ALL samples for the comparison with AMLL. The AML, ALL, and AMLL samples exhibited the proliferation of blastic cells accounting for more than 80% of all bone marrow cells. The patients were not infected with viruses including HTLV-1 and had not been treated with therapeutic drugs prior to the study.

The procedures followed were in accord with the ethical standards established by the ethics committee of Tokyo Medical and Dental University.

Double Staining for Myeloid and Lymphoid Cell Markers

The phenotype of leukemic cells in AMLL was confirmed by double immunostaining using the formalin-fixed paraffin-embedded bone marrow samples. Sections were deparaffinized and incubated with monoclonal antibody against CD20 or CD79a (DAKO, Glostrup, Denmark) and polyclonal antibody against myeloperoxidase (DAKO). Next, the sections were treated with peroxidase-conjugated anti-mouse IgG followed by a DAB development system and then with alkaline phosphatase-conjugated anti-rabbit IgG (DAKO) followed by development with an alkaline phosphatase–nitroblue tetrazolium chloride–5-bromo-4-chloro-3-indolyl phosphatase development system (DAKO).

Identification of Apoptotic Cells

To identify apoptotic cells, the terminal deoxy-transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method was used as previously described [10]. Briefly, tissue sections were deparaffinized and incubated with proteinase K (prediluted, DAKO) for 15 min at room temperature. After the tissues were washed, TdT, fluorescein isothiocyanate (FITC)-dUTP and -dATP (Boehringer Mannheim, Mannheim, Germany) were applied to the sections, which were then incubated in a moist chamber for 60 min at 37°C. Anti-FITC-conjugated antibody-peroxidase (POD converter, Boehringer Mannheim) was employed to detect FITC-dUTP labeling, and color development was achieved with DAB containing 0.3% hydrogen peroxide solution. The sections were then observed under a microscope and the proportion of TUNEL-positive cells was determined by dividing the number of positively stained cells by the total cell number (count of more than 1,000 cells).

Preparation of RNA and Quantitative Assay for IAP-Family Proteins Using TaqMan RT-PCR

RNA was extracted from frozen bone marrow samples of control subjects with no hematological disorders, AML, ALL and AMLL patients using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. For quantitative RT-PCR, fluorescent hybridization probes and a TaqMan PCR Core Reagents Kit with AmpliTaq Gold (PerkinElmer Cetus, Norwalk, CT) were used with an ABI Prism 7900HT Sequence Detection System (PerkinElmer, Foster City, CA). Oligonucleotides as specific primers and TaqMan probes for the IAP-family and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized at a commercial laboratory (PerkinElmer Cetus). The primers and TaqMan probes used were as follows. The sequence of the forward primer for survivin mRNA was 5′-TGCCTGGCAGCCCTTTC-3′ and that of the reverse primer was 5′-CCTCCAAGAAGGGCCAGTTC-3′; the TaqMan probe was 5′-CAAGGACCACCG CATCTCTACATTC-3′. For cIAP1 mRNA, the forward primer was 5′-CAGCCTGAGCAGCTTGCAA-3′ and the reverse primer was 5′-CAAGCCACCAT CACAACAAAA-3′; the TaqMan probe was 5′-TTT ATTATGTGGGTCGCAATGATGATGTCAAA-3′. For cIAP2 mRNA, the forward primer was 5′-TCCGTCAAGTTCAAGCCAGTT-3′ and the reverse primer was 5′-TCTCCTGGGCTGTCTGATGTG-3′; the TaqMan probe was 5′-CCCTCATCTACTTGAA CAGCTGCTAT-3′. The forward primer for NAIP mRNA was 5′-GCTTCACAGCGCATCGAA-3′ and the reverse primer was 5′-GCTGGGCGGATGCT TTC-3′; the TaqMan probe was 5′-CCATTTAAAC CACAGCAGAGGCTTTAT-3′. The forward primer for XIAP mRNA was 5′-AGTGGTAGTCCTGTTT CAGCATCA-3′ and the reverse primer was 5′-CCGCACGGTATCTCCTTCA-3′; the TaqMan probe was 5′-CACTGGCACGAGCAGGGTTTCTT TATACTG-3′. Finally, the forward primer for GAPDH mRNA was 5′-GAAGGTGAAGGTCG GAGT-3′ and the reverse primer was 5′-GAA GATGGTGATGGGATTTC-3′; the TaqMan probe was 5′-CAAGCTTCCCGTTCTCAGCC-3′. The conditions for one-step RT-PCR were as follows: 30 min at 48°C (stage 1, reverse transcription), 10 min at 95°C (stage 2, RT inactivation and AmpliTaq Gold activation), and then 40 cycles of amplification for 15 sec at 95°C and 1 min at 60°C (stage 3, PCR). The expression of survivin and other IAP-family proteins was quantitated according to a method described elsewhere [13]. Briefly, the intensity of the reaction was evaluated from the quantity of total RNA in Raji cells (ng) corresponding to the initial number of PCR cycles to reveal the linear increase in reaction intensity (threshold cycle) for each sample on a logarithmic standard curve. Data on the quantity of RNA (ng) for the IAP family was normalized using the data for GAPDH in each sample, and then the ratio to the mean value of control subjects was calculated and compared.

