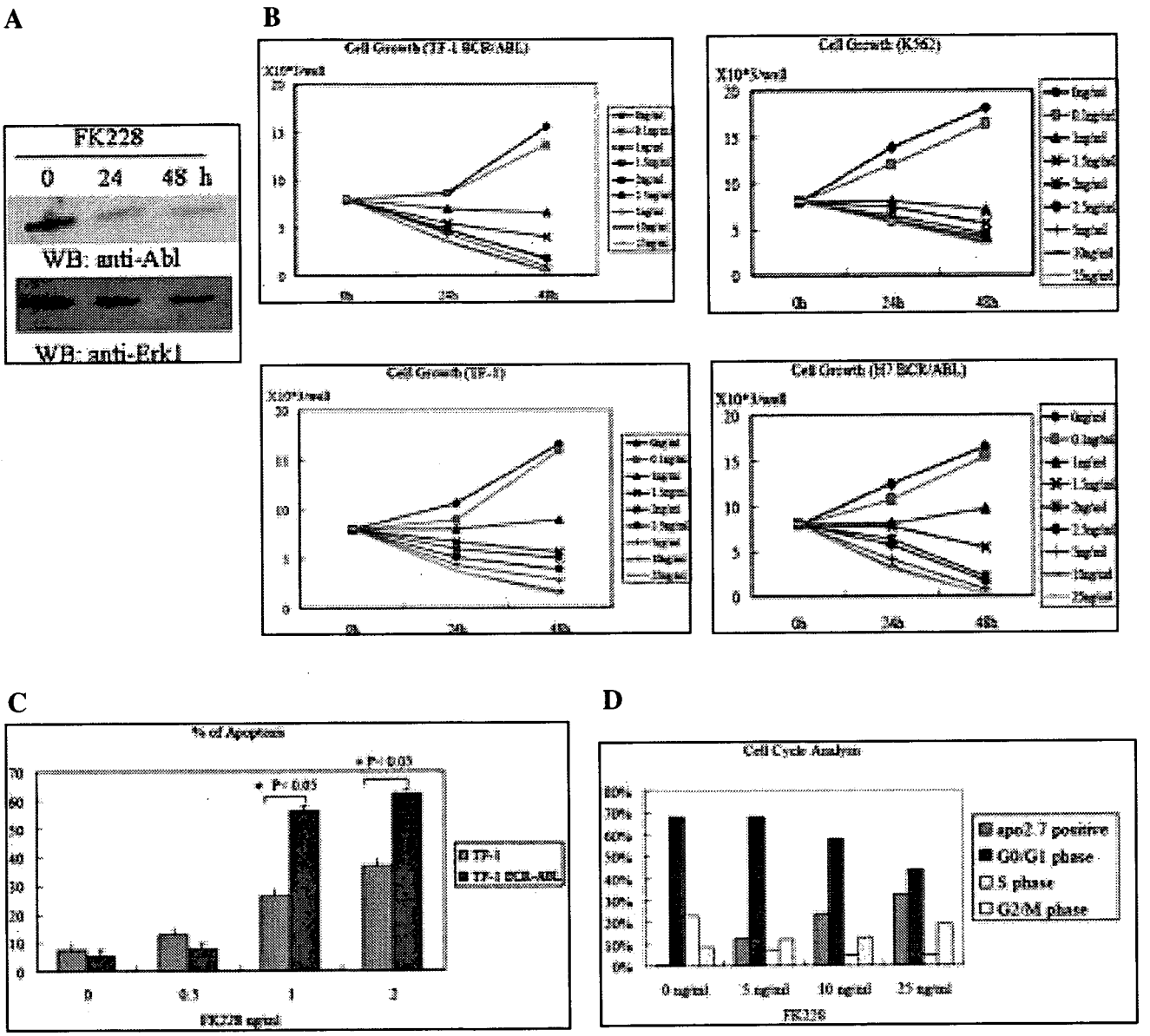


FIG. 1. FK228 inhibits BCR/ABL protein in TF-1 BCR/ABL cells, and induces apoptosis, decreased cell growth, and cell cycle arrest in G_2/M phase. (**A**) TF-1 BCR/ABL cells were treated with 10 ng/ml FK228 for the indicated times; total extracts were analyzed by immunoblot analysis with anti-Abl and anti-Erk-1 antibodies. These results are representative of three separate experiments. (**B**) Parental TF-1, TF-1 BCR/ABL, K562, and H7 BCR/ABL cells were cultured at a concentration of 8×10^4/ml in the presence or absence of FK228 for 24 and 48 h. Viable cell numbers were calculated. Results are representative of three separate experiments. (**C**) TF-1 BCR/ABL cells and parental TF-1 cells were treated with FK228 at the indicated concentrations for 48 h. The percentage of apoptotic cells was examined. Results are representative of three separate experiments. The asterisk (*) designates, $p < 0.05$. (**D**) TF-1 BCR/ABL cells were treated with FK228 at the indicated concentrations. Cell cycle analysis was performed. Similar results were found in five separate experiments.

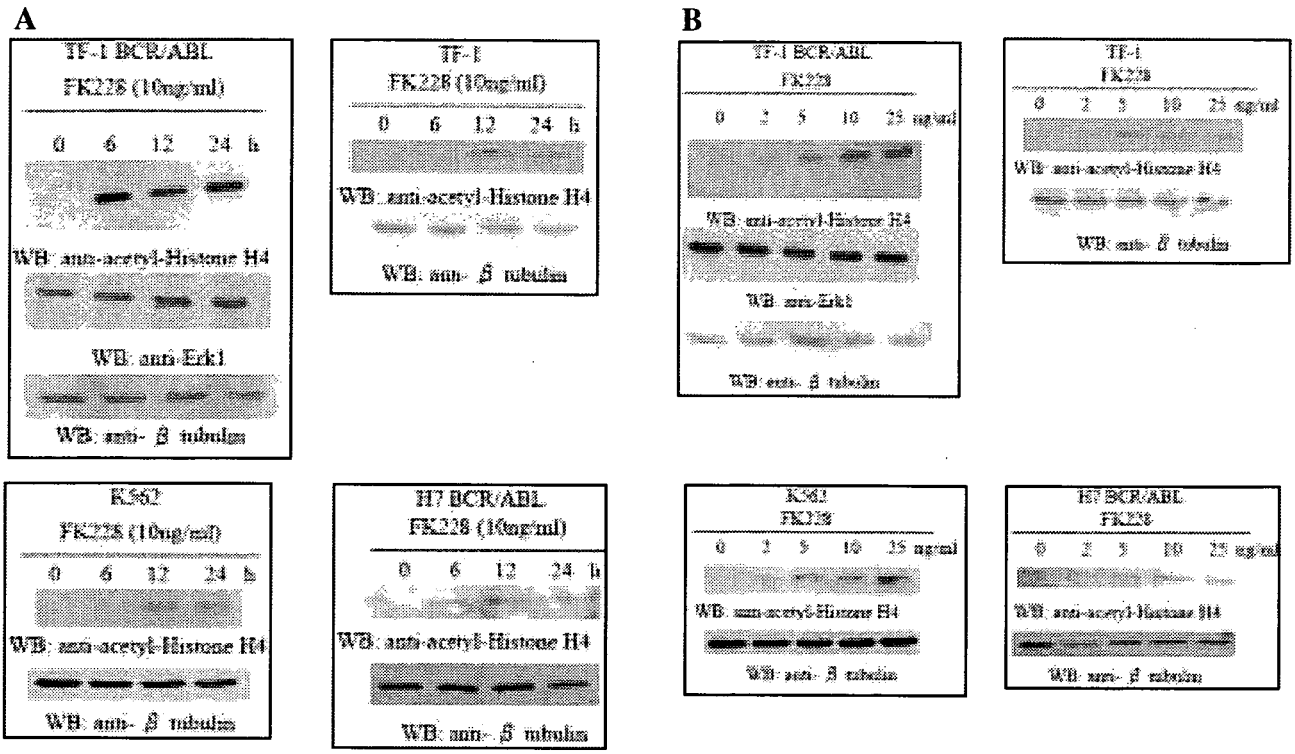


FIG. 2. FK228 induces acetylation of histone H4 and intracellular proteins. BCR/ABL-expressing cell lines and TF-1 cells were treated with FK228 for the indicated times (**A**) and at various doses (**B**). Lysates were immunoblotted with anti-acetyl histone H4, Erk-1, and β-tubulin. (**C**) The AntibodyArray was incubated with cell lysates after 24 h with 10 ng/ml FK228 treatment and immunoblotted with HRP-conjugated acetylated lysine Ab. These results represent one of two similar experiments. (**D**) Cell lysates were immunoprecipitated with anti-acetyl-lysine Ab and immunoblotted with the indicated Abs. These results are representative of three separate experiments.

Reverse transcriptase PCR

Total RNA was isolated from cells using the RNA isolation kit, Isogen (WAKO, Tokyo, Japan) according to the manufacturer's instructions, and incubated with 1 U of DNase for 30 min at 37°C to eliminate genomic DNA. Total RNA (100 ng) was reverse-transcribed using a reverse transcriptase (RT) kit (Amersham). PCR primers for amplification of inhibitor of apoptosis proteins (IAPs) have been reported previously (19). PCR primers for amplification of IAPs were: survivin, 5′-GAGCTGCAGGTTCCTTATC-3′ and 5′-ACAGCATCGAGCCAAGTCAT-3′; livin, 5′-TGAGGTGCTTCTTCTGCTAT-3′ and 5′-TTTCAGACTGGACCTCTCTC-3′; XIAP, 5′-GAAGACCCTTGGGAACAACA-3′ and 5′-GTCCTTGAAACTGAACCCCA-3′; c-IAP1, 5′-GCCTTTCTCCAAACCCTCTT-3′ and 5′-CATTCGAGCTGCATGTGTCT-3′; c-IAP2, 5′-CAGTGGATATTTCCGTGGCT-3′ and 5′-ATTTTCCACCACAGGCAAAG-3′; NAIP, 5′-CCGAACAGGAACTGCTTCTC-3′ and 5′-AAATTTGGCAAACTGGCAAC- 3′; Apollon, 5′-AAGTGGCACCCGTAAATCTG-3′ and 5′-CCTGCCTCAAAGAAGCAAAC-3′. PCR products were shown in 1.5% agarose gels stained with etidium bromide.

Enzyme-linked immunosorbent assay

Analysis of telomerase activity was performed using the Telomerase PCR ELISA kit (Roche, Tokyo, Japan), which contains all needed reagents. The kit is based on the TRAP assay and is combined with nonradioactive techniques for detection of the PCR product. A colored reaction product, if formed, is measured at 450 nm, with a reference wavelength of 690 nm, using an enzyme-linked immunosorbent assay (ELISA) microtiter plate reader.