Immunohistochemistry for Survivin and Proliferating Cells

Tissue sections (4 μm thick) of bone marrow from the control, AML, ALL, and AMLL cases were cut on slides covered with adhesive. The sections were deparaffinized, and endogenous peroxidase was quenched with 1.5% hydrogen peroxide in methanol for 10 min. Antibodies were applied to identify survivin and to characterize proliferating cells. The primary antibodies included polyclonal rabbit antibody against human survivin (SURV 11-A, Alpha Diagnostic International, Inc., San Antonio, TX) and monoclonal antibody Ki-67 (DAKO). All sections were developed using biotin-conjugated secondary antibodies against rabbit IgG or mouse IgG followed by a sensitive peroxidase-conjugated streptavidin system (DAKO) with DAB as the chromogen. Negative control staining was performed using rabbit or mouse immunoglobulin of irrelevant specificity substituted for the primary antibody. The proportion of Ki-67-positive cells was determined in the same way as the proportion of TUNEL-positive cells.

Statistical Analysis

Statistically significant differences in the quantitative analysis were determined using the Mann–Whitney *U*-test for comparisons between the control, AML, ALL, and AMLL samples.

RESULTS

Clinicopathological Characteristics of Cases With Acute Mixed Lineage Leukemia

To determine the clinicopathological characteristics of cases with AMLL, the clinical data for cases including laboratory findings are summarized in Table I. As indicated by the flow-cytometric data, bone marrow blasts in these cases exhibited a high frequency of B-cell lineage antigen (CD19) and myeloid cell marker (CD13 and/or CD 33) expression. Thus, blasts of these cases were "biphenotypic." Chromosomal abnormalities were identified in 5 cases (cases 1, 3, 6, 7, and 8), and the Philadelphia chromosome was identified in two cases (cases 1 and 7). Although abnormalities involving chromosome 11q were identified in two cases (cases 1 and 8), the molecular rearrangement of the *mixed lineage leukemia* (*MLL*) gene located on chromosome 11q23 [32,33] was not observed at the chromosome level.

In spite of AML- and ALL-directed therapy (cytarabine, vincristine, etoposide, adriamycin, predonin, etc.), five patients failed to exhibit complete hematological remission, having blast persistence in bone marrow above 10%. Although complete remission could be induced by chemotherapy in four cases (cases 3, 6, 7, and 8), relapse with leukemic blast proliferation occurred within 6 months in two cases (cases 3 and 6, Table II). Overall, most cases exhibited a poor prognosis and the survival times after diagnosis were shorter than 14 months for 5 cases. However, one patient who received a bone marrow transplant (case 6) and the other patients who received chemotherapy (cases 7 and 8) lived.