Statistics

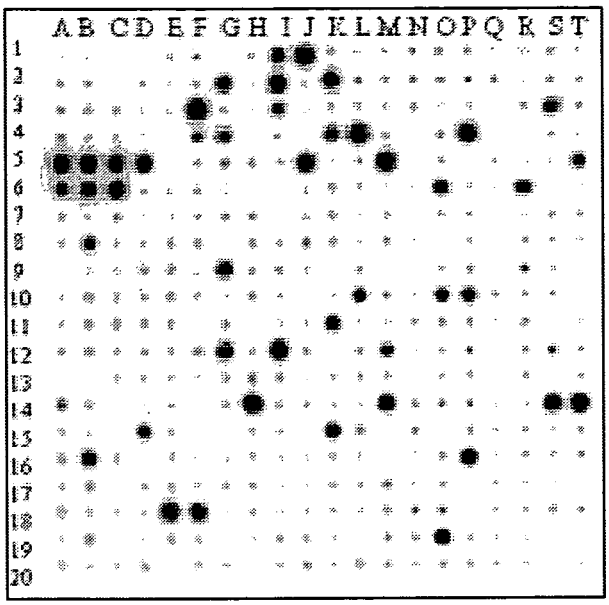
Data are given as mean ± SD. Comparisons between two groups were assessed with Student *t*-test.

RESULTS

FK228 induces cell growth arrest, apoptosis, and cell cycle arrest

Effects of FK228 on proliferation of BCR/ABL-expressing cell lines were examined by counting the

C



Acetylated proteins

Tumor Suppressors/ Apoptosis	Cell Cycle Proteins	Kinase and Phosphatases	Cell Adhesion Proteins
Bcl-2	Cdk1/Cdc2	FAK	E-Cadherin
Bcl-xS/L	Cdk2	fyn	Pan-Cadherin
Mcl-1	Cdk4	Lck	a-Catenin
Caspase4	Cdk6		b-Catenin
MGMT	CyclinA		Connexin43
p53	CyclinB		Integrin-alpha1
PTEN	CyclinD3		Integrin-b1
Rad51	CUL-1		Integrin-b3
Rb	PCNA		L1
TOSO			paxillin
Transport and Trafficking	**Synthesis and Degradation**	**Transcription Regulators**	**Membrane Receptor**
Bin1	MMp-9	NF-kappaB52	DcR2
	Phospholipase D		DR5
Structural proteins	**Signaling Intermediates**	**CD Markers**	
Ankyrin	Grb2	CD3 epsilon	
Clathrin	NCK	CD27	
	p130Cas	CD45	

D

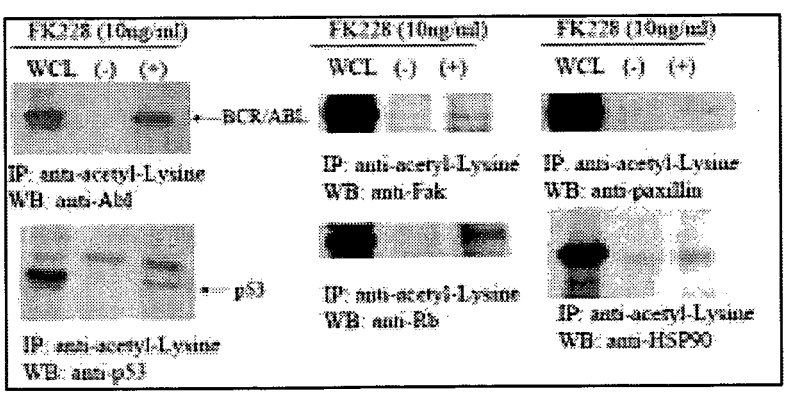


FIG. 2. (*continued*).

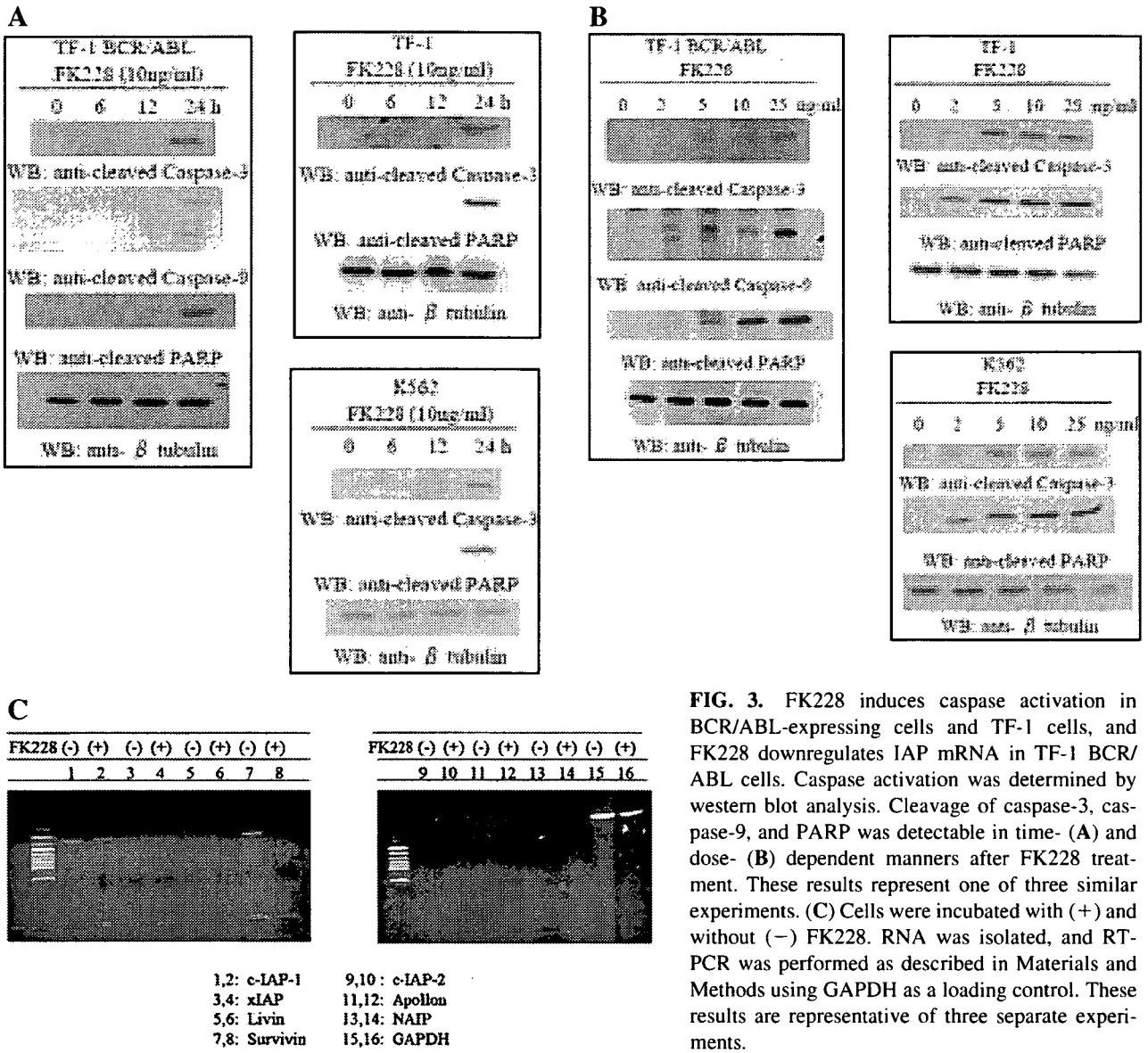


FIG. 3. FK228 induces caspase activation in BCR/ABL-expressing cells and TF-1 cells, and FK228 downregulates IAP mRNA in TF-1 BCR/ABL cells. Caspase activation was determined by western blot analysis. Cleavage of caspase-3, caspase-9, and PARP was detectable in time- (**A**) and dose- (**B**) dependent manners after FK228 treatment. These results represent one of three similar experiments. (**C**) Cells were incubated with (+) and without (−) FK228. RNA was isolated, and RT-PCR was performed as described in Materials and Methods using GAPDH as a loading control. These results are representative of three separate experiments.

number of viable cells and apoptosis after incubation with increasing concentrations of FK228 for 24 or 48 h. TF-1 BCR/ABL cells demonstrated chromatin condensation by 48 h after FK228 treatment (data not shown). Exposure of TF-1 BCR/ABL cells for 48 h to FK228 reduced the BCR/ABL protein level (Fig. 1A). Treatment with FK228 for 24 and 48 h reduced the growth of BCR/ABL-expressing cell lines TF-1 BCR-ABL, K562, and H7 BCR/ABL with a half-maximal inhibitory concentration (IC_{50}) from 0.75 to 1.5 ng/ml FK228 (Fig. 1B). TF-1 BCR/ABL cells were more sensitive to FK228 suppression than parental cell line, TF-1 (Fig. 1C). Treatment with FK228 for 24 h induced accumulation of cells in G2/M phase (Fig. 1D).

FK228 induces histone and intracellular protein acetylation in BCR-ABL-transfected cell lines

HDAC inhibitors induce histone acetylation and apoptosis in a number of cell types (10). To determine effects of FK228 on BCR-ABL cell lines, we first examined histone acetylation in TF-1 BCR/ABL, K562, and H7 BCR/ABL cells and on the parental cell line TF-1. BCR/ABL cell lines and TF-1 cells were treated with various concentrations of FK228 for 24 h. Acetylated histone H4 is shown from 6 to 24 h (Fig. 2A). Figure 2B shows that FK228-induced histone H4 acetylation was concentration-dependent. Because β-tubulin and Erk-1 protein were the same throughout, we also used the Erk-1 antibody as a loading control in this experiment. All

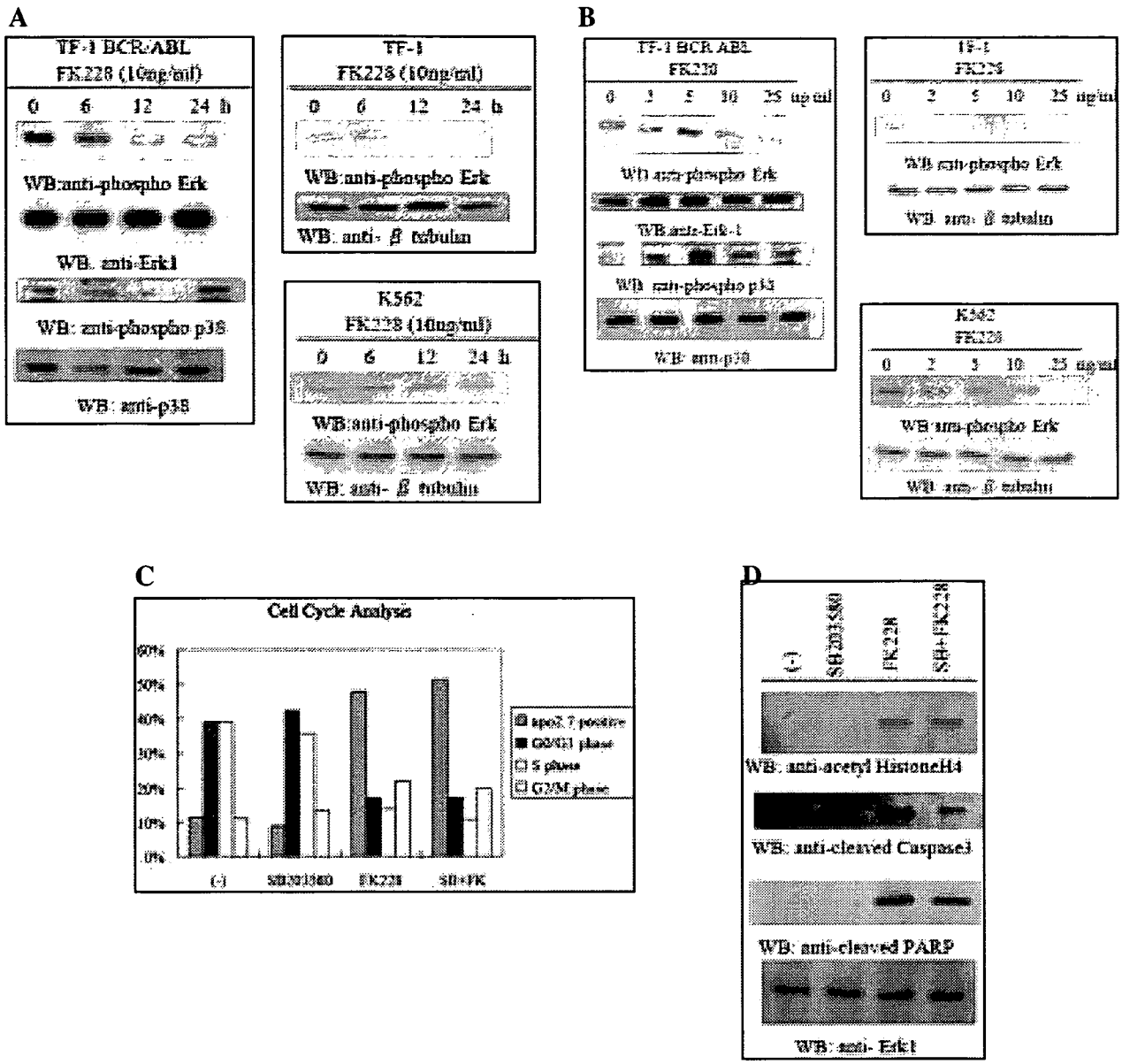


FIG. 4. FK228 regulates MAPK and effects of p38 MAPK inhibitors on FK228 treatment effects. BCR/ABL-expressing cells and TF-1 cells were treated with FK228 for the indicated times (**A**) and doses (**B**). Total extracts were analyzed by immunoblotting with phospho-specific Erk and p38 Abs. These results are representative of three separate experiments. (**C**) TF-1 BCR/ABL cells were treated with FK228 and/or 10 μM specific p38 inhibitor SB203580 for 24 h. Cell cycle analysis was performed. Results represent one of three similar experiments. (**D**) TF-1 BCR/ABL cells were treated with FK228 and/or a specific 10 μM p38 inhibitor SB203580 for 24 h. Total cellular lysates were immunoblotted with anti-acetyl histone H4, cleaved caspase-3, PARP, and Erk-1 Abs. These results are representative of three separate experiments.

four lines demonstrated acetylated histone H4 in response to FK228, but acetylation occurred sooner and was more apparent in TF-1 BCR/ABL than in parental TF-1 cells.

We proceeded to determine whether other intracellular proteins were acetylated or not. For screening of protein acetylation, we used the AntibodyArray system. Twenty four hours after treatment with 10 ng/ml FK228, cell lysates were immunoblotted with HRP-conjugated acetylated lysine Ab. A number of proteins were acetylated (Fig. 2C), and these are listed in the top right panel. To confirm protein acetylation, lysates were immunoprecipitated with acetyl-lysine Ab, and blotted with several different Abs. Figure 2D shows that BCR/ABL, p53, FAK, Rb, paxillin, and HSP-90 were acetylated. Other proteins such as cyclin D1, Cdk2, Bcl-2, Bcl-xL, p130Cas, fyn, Nck, and integrin β1 were not acetylated (data not shown).

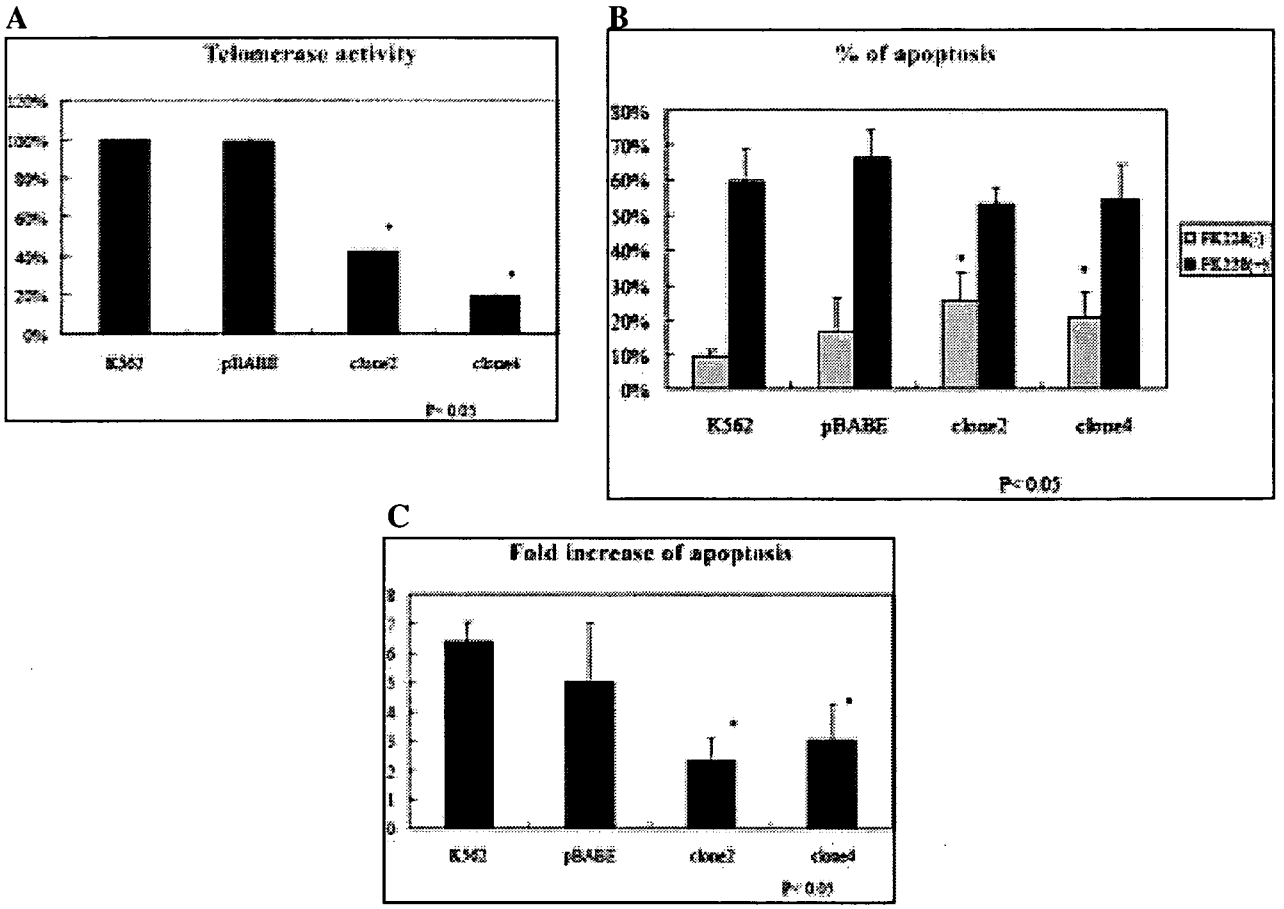


FIG. 5. Effects of hTERT on apoptosis by FK228 in the CML cell line K562. (**A**) Telomerase activities were determined by ELISA. These results represent three separate experiments. (**B** and **C**) Percent of apoptosis was determined by fluorescence-activated cell sorting (FACS) analysis. Apoptosis was quantified by the percent of apo 2.7-positive cells. The noted p values refer to a comparison of control K562 an hTERT shRNA transfectant cells and represent the mean ± SD of at least 3 experiments. $*p < 0.05$.

FK228 activates caspases in BCR/ABL-expressing cells and TF-1 cells and reduces expression of IAP in TF-1 BCR/ABL cells

Activation of apoptosis pathways is a key mechanism by which tumor cells are killed by cytotoxic drugs. Apoptosis can be initiated by extracellular or intracellular stimuli, leading to the activation of caspases. We checked caspase activation by FK228 in BCR/ABL-expressing cells and parental TF-1 cells. Cleaved caspase-3, caspase-9, and PARP were detected after 24 h FK228 treatment (Fig. 3A). Caspase-3, caspase-9, and PARP were dose-dependently cleaved (Fig. 3B). These results indicate that apoptosis of TF-1 BCR/ABL, TF-1, and K562 cells in response to FK228 treatment involves caspase activation. No apparent differences were detected between the BCR-ABL-expressing and TF-1 cells. Inhibitors of apoptosis proteins are expressed in most cancers and leukemias. IAP families are overexpressed in cancer cell lines, suggesting that IAP may play a role in cancer progression (19,20). IAPs are thought to inhibit caspases endogenously by promoting their ubiquitination and degradation (21). There are seven IAP family proteins: c-IAP-1, c-IAP-2, NAIP, XAIP, survivin, livin, and apollon. We examined expression of IAPs by RT-PCR after treatment of TF-1 BCR/ABL cells with FK228. After 24 h FK228 treatment, mRNAs of IAP family members, especially survivin, cIAP-1, and apollon, were reduced when compared to untreated cells (Fig. 3C). These results indicate that IAP family member proteins are reduced by FK228.

FK228 regulates MAPKs in BCR/ABL-expressing cells and TF-1 cells, but p38 is not involved in either G_2/M arrest or apoptosis in TF-1 BCR/ABL cells

Erk is activated principally in response to mitogenic stimulation and regulates cell growth. p38MAPK is activated by stress and other stimuli. Control and FK228-

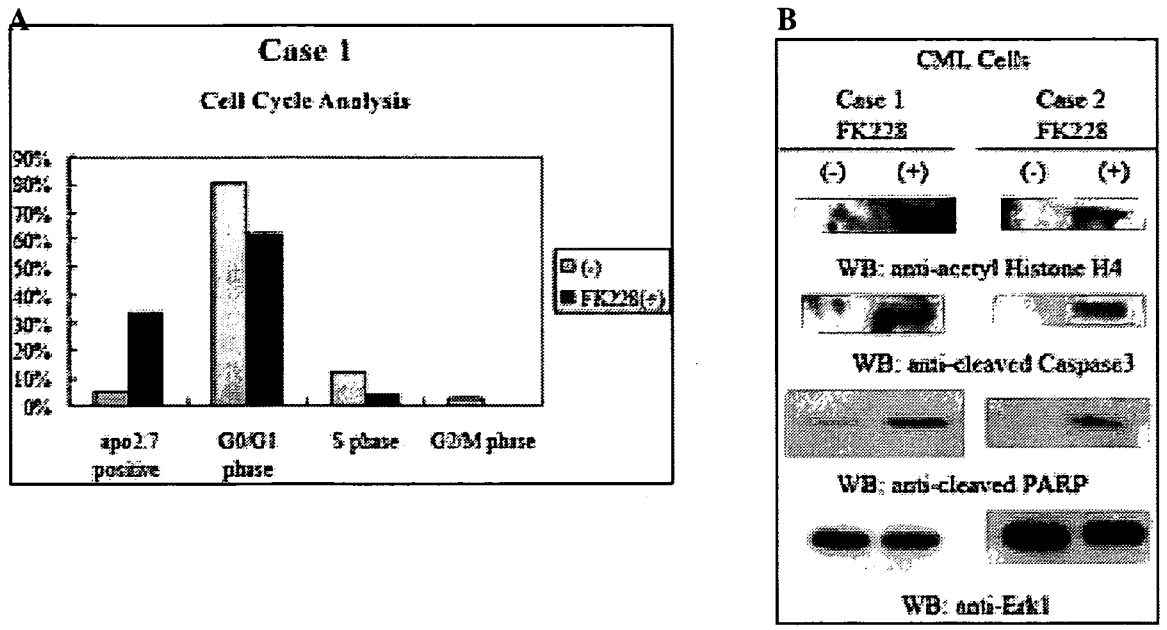


FIG. 6. Effects of FK228 on CML blast crisis cells. (**A**) CML blast crisis cells were treated with 5 ng/ml FK228. Cell cycle analysis was performed. (**B**) Lysates from CML blast crisis cells that were treated with or without 5 ng/ml FK228 for 24 h and were immunoblotted using the indicated antibodies.

treated TF-1 BCR/ABL cells were harvested, and the kinase activity of Erk, and p38MAPK was evaluated. Erk activities were time (Fig. 4A) and dose-dependently (Fig. 4B) reduced after FK228 treatment, but p38MAPK activity increased.

We examined the role of p38 in FK228-mediated effects because p38 was activated after FK228 treatment and p38 regulates G_2/M checkpoint activation (22). TF-1 BCR/ABL cells were treated with a specific p38 inhibitor, SB203580, and/or FK228, and cell cycle status was checked. SB203580 (10 μM) was not toxic to the cells, nor did it inhibit apoptosis, or the percent of cells in the G_2/M phase, compared to that of FK228 alone (Fig. 4C). Moreover, acetylation of histone H4 was not enhanced, and caspase activity was not inhibited by SB203580 (Fig. 4D). These results indicate that p38 does not regulate aceylation of histone H4, the G_2/M checkpoint, or apoptosis induced by FK228.

hTERT activity influences apoptosis induced by FK228

IC_{50} values of FK228 for human tumor cells are in the order of several ng/ml, whereas they are in the range of 1,000 ng/ml for human normal fibroblast cells (23). We noted above that TF-1 BCR/ABL cells were more sensitive to induction of apoptosis by FK228 than parental cell line, TF-1 (Fig. 1C). hTERT, the catalytic subunit of telomerase, is highly expressed in most human tumors (24), and ectopic expression of hTERT has been shown to facilitate immortalization of human cells (25). Moreover, transcription of the *hTERT* gene is regulated though recruitment of HAT and HDAC (26). We examined the relationship between hTERT and the HDAC inhibitor, FK228, as the E2F-pocket protein HDAC complex regulates activation of hTERT (27). ShRNA of hTERT was transfected into the BCR/ABL-expressing CML cell line, K562. These cells showed reduced telomerase activity (Fig. 5A), and increased apoptosis (clones 2 and 4 were PD10) (Fig. 5B), in comparison to the parental cells. shRNA-transfected K562 cells and wild-type cells were treated with 5 ng/ml FK228 for 24 h and checked for apoptosis and acetylation of histone H4. Although there was no difference in the percentage of apoptotic cells between hTERT ShRNA-transfected cells and control-transfected K562 cells (Fig. 5B), the previously noted elevated apoptosis rate was reduced in cells transfected with hTERT shRNA (Fig. 5C). Thus, hTERT acts to attenuate apoptosis induced by FK228.

FK228 induces apoptosis of blast crisis cells from patients with CML

To investigate effects of FK228 on primary CML cells, cells were isolated from peripheral blood of 2 patients with imatinib-resistant blast crisis CML. Mutation of codon 244ATG(Met) to GTG(Val) was found in cells of patient 1 and no mutation was found in cells of patient 2. Cells were incubated with or without 5 ng/ml FK228 for 24 h. Apoptosis was induced in cells of the CML pa-

tient (Case 1) in blast crisis after treatment with FK228 (Fig. 6A). We also found that histone H4 was acetylated and caspase 3 and PARP were cleaved (Fig. 6B). These results demonstrate that FK228 is also effective as an inhibitor of primary imatinib-resistant blast crisis cells from patients with CML, and that effects on intracellular signaling were similar to that seen with CML cell lines.

DISCUSSION

There are a few reports that other HDAC inhibitors, such as suberoylanilide hydroxamic acid (SAHA), LAQ824, and LBH589, are effective against CML cells (28–30). Our study demonstrates that treatment of BCR/ABL-expressing cells with FK228 strongly inhibits cell growth by arresting cells in the G_2/M phase. Western blot analysis revealed that FK228 induced histone H4 acetylation and decreased BCR/ABL protein concentration in BCR/ABL-expressing cell lines. By using the AntibodyArray system, we found that FK228 induced acetylation of several proteins (Fig. 2C). These acetylated proteins were categorized as cell cycle-related proteins and cell adhesion proteins. BCR/ABL, p53, FAK, Rb, paxillin, and HSP-90 were acetylated in response to FK228. Thus, destabilization of BCR/ABL protein, as well as acetylation of intracellular proteins, may be related to inhibition of cell growth and apoptosis in TF-1 BCR/ABL cells. The apoptosis-inducing effect of FK228 was confirmed by experiments using cells from imatinib resistant patients with CML in the blast crisis phase of their disease.

Gene expression of IAP family members, such as cIAP-1, survivin, and apollon, were down-regulated by 24 h of FK228 treatment. IAPs are a family of antiapoptotic proteins that bind to and inhibit caspases-3, -7, and/or -9; they also modulate cell division and cell cycle progression (31). IAP family members are implicated in both the control of apoptosis and the regulation of cell division; hence, they may have the potential to function as a target for cancer treatment (21). We observed that apoptotic cell death significantly increased after FK228 treatment in a time and dose-dependent manner by measuring poly(ADP-ribose) polymerase and caspase-3 and caspase-9 cleavage. Expression of IAP family members was clearly reduced by FK228. Thus, FK228 decreased concentrations of IAP family members and increased activation of caspases and subsequent apoptosis of TF-1 BCR/ABL cells.

Stress causes activation of complex signal transduction pathways, which eventually shape responses of cells and organisms. FK228 reduced Erk activation, but increased activation of p38MAPK. It has been reported that the proteasome inhibitor, bortezomib, induces JNK and p38MAPK activation. It also down-regulates Erk activation in CML cell lines (32). In our system, different members of the MAPK family participated in generation of FK228 responses. Topoisomerase II and histone deacetylase inhibitors delay G_2/M transition by triggering the p38MAPK checkpoint pathway (33). We found that FK228 induced p38MAPK activation, but observed no differences in the cell cycle status or apoptosis using specific p38MAPK inhibitor, SB203580. Thus, even though FK228 induced G_2/M arrest and activated p38MAPK; other molecules may be involved in cell cycle arrest by FK228.

We noted that increases in apoptosis were reduced in hTERT shRNA-transfected CML cells after FK228 treatment, and that FK228 was more effective on TF-1 BCR/ABL cells than on the parental cell line TF-1. This suggests that FK228 has relatively more selective, suppressive effects on cells expressing BCR/ABL.

Changes in telomere functions and associated chromosomal abnormalities have been implicated in human aging and cancer (34). hTERT is the key determinant of enzymatic activity of human telomerase. Overexpression of hTERT may act as a signal of over-proliferation; most cancer cells express hTERT and are telomerase positive (35). Histone acetylation regulates *hTERT* transcription, because treatment of human cells with the HDAC inhibitor, Trichostatin A (TSA), activated *hTERT* promoter activity and induced hTERT mRNA expression. This suggests that HDAC plays a central role in regulation of hTERT (36–39). Moreover, the Sp1 transcription factor has been identified as a potential activator of *hTERT* transcription, and Sp1 also recruits HDAC or HAT activity to the *hTERT* promoter (40,41). In our experiments, acetylation of histone H4 was not noticeably different between the cell line transfected with hTERT ShRNA and control cells after treatment with FK228. However, we did observe that the high hTERT activity cell line was more sensitive to FK228 inhibition. ShRNA mediated-suppression of hTERT reduced the sensitivity of the CML cell line K562 to FK228 without affecting the levels of histone H4 acetylation. Our results indicate that expression of telomerase may predispose CML cells to apoptosis induced through HDAC inhibition.

The interaction of cell survival and death pathways suggests that methods to manipulate them may be useful in therapy. We found that an HDAC inhibitor, FK228, decreased growth and increased apoptosis in the CML cell line TF-1 BCR/ABL. The same was true for primary cells from patients with CML who were resistant to imatinib. Clinically, 10–15% of CML patients treated with imatinib as first-line therapy will have disease progression. Imatinib resistance may be multifactorial, including BCR/ABL mutations of the kinase domain interfering with imatinib binding. BCR/ABL mutations are found more frequently in accelerated phase and blast crisis (42,43). We demonstrated that FK228 is also effective in enhancing apoptosis of primary cells from imatinib-resistant blast crisis CML. These results may be of potential value for the therapy of CML

and will support future investigations of mechanisms by which HDAC inhibitors act.

ACKNOWLEDGMENTS

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Uncontrolled thrombocytosis in polycythemia vera is a risk for thrombosis, regardless of JAK2^{V617F} mutational status

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The JAK2^{V617F} mutation is found in the vast majority of patients with polycythemia vera (PV) and this change is proposed to be one of diagnostic tools for PV,[1] while the presence of idiopathic erythrocytosis with other JAK2 mutations is currently demonstrated.[2] PV patients with JAK2^{V617F} have significantly higher leukocytes and platelets, and a higher frequency of palpable splenomegaly at the time of diagnosis.[3,4] It is reported that thrombotic events in PV patients are not linked to JAK2^{V617F} mutational status.[3,5] Moreover, although thrombosis is a major complication in PV patients,[1] no hematologic parameters during followup could be shown to predict thrombosis.[1,6] We, therefore, assessed the association between thrombosis and hematologic indicators both at diagnosis and during the courses of PV patients, in combination with JAK2^{V617F} mutational status, to find out the possible risk factor for thrombosis in PV patients.

We analyzed 33 patients with PV (followed for at least 1 year) based on the PV Study Group criteria: seven patients were non-WHO PV and the remaining 26 met the World Health Organization (WHO) criteria. The JAK2^{V617F} mutation was determined by using the sequence-specific primer-single molecule fluorescence detection assay.[3] As reported previously, PV patients with JAK2^{V617F} ($n=24$) had a significantly higher leukocyte count ($P<0.0001$) and platelet ($P<0.0001$) at the time of PV diagnosis, while there was no significant difference in the frequency of thrombotic episodes (1/9 versus 5/24: $P=0.5190$) (Supplementary Table 1).[3] None of the 33 PV patients showed MPL 515 mutation (data not shown). The frequency of thrombosis (3/17 versus 2/7: $P=0.5492$) and initial hematologic parameters did not show any significant difference between PV patients with heterozygous and homozygous JAK2^{V617F}. We next compared the maximal levels of hematologic data during followup in PV patients with or without thrombotic episodes. Notably, PV patients with thrombosis ($n=6$) had a significantly higher platelet count during their courses ($987\pm460\times10^9$/l versus $604\pm335\times10^9$/l: $P=0.0349$), while the initial platelet count between these two groups did not show any significant difference ($P=0.3722$) (Table 1). This tendency was also evident in PV patients with JAK2^{V617F} ($P=0.0612$). The frequency of thrombosis was not influenced by the anti-thrombotic treatment ($P=0.0742$ in total PV, and $P=0.2611$ in PV with JAK2^{V617F}).

The presence of JAK2^{V617F} mutation was related to initial platelet counts in PV patients,[3] but not to thrombotic episodes. In the current study, the increased amount of platelets during follow-up (delta platelets = maximal platelets during follow-up – initial platelets) correlated with thrombotic episodes ($P=0.0318$), but did not show any significant association with the JAK2^{V617F} mutational status (Table 1). Five of six patients with thrombosis had additive elevated platelet counts of $>250\times10^9$/l during the follow-up period from the base-line platelets. This aspect requires further confirmation using large cohort studies, since we encountered only 6/33 PV patients with thrombosis. Our data indicate that uncontrolled thrombocytosis, despite administration of cytoreductive chemotherapy, may be a risk factor for developing thrombosis.

Management for PV patients focused on the reduction of hematocrit level for less than 45% in men and less than 42% in women by either phlebotomy or administration of hydroxyurea.[1] Although aspirin administration is recommended for PV patients,[1] aspirin therapy might be insufficient to prevent thrombosis in some PV patients with prominent thrombocytosis. Moreover, the timing of thrombosis in PV patients did not coincide with the maximal platelet count during followup (data not shown), in agreement with other reports.[6] Finazzi *et al.*[7] reported that the risk of thrombosis in JAK2^{V617F}-positive PV was 3.63-fold that of those with wild-type JAK2 essential thrombocythemia (ET), while Tefferi *et al.*[5] found no association between JAK2^{V617F} mutational status and bleeding or thrombotic history. Vannucchi *et al.*[8] also confirmed the lack of any difference in the frequency of thrombosis between PV patients with heterozygous and homozygous JAK2^{V617F}. Some JAK2^{V617F}-positive PV cases show hematologic transformation among CMPD and tend to exhibit thrombosis or myelofibrosis during their courses;[1,3,4] Di Nisio *et al.*[6] did not find any association between platelet count during follow-up and thrombotic events

Table 1 Clinical and hematologic features of polycythemia vera associated with thrombosis

	Thrombosis	*No thrombosis*	*P-value*
Total PV patients ($n=33$)	6 (18.2%)	27 (81.8%)	
Age (years)	56.2±6.9	56.6±13.1	0.9343
Gender (M/F)	2/4	19/8	0.088
Initial hematologic findings			
Leukocytes ($\times 10^6$/l)	11260±3659	12455±5698	0.6567
Hemoglobin (g/dl)	19.0±1.5	18.7±2.1	0.7476
Hematocrit (%)	58.1±2.0	56.5±7.3	0.6349
Platelets ($\times 10^9$/l)	555±275	446±240	0.3722
Maximum hematologic level during course			
Leukocytes ($\times 10^6$/l)	15420±5278	13740±6431	0.5884
Hemoglobin (g/dl)	19.1±1.4	19.0±1.6	0.8452
Hematocrit (%)	58.1±2.0	56.3±7.9	0.6209
Platelets ($\times 10^9$/l)	**987±460**	**604±335**	**0.0349**
Delta platelets[a]	**433±352**	**155±230**	**0.0318**
Palpable splenomegaly	4/6	12/27	0.3245
Requirement of chemotherapy	4/6	22/27	0.422
Prophylactic anti-coagulant therapy	6/6	17/27	0.0742
Abnormal karyotypes	**2/6**	**1/24**	**0.0332**
Evolution of myelofibrosis	2/6	3/27	0.1697
Evolution of acute leukemia	**2/6**	**0/27**	**0.002**
$JAK2^{V617F}$ (wild/hetero/homo)	1/3/2	8/14/5	0.6658
K2(T)%/K2(G)% × 100 (mean±s.d.)[b]	214.1±199.6	119.0±89.4	0.0753
PV with $JAK2^{V617F}$ ($n=24$)	5 (21%)	19 (79%)	
Age (years)	55.2±7.2	59.5±12.8	0.4833
Gender (M/F)	1/4	11/8	0.1316
Initial hematologic findings			
Leukocytes ($\times 10^6$/l)	12775±1597	15062±4581	0.3429
Hemoglobin (g/dl)	18.4±0.9	18.6±2.1	0.8804
Hematocrit (%)	57.5±1.6	57.9±5.8	0.8805
Platelets ($\times 10^9$/l)	662±155	552±211	0.3408
Maximum hematologic level during course			
Leukocytes ($\times 10^6$/l)	16950±4641	16231±6117	0.8282
Hemoglobin (g/dl)	18.6±0.6	18.8±1.5	0.796
Hematocrit (%)	57.9±1.1	58.2±5.9	0.7604
Platelets ($\times 10^9$/l)	1104±437	753±294	0.0612
Delta platelets[a]	442±406	200±268	0.1547
Palpable splenomegaly	4/5	10/19	0.2694
Requirement of chemotherapy	**4/5**[c]	**19/19**	**0.0464**
Prophylactic anti-coagulant therapy	5/5	15/19	0.2611
Abnormal karyotypes	**2/5**	**1/19**	**0.0431**
Evolution of myelofibrosis	2/5	3/19	0.2356
Evolution of acute leukemia	**2/5**	**0/19**	**0.0040**
K2(T)%/K2(G)% × 100 (mean±s.d.)[b]	256.3±191.0	165.1±62.9	0.0812

Abbreviations: F, female; M, male; PV, polycythemia vera.
Bold values indicate statistical significance.
[a]Delta platelets = maximal platelets during follow-up minus initial platelets.
[b]Calculated by the sequence-specific primer-single molecule fluorescence detection assay.
[c]One patient refused cytoreductive medication.

in PV. It is recommended to control the platelet count to under 400×10^9/l even in PV patients,[1] however, cytoreductive treatment sometimes fails to control platelets at any appropriate level, due to paradoxical over-reduction of red blood cells. These data indicate that control of platelet counts during followup by some approaches, for example, combination of hydroxyurea and interferon or switching to interferon, might be the next issue to examine concerning the prevention of thrombosis in PV patients, regardless of the $JAK2^{V617F}$ mutational status. The risk of thrombosis in PV might be linked to elevated platelet level during the course, but to neither initial platelet count nor $JAK2^{V617F}$ mutational status. This situation is different from ET, in which $JAK2^{V617F}$ mutation is a key change predictive of thrombosis. Further studies are required to clarify the candidates linking uncontrolled thrombocytosis to thrombosis in PV patients.

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achieved morphologic and cytogenetic remission after 2 months of treatment with lenalidomide therapy and a second patient who achieved a platelet response after only 1 month of therapy before receiving an unrelated allogeneic stem cell transplant. Both patients remain without any evidence of relapse with a maximum follow-up of 8 months.

Both radiation therapy and traditional DNA-interactive antineoplastics, such as alkylating agents and topoisomerase II inhibitors, are known genotoxins with the potential to induce MDS or acute myeloid leukemia (AML) that commonly harbors a chromosome 5q deletion with high frequency of evolution to AML and short overall survival.[4–6] Our findings indicate that lenalidomide has therapeutic potential in patients with secondary MDS with complex karyotype accompanied by chromosome 5q deletion.

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Recurrent chromosomal aberration at 12q15 in chronic idiopathic myelofibrosis with or without JAK2^{V617F} mutation

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Mutation of JAK2^{V617F} is currently known to play a potential role in the development of chronic myeloproliferative disorders (CMPD);[1] 78% (393/506) of polycythemia vera (PV) cases have JAK2^{V617F}, while only 43% (55/127) of reported chronic idiopathic myelofibrosis (CIMF) cases have JAK2^{V617F}.[2] Thus the question naturally arises whether the CIMF patients without JAK2^{V617F} might have another pathway towards myelofibrosis or a common pathogenic factor may exist with or without the

Table 1 JAK2-V617F status and cytogenetic results at the time of myelofibrosis

Case no.	*Age at diagnosis/sex*	*Cytogenetics at the time of myelofibrosis*	*JAK2 V617F*
Idiopathic myelofibrosis			
JAK2_0048	41/male	46,XY,**t(1;12)(p34;q15)**[10]	G/G
JAK2_0039	56/male	46,XY,del(11)(q13)[18]/46,XY[3]	G/G
JAK2_0057	78/female	46,XX[21]	G/G
JAK2_0098	67/male	46,XY,del(20)(q11)[5]/46,XY[3]	G/G
JAK2 0112	33/male	46,XY[23]	G/G
JAK2_0163	63/female	46,XX,**t(12;20)(q15;q11)**[7]/47,XX,+9[10]	G/T
JAK2_0036	54/male	46,XY,del(20)(q11)[2]/ 46,XY,idem,t(2;17)(q24;q22)[13]/ 46,XY,idem,i(17q)[5]	G/T
JAK2_0105	70/male	46,XY,add(9)(p21)[16]	G/T
JAK2_0148	56/male	46,XY[21]	T/T
Myelofibrosis with prior history of myelodysplastic syndrome			
JAK2_0021	70/male	46,XY,**t(4;12)(q27;q15)**[22][a]	G/T
Polycythemia vera developing myelofibrosis			
JAK2_0042	55/female	46,XX,del(7)(q22)[9]/45,X,add(X)(p22),−18[6]/ 46,XX[5]	G/T
JAK2_0061	60/female	46,XX,tan(1q12–1qter)[8]/46,XX[1]	G/T
JAK2_0065	43/female	43,XX,−1,−3,−7,−9,−10,−12,−13, −16,+5m[13]/46,XX[8]	T/T
JAK2_0118	46/female	46,XX[20]	T/T
JAK2_0141	62/male	46,XY[20]	T/T

Table 1 *Continued*

Case no.	*Age at diagnosis/sex*	*Cytogenetics at the time of myelofibrosis*	*JAK2 V617F*
Essential thrombocythemia developing myelofibrosis			
JAK2_0013	58/female	46,XX[13]	G/G
JAK2_0035[b]	49/male	46,XY,t(2;5)(p16;q14),add(11)(q23)[23]	G/G
JAK2_0005[b]	57/male	46,XY,+1,der(1;7)(q10;p10),del(20)(q11)[20]/ 46,XY,idem,add(18)(p11)[2]/46,XY[4]	G/T
JAK2_0034	67/male	46,XY,i(7q)[6]/48,XY,+8,+21[9]/46,XY[6]	G/T
JAK2_0054[b]	59/male	46,XY,+1,der(1;7)(q10;p10)[9]/46,XY[3]	G/T
JAK2_0055	76/male	46,XY,add(18)(p11)[20]	G/T
JAK2_0158	46/female	46,XX[17]	G/T

[a]This case was reported as myelodysplastic syndrome developing myelofibrosis with a 6-year interval;[5] however, the continuity of the disease is uncertain.[3]
[b]JAK2_0035 was UPN-12, JAK2_0005 was UPN-6 and JAK2_0054 was UPN-8 in Hsiao *et al.*[6]
Bold signifies chromosomal translocations involving 12 of 15 region.

presence of $JAK2^{V617F}$. During the series of mutational assay of $JAK2^{V617F}$ in CMPD using the sequence-specific primer-single molecule fluorescence detection assay,[3] we studied cytogenetic changes in nine patients with CIMF, 51 with essential thrombocythemia (ET) and 34 with PV. Myelofibrosis developed in seven of 51 patients (13.7%) with ET and five of 34 patients (14.7%) with PV, and we compared clinical and cytogenetic changes between patients with CIMF and those with myelofibrosis developing in PV/ET.

The group of patients with myelofibrosis associated with PV/ET had high incidences of history of thrombosis (4/12 versus 0/9; $P=0.0542$), requirement of cytoreductive chemotherapy (12/12 versus 5/9; $P=0.0103$) and acute leukemia development (7/12 versus 1/9; $P=0.0274$) compared with the CIMF group. Depending on the status of $JAK2^{V617F}$, the group of patients with myelofibrosis associated with PV/ET with GT/TT mutation of JAK2 had a high incidence of chemotherapy requirement (10/10 versus 2/4; $P=0.0157$) and tended to have a frequent thrombosis history (4/10 versus 0/4; $P=0.1345$) (Supplementary Table 1). However, there was no particular difference in the percentage of abnormal karyotypes at the time of myelofibrosis according to CIMF diagnosis or the mutational status of $JAK2^{V617F}$. We also noted a high frequency of myelofibrosis development in patients with $JAK2^{V617F}$ in PV (wild-type JAK2/heterozygous $JAK2^{V617F}$/homozygous $JAK2^{V617F}$: 0/9 versus 2/18 versus 3/7: $P=0.0460$), but not in ET (2/18 versus 5/30 versus 0/1: $P=0.7970$), in agreement with the report by Kralovics *et al.*[4]

In myelofibrosis patients, we noticed that two of the nine patients with CIMF had chromosome abnormalities at the 12q15 region; one had t(1;12)(p34;q15), while the other had t(12;20)(q15;q11?) (Table 1 and Figure 1). Another patient (JAK2_0021) reported as showing t(4;12)(q31;q21) had a prior history of myelodysplastic syndrome (MDS)-refractory anemia with a normal karyotype 6 years before myelofibrosis.[5] Re-assessment by the spectral karyotypic analysis revealed that this anomaly was t(4;12)(q27;q15) (Supplementary Figure 1). In contrast to the results of CIMF, no patients with myelofibrosis developing from PV/ET had 12q15 anomaly. Of the 12 patients with myelofibrosis associated with PV/ET, four had −7/7q−, including two with der(1;7)(q10;p10); both patients with der(1;7)(q10;p10) had a prior ET diagnosis[6] with heterozygous $JAK2^{V617F}$ (Table 1). Of the nine patients with CIMF and one myelofibrosis associated with MDS, four had heterozygous and

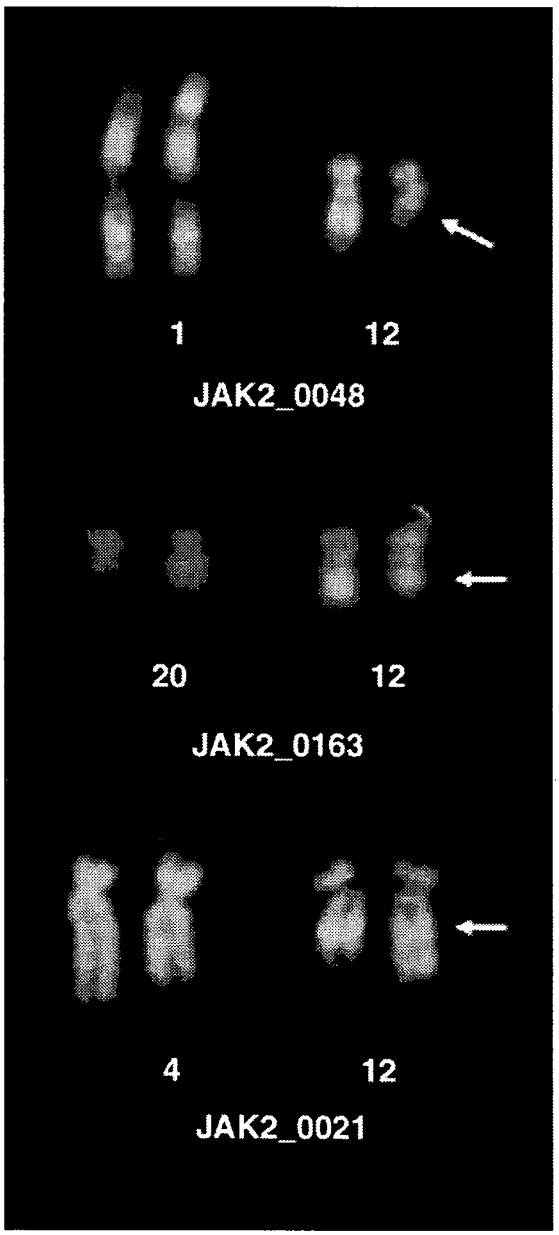

Figure 1 Partial quinacrine-banded karyotypes obtained from two patients with chronic idiopathic myelofibrosis (JAK2_0048 and JAK2_0163) and one patient with myelofibrosis with prior history of myelodysplastic syndrome (JAK2_0021) showing 12q15 anomalies, that is, t(1;12)(p34;q15), t(12;20)(q15;q11?) and t(4;12)(q27;q15). Arrows indicate possible breakpoint of 12q15.

one had homozygous $JAK2^{V617F}$; the 12q15 anomaly was detected in one patient with wild-type JAK2, while two had heterozygous $JAK2^{V617F}$.

Cytogenetic changes in CIMF are well documented: +8, del(20q), −7/7q−, del(11q) and del(13q) are known to be recurring nonspecific cytogenetic abnormalities, and some of them are also detectable in PV or ET patients. In the literature, Andrieux *et al.*[7] reported a possible role in the association between *HMGA2* and translocation involving 12q15 in CIMF. In the current study, we found that 12q15 anomaly does not depend on the JAK2 mutational status; thus genetic anomaly, independent to $JAK2^{V617F}$, may exist in CIMF, and molecular study on the 12q15 region, including *HMGA2*,[8] may disclose another pathogenetic pathway in CIMF. The 12q15 chromosomal abnormality was recurrently detected in patients with CIMF, while der(1;7)(q10;p10) was only noted in ET patients who had myelofibrosis with $JAK2^{V617F}$.[6] These findings clearly indicate that myelofibrosis among CMPD might be cytogenetically heterogeneous.

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Neighboring adipocytes participate in the bone marrow microenvironment of multiple myeloma cells

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Multiple myeloma (MM) is a hematological malignancy, characterized by the accumulation of monoclonal plasma cells in the bone marrow (BM). MM disease progression has been recently recognized as the result of an evolving crosstalk between different cell types within the BM. Although genetically abnormal plasma cells define the tumor compartment itself, the surrounding and interwoven stroma provides the supporting framework of the tumor. This framework includes extracellular matrix proteins, secreted growth factors and cellular interactions with fibroblasts, macrophages, endothelial cells, osteoblasts and osteoclasts.[1] Little attention has been given to another cell type present in the BM cavity: namely the adipocyte. These are absent in the BM of a new-born individual; however, their number increases with advancing age, resulting in adipocytic deposits occupying up to 70% of the BM cavity in elderly persons. MM is typically a disease of the elderly with a median age of diagnosis of 65 years and the incidence increases with age. Knowing that with advancing age, the BM cavity is filled with adipocytes and that MM cells closely interact with their neighboring cells, we assumed functional interactions between BM adipocytes and MM cells. We studied these interactions using the 5T33MM model and the human MM5.1 cell line. In this study, we further tried to characterize the secreted cytokines and explored the potential role of leptin in mediating the effects of adipocytes. We finally evaluated the expression of leptin receptor on both murine and human MM cells and tried to correlate this with different clinical parameters.

From the observation that MM cells, at interstitial disease stages, can be found in close contact with adipocytes, functional interactions between these cells are reasonable and prompted us to start *in vitro* tests. The murine BM adipocytic cell line 14F1.1 (obtained from Professor Zipori D, Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot, Israel) and primary isolated human adipocytes were used. The 14F1.1 cells initially have a

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Detection of *NOTCH1* Mutations in Adult T-Cell Leukemia/Lymphoma and Peripheral T-Cell Lymphoma

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Abstract

We analyzed *NOTCH1* gene mutation in 53 adults with mature T-cell leukemia/lymphoma: 21 patients with adult T-cell leukemia (ATL), 25 with T-cell non-Hodgkin's lymphoma (T-NHL), and 7 with T-cell prolymphocytic leukemia. We detected a nonsense mutation, C7249T (resulting in Q2417X, where X is a termination codon) in the PEST domain of *NOTCH1* in an ATL patient and detected a 3-bp deletion (positions 7234-7236) that resulted in deletion of a proline codon at codon 2412 in the PEST domain of *NOTCH1* in a patient with a T-NHL, peripheral T-cell lymphoma–unspecified (PTCL-u). We also analyzed the expression of *NOTCH1* target genes (*HES1*, *CCND1*, and *MYC*), all of which were expressed in the sample of the PTCL-u patient with the *NOTCH1* mutation, but found only *MYC* to be expressed in the sample from the ATL patient. These findings suggest that nonsense mutation in the PEST domain in the ATL case was associated with NOTCH1 signaling through a pathway different from that for T-cell acute lymphoblastic leukemia (T-ALL). Although *NOTCH1* mutation occurs infrequently in mature T-cell leukemia/lymphoma, *NOTCH1* may be involved in leukemogenesis associated with various forms of T-cell leukemia/lymphoma rather than only with T-ALL.

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Key words: NOTCH1; Mutation; T-cell leukemia/lymphoma; Adult T-cell leukemia/lymphoma; Peripheral T-cell lymphoma–unspecified

1. Introduction

T-cell non-Hodgkin's lymphomas (T-NHLs) are relatively uncommon malignancies that represent approximately 12% of all lymphomas [1]. The current classification of the World Health Organization (WHO)/European Organization for Research and Treatment of Cancer recognizes 9 clinicopathologically distinct peripheral T-NHLs. In Japan, the frequency of T-NHL is higher, because adult T-cell leukemia/lymphoma (ATL) accounts for as many as 7.5% of all NHLs in this country [2]. The identification of genetic abnormalities and chromosomal rearrangements has been helpful for recognizing and defining lymphoma entities according to the WHO classification. Nonrandom cytogenetic abnormalities have been detected, with the notable exceptions of the t(2;5)(p23;q35) creating the *NPM-ALK* fusion gene and its variants in anaplastic large cell lymphoma (ALCL) and the inv(14)(q11q32) expressing and activating the *TCL1* gene in T-cell prolymphocytic leukemia (T-PLL), another type of mature T-cell leukemia/lymphoma [3,4]. Such rearrangements involve 1p, 1p22, 2q, 3q, 14q, and 14q32 in ATL, and trisomies of chromosomes X and 3 in angioimmunoblastic T-cell lymphoma [5-9]; however, which molecular changes play a role in leukemogenesis and progression is still not known.

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NOTCH1, a transmembrane receptor in hemopoiesis that regulates lymphoid progenitor cells and T-cell fate [10-14], was discovered as a partner gene in t(7;9)(q34;q34), which is found in <1% of T-cell acute lymphoblastic leukemia (T-ALL) cases [15]. In T-ALL, chromosomal translocations involving the T-cell receptor α/δ locus on chromosome 14 and the T-cell receptor β locus on chromosome 7 have been observed, but only in a relatively small patient population. NOTCH1 is also essential for various tissues and organs because it affects cell differentiation, proliferation, and apoptotic programs [16,17].

Notch1 is required at multiple stages of early T-cell development, including the decision to develop along either the T-cell or the B-cell lineage [12], and has been implicated in later stages of T-cell development, mostly in the transition from $CD4^-CD8^-$ to $CD4^+CD8^+$ cells [10]. Furthermore, Notch1 promotes αβ-lineage T-cell development at the expense of γδ-lineage T-cells and is involved in the selection of CD4 or CD8 single-positive thymocytes [11]. Recently, it has become increasingly apparent that Notch can have a profound effect on cell fate determination during the differentiation of mature T-cells. Of the Notch ligand families, Delta reportedly induces type 1 helper T-cells (Th1), and Jagged induces the alternative Th2 fate [18]. γ-Secretase inhibitor has been reported to block Th1 polarization [19].

Weng et al showed that the enforced NOTCH1 pathway is an effective inducer of T-ALL in mice [20] and reported that more than 50% of human T-ALL cases possess activating mutations in the extracellular heterodimerization domain (HD) and in the terminal PEST domain of *NOTCH1* [21]. Recently, *NOTCH1* mutation has been found to be an important prognostic marker for T-ALL [22,23]. To date, however, *NOTCH1* mutation has not been investigated in other hematologic malignancies, including mature T-cell leukemia/lymphoma. Although mature T-cell leukemia/lymphomas differ from T-ALL in terms of maturation, NOTCH1 regulates T-cell fate and may be associated with the development of both mature and immature T-cell leukemia/lymphoma. In this study, we demonstrated that *NOTCH1* mutations are associated with ATL and T-NHL cases of mature T-cell leukemia/lymphoma, as well as with T-ALL of immature T-cell leukemia.

2. Materials and Methods

2.1. Patients

We studied 53 patients with mature T-cell leukemia/lymphoma (21 with ATL, 7 with T-PLL, and 25 with T-NHL, the latter comprising 4 cases of angioimmunoblastic T-cell lymphoma, 1 of ALCL, 13 of peripheral T-cell lymphoma-unspecified [PTCL-u], 5 of cutaneous T-cell lymphoma [CTCL], and 2 of natural killer/T cell lymphoma). Samples of lymph nodes, peripheral blood, bone marrow, ascites, and pleural effusions containing tumor cells were obtained from these 53 patients, who were treated at Kyoto Prefectural University of Medicine Hospital, Kyoto First Red Cross Hospital, Kagoshima University Hospital, Otsu Municipal Hospital, and Kyoto Prefectural Yosanoumi Hospital. The diagnosis of ATL was based on clinical features, hematologic characteristics, immunophenotype, the presence of serum antibodies to ATL-associated antigens, and monoclonal integration of human T-cell lymphoma/leukemia virus type I proviral DNA. The diagnosis of T-NHL was based on clinical features, pathologic findings, immunophenotype, and rearrangement of the T-cell receptor β gene. Informed consent was obtained from all patients, and the ethics committee of Kyoto Prefectural University of Medicine approved this project.

2.2. DNA Extraction

High molecular weight DNA was extracted from samples of T-cell tumors with the aid of the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA).

2.3. Mutational Analysis

The hot spots of *NOTCH1*-activating mutation in T-ALL cases are located in exons 26 and 27, which encode the N-terminal and C-terminal regions of the HD (ie, HD-N and HD-C), and in exon 34, which encodes the PEST domain and transcriptional-activation domain (TAD). Mutation in these 2 locations was detected by means of polymerase chain reaction (PCR)-based denaturing high-performance liquid chromatography (dHPLC) using a WAVE DNA fragment analysis system equipped with a DNASep HT cartridge (Transgenomic, Omaha, NE, USA). The primer combination for generating amplicons for mutation analysis and the procedures used have been described previously [21].

2.4. Nucleotide Sequence

The PCR products of positive cases detected by PCR-based dHPLC were purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Sequencing by means of fluorescent-dye chemistry was performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with the same primers used for amplification. The PCR products that did not yield valid results were subcloned with a TOPO cloning kit (Invitrogen, Carlsbad, CA, USA) and then sequenced.

2.5. Reverse Transcriptase–PCR Analysis

We obtained tumor RNA from 38 of the 53 T-cell tumors for reverse transcriptase (RT)-PCR analysis. RNA was extracted from the samples of T-cell tumors with the aid of the Isogen LS kit (Nippon Gene, Osaka, Japan), and complementary DNA (cDNA) was generated from 4 μg total RNA with the aid of the Ready-to-Go You-Prime First-Strand Beads kit (GE Healthcare, Piscataway, NJ, USA). The following primers were used: *HES1* sense, AAAAT GCCAGCTGATATAATGGAG; *HES1* antisense, GGTCT GTGCTCAGCGCAGCCGTCA [24]; *MYC* sense, TGAG GAGACACCGCCCAC; *MYC* antisense, ACCCTCTTG GCAGCAGGATAG; *CCND1* sense, CCGTCCATGCG GAAGATC; *CCND1* antisense, CCACTTGAGCTTGTTC ACCA; *NOTCH1* sense, AGACTGGCCCACCTCGTCTCT;

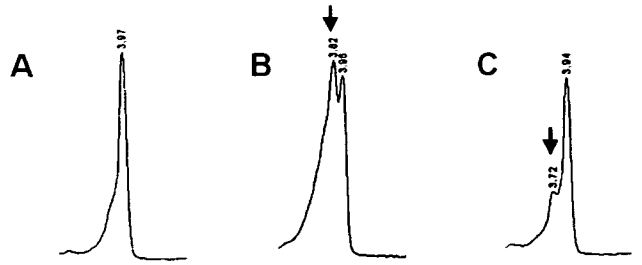

Figure 1. Polymerase chain reaction–based denaturing high-performance liquid chromatography analysis of exon 34 of *NOTCH1*. A, A single peak for wild-type *NOTCH1*. B, Additional peak (arrow) observed in the adult T-cell leukemia patient with mutated *NOTCH1* (case 9). C, Additional peak (arrow) observed in the patient with peripheral T-cell lymphoma–unspecified with mutated *NOTCH1* (case 38).

NOTCH1 antisense, GCTCTCCACTCAGGAAGCTC [21]. β-actin was used as an internal control. PCR products were separated, detected, and quantified with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) in conjunction with DNA 1000 LabChip kits (Agilent Technologies) for the detection of cDNA fragments, as has previously been described [25].

3. Results

3.1. NOTCH1 Mutation Was Detected in an ATL Patient and a PTCL-u Patient

Mutation screening by dHPLC identified 4 positive cases: 1 ATL (case 9), 2 of PTCL-u (cases 37 and 38), and 1 CTCL (case 43) (Figure 1). Nucleotide sequencing of these samples revealed that the patient in case 9 possessed a nonsense mutation, C7249T (resulting in Q2417X, where X is a termination codon), in the PEST domain (Figures 2 and 3) and that case 38 featured a 3-bp deletion (nucleotides 7234-7236) that produced a deletion of a proline residue at codon 2412 in the PEST domain (Figures 2 and 3). Cases 37 and 43 showed no mutation. Furthermore, a heteroallelic single-nucleotide polymorphism, C5097T, was observed in the HD-C domain in 12 (22.6%) of the 53 cases; the homoallelic C5097T single-nucleotide polymorphism was observed in 39 cases (73.6%).

3.2. Expression of NOTCH1 and NOTCH1 Target Genes HES1, MYC, and CCND1, in Mature T-Cell Leukemias/Lymphomas

We used semiquantitative RT-PCR to analyze the expression of *NOTCH1*, *HES1*, *MYC*, and *CCND1* genes in ATL and PTCL-u patient samples with *NOTCH1* mutations and compared the results with those for samples from T-ALL patients with *NOTCH1* mutation and for samples of other T-cell tumors (Figure 4). Expression of *NOTCH1* and *MYC* genes was detected in all of the samples from patients with mature T-cell tumors, although the frequencies of *HES1* and *CCND1* gene expression were relatively low (Table 1). All *NOTCH1* target genes were expressed in the sample from the PTCL-u patient with *NOTCH1* mutation, but only *MYC* was expressed in the corresponding sample from the ATL patient with *NOTCH1* mutation (Figure 4).

4. Discussion

Although the frequent occurrence of *NOTCH1* mutation in T-ALL has been reported [21-23,26,27], including in mouse models of this disease [28,29] (Figure 3), there have been no reports of *NOTCH1* mutation in cases of mature T-cell tumors. In the present study, we identified *NOTCH1* mutations in an ATL patient and a PTCL-u patient.

The deletion of the single amino acid residue observed in the PTCL-u patient was within the PEST domain, and this type of mutation has not been reported previously. In addition, both *NOTCH1* and *HES1* genes were expressed in this patient's lymphoma cells. Expression analysis by semiquantitative RT-PCR demonstrated *NOTCH1* gene expression in all PTCL-u patients examined, but more than half of these PTCL-u patients did not express the *HES1* gene. These findings suggest that *NOTCH1* signaling to *HES1* generally

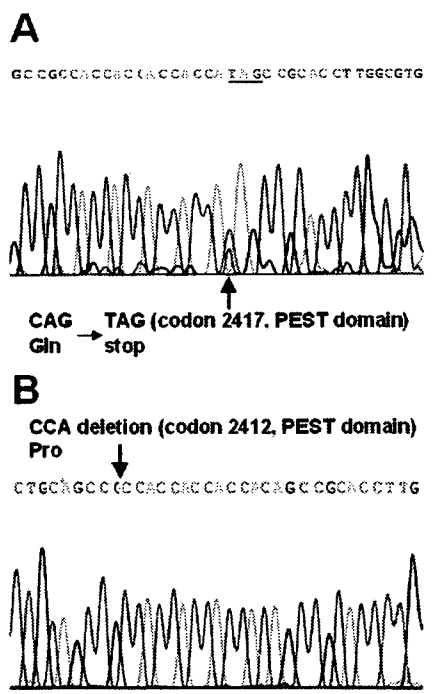

Figure 2. A, Chromatogram of sequencing with fluorescent-dye chemistry for unfractionated genomic DNA from an adult T-cell leukemia patient with a heterozygous C-to-T mutation (arrow) in exon 34 of *NOTCH1* (case 9). B, Sequencing chromatogram of genomic-DNA subcloned from tumor cells from a patient with peripheral T-cell leukemia–unspecified (case 38), showing a 3-bp CCA deletion in exon 34 of *NOTCH1*. Arrow indicates the deleted position.

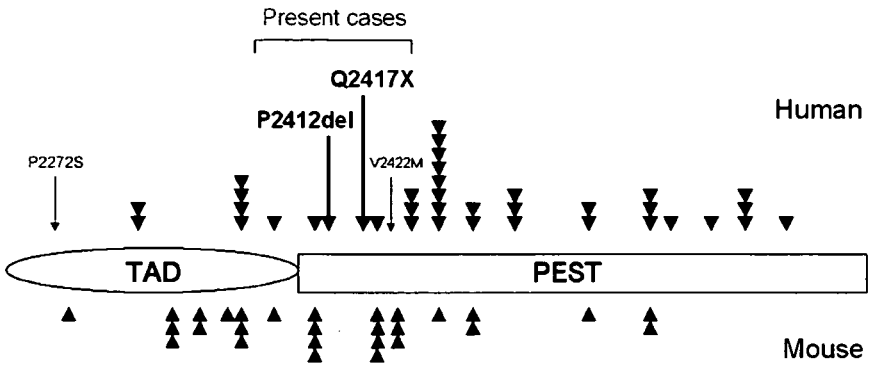

Figure 3. Comparison with previously reported mutations, including those in mouse models. Arrowheads above the schema of part of the NOTCH1 protein indicate the positions of insertions, deletions, and point mutations resulting in premature stop codons that were detected in previous studies [21-23,26,27]. Two missense mutations (P2413S and V2422M) previously reported in human T-cell acute lymphoblastic leukemia (T-ALL) cases [23] are indicated by small arrows. Arrowheads below this schema indicate the positions of mutations observed in murine T-ALL [28,29]. TAD indicates transcriptional-activation domain; PEST, PEST domain.

does not play a critical role in PTCL-u, although NOTCH1 signaling to HES1 may have been activated by the deletion of the single amino acid residue in the PEST domain in the PTCL-u patient with *NOTCH1* mutation.

On the other hand, the nonsense mutation observed in the ATL patient was considered to represent an activating mutation, and such mutations have previously been described [21]. No *HES1* gene expression could be detected, but the *NOTCH1* gene was expressed in the same ATL sample. *NOTCH1* was expressed in all of the ATL patients examined, whereas the frequency of *HES1* gene expression was less than that in the PTCL-u patients. One possible explanation suggested by these findings is that the nonsense mutation observed in the ATL case with *NOTCH1* mutation was not an activating mutation as observed in T-ALL. Another explanation is that an alternative activating pathway of NOTCH signaling may be critical in ATL. The ATL sample with *NOTCH1* mutation in our study was obtained at diagnosis. Interestingly, the patient in this case developed therapy-related myelodysplastic syndrome (MDS) 35 months

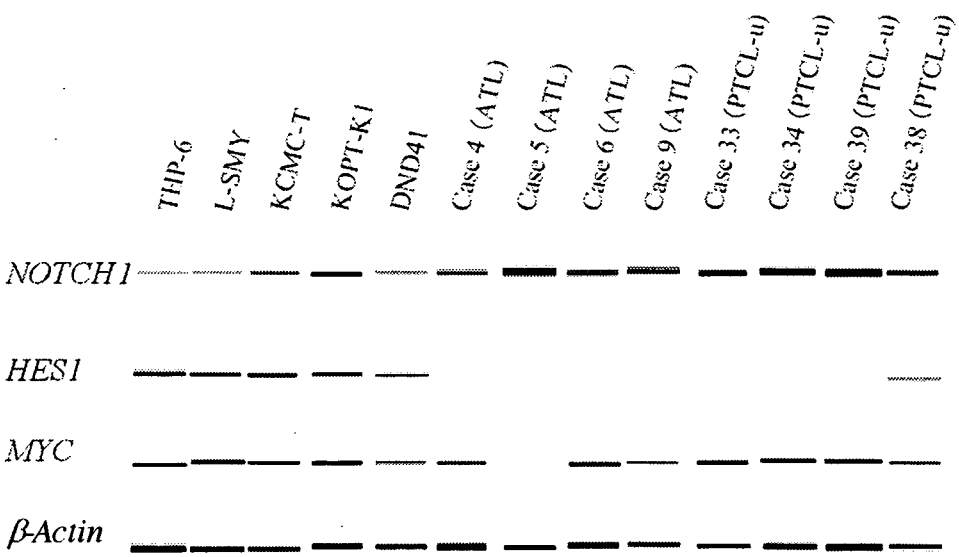

Figure 4. Expression of the *NOTCH1*, *HES1*, *MYC*, and *CCND1* genes by reverse transcriptase–polymerase chain reaction analysis. Two T-cell acute lymphoblastic leukemia (T-ALL) cell lines (KOPT-K1 and DND41) with *NOTCH1* mutation in the PEST domain, case 9 (adult T-cell leukemia [ATL]), and case 38 (peripheral T-cell leukemia–unspecified [PTCL-u]) were examined. The β-actin gene was used as a complementary DNA control. The data for other T-ALL cell lines, ATL patients, and PTCL patients without mutation are also shown. The electrophoresis gel image for the samples was generated with the Agilent 2100 Bioanalyzer in conjunction with the DNA 1000 LabChip kits (Agilent Technologies).

Table 1.
Expression Frequencies of *NOTCH1* Target Genes in Mature T-Cell Tumors (n = 38)*

Disease	Cases, n	Cases Expressed, n (%)			
		NOTCH1	*CCND1*	*HES1*	*MYC*
ATL	13	13 (100)	4 (31)	2 (15)	11 (77)
PTCL-u	11	11 (100)	5 (45)	5 (45)	11 (100)
Others	14	14 (100)	10 (71)	8 (57)	14 (100)
AILT	3	3	3	3	3
ALCL	1	1	0	1	1
CTCL	4	4	2	1	4
NK/T	2	2	2	0	2
T-PLL	4	4	3	3	4

*ATL indicates adult T-cell leukemia/lymphoma; PTCL-u, peripheral T-cell lymphoma–unspecified; AILT, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large T-cell lymphoma; CTCL, cutaneous T-cell lymphoma; NK/T, natural killer/T cell lymphoma; T-PLL, T-cell prolymphocytic leukemia.

after diagnosis; however, the *NOTCH1* mutation in the MDS cells unfortunately could not be analyzed [30]. The remaining 20 ATL cases in our study showed no *NOTCH1* mutation, nor did any other cancer. At present, whether this mutation correlates with the occurrence of MDS or of ATL is not yet clear.

A few cases have shown missense mutation in the TAD or the PEST domain [23], but the biologic significance of these cases has not been determined. These missense mutations are considered to be activating mutations, like the previously reported *NOTCH1* mutations, which caused premature termination and truncation of the PEST domain [21]. Only 1 study (Weng et al [21]), which used transcriptional-reporter assays with NOTCH-sensitive reporters, has found that HD and PEST domain mutations in T-ALL patients activated NOTCH1 function. There have been no reports of a functional analysis of missense mutations in the TAD or the PEST domain [21].

Amplification of *NOTCH1* has also been reported to be involved in enteropathy-type T-cell lymphoma [31]. Furthermore, NOTCH1 up-regulation by ligands Jagged1 and JAG2 has been detected in Hodgkin's lymphoma, ALCL, and multiple myeloma [32,33]. Overexpression of NOTCH ligand Dll4 has been found to induce T-ALL in mice [34].

Although somatic mutations frequently occur in a "hot spot" of the HD domain in T-ALL cases [21-23,26,27], none of the cases in our study showed mutations in this region. In 1 study, the HD domain was found to be conserved and required for stabilizing the association of the extracellular and intracellular subunits, which prevents ligand-independent cleavage [35,36]. A recent analysis showed that T-ALL–associated HD-domain mutations activated *NOTCH1* signaling through an increase in metalloprotease sensitivity and a reduction in heterodimer stability [37]. On the other hand, point mutations, deletions, and insertions in various regions have been found in the PEST domain and the TAD of intracellular *NOTCH1* [21-23,26,27]. The C-terminal PEST-like domain of NOTCH has been reported to contribute to the instability and degradation of intracellular NOTCH by the ubiquitin-proteasome pathway, thus preventing potentially deleterious transcription [38,39]. In mice, a Notch1 C-terminal deletion mutant accelerated lymphoid oncogenesis, with the resulting loss of PEST sequences reducing the rate of Notch1 turnover and elevating Notch1 activity [40]. Another study found that the ankyrin-repeat domain and TAD are required for induction of T-ALL in a murine model [41]. NOTCH1 is also considered to contribute to tumorigenesis via nuclear localization of intracellular NOTCH [42]. Intracellular Notch reportedly must accumulate in the nucleus to induce transformation of the E1A-immortalized baby rat kidney cell line (RKE), whereas transformation is likely to be independent of CBF1 activation [42].

NOTCH1 germline mutation was recently detected in aortic valve disease [43]. NOTCH1 mutation was found to cause a spectrum of developmental aortic valve anomalies and severe valve calcification in nonsyndromic autosomal-dominant human pedigrees. This mutation was located in the extracellular domain and was considered to constitute a loss-of-function mutation. Aortic valve calcification is associated with repression of the activity of RUNX2, a central transcriptional regulator of osteoblast cell fate determined by NOTCH1, and is considered to be a result of the inappropriate activation of osteoblast-specific gene expression.

In conclusion, although *NOTCH1* mutation is rare in T-cell tumors besides T-ALL, 1 ATL patient and 1 PTCL-u patient of the 53 T-cell leukemia/lymphoma cases in our study presented *NOTCH1* mutations that have not previously been reported. Analysis of *NOTCH1*, *HES1*, *MYC*, and *CCND1* expression could not clarify whether these mutations were activating. However, *NOTCH1* mutations found in T-cell leukemia/lymphoma cases merit further investigation in a more detailed study on a larger scale to clarify the association between *NOTCH1* mutation and T-cell leukemogenesis. In addition, further functional analysis of these novel mutations is needed with a variety of cell types.

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