Double Immunostaining for Myeloid and Lymphoid Cell Markers on AMLL Cells

To confirm the biphenotypic nature of blasts in the AMLL samples, double immunostaining for myeloid and lymphoid cell markers was performed. The majority of AMLL cells exhibited positive signals for B-cell markers such as CD20 or CD79a, while the myeloid cell marker (myeloperoxidase) was partially observed for many of the cases examined

TABLE II. Treatment and Outcome of Cases With Adult AMLL*

Case no.	First treatment	Response and status	Second treatment	Response status	Survival (months)
1	A-VVV	Failure	H-CPM/VP-16	Failure	3
2	H-CPM/VP-16, H-AraC+MIT	Failure	TBI + CPM	Failure	5
3	DCM, H-AraC+MIT	CR, relapse	A-VVV, H-AraC	Failure	6
4	L-AdVP, MVP	Failure	B-VVV, H-CPM/VP-16, H-AraC, L-AdVP	Failure	11
5	AdVP	Failure	A-VVV, VP-16, CAG	Failure	14
6	DC, A-VVV, H-CPM/VP-16	CR, relapse	H-AraC + MIT, BMT	CR and alive	>6
7	A-VVV	CR	H-AraC + MTX	CR and alive	>6
8	CAG	CR	DC	CR and alive	>9

*Abbreviations: A-VVV, AraC (cytarabine) + VCR (vincristine) + VLB (vinblastine) + VP-16 (etoposide); H-CPM, high-dose CPM (cyclophosphamide); H-AraC, high-dose AraC; MIT, mitoxantrone; TBI, total body irradiation; DCM, DNR (daunorubicin) + AraC + 6-MP (mercaptopurine); CR, complete remission; L-AdVP, L-Asp (L-asparaginase) + ADR (doxorubicin) + VCR + PDN (predonin) + CPM; MVP, MIT + VP-16 + PDN; B-VVV, BHAC (enocitabine) + VCR + VLB + VP-16; CAG, AraC + ACR (acurabicin) + G-CSF (lenograstim); DC, DNR + AraC; BMT, bone marrow transplantation; MTX, methotrexate.

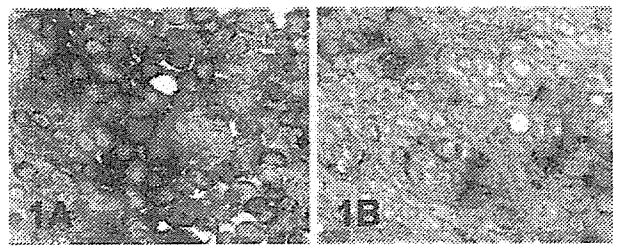

Fig. 1. Double immunostaining for a B-cell marker (CD79a) and myeloid cell marker (myeloperoxidase) in cases with AMLL (A, case 8; and B, case 6; original magnification 400×). Note that the majority of blasts stained positively for CD79a (brown) and a portion of them also stained positive for myeloperoxidase (blue) in both cases. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Fig. 1A for case 8 and Fig. 1B for case 6). These findings were consistent with the flow-cytometric analytical data shown in Table I.

Apoptotic Frequency and Proliferation Activity of Acute Mixed Lineage Leukemia

To identify the apoptotic and proliferative cells present in the bone marrow samples, the TUNEL method and immunohistochemistry for Ki-67 were performed on paraffin-embedded sections. As expected from our previous studies [7,10], the frequency of apoptosis was significantly lower in AML (median, 0.769; range, 1.06–0.219) ($P < 0.001$) and ALL bone marrow cells (median, 0.543; range, 1.18–0.072) ($P < 0.01$) than control cells (median, 2.03; range, 2.81–0.848), and the proliferative cell ratio in AML/ALL bone marrow (median, 39.7; range, 47.8–32.4/median, 45.9; range, 71.9–34.2) was significantly higher than that in control cases (median, 19.2; range, 24.3–10.0) ($P < 0.0001$ and $P < 0.001$, respectively). As shown in Table III, AMLL cells exhibited a tendency similar to AML and ALL cells in that the apoptotic ratio (median, 0.176; range, 1.69–0.021) was significantly lower than the control ($P < 0.01$) and the proliferative cell ratio (median, 26.7; range, 49.1–18.3) was significantly higher ($P < 0.01$). However, AMLL cells exhibited a relatively lower apoptotic index and also significantly lower proliferative index compared with the AML ($P < 0.05$) or ALL samples ($P < 0.05$).

Expression of IAP-Family Proteins Determined by Real-Time Quantitative PCR

To quantitate the mRNA expression levels of the IAP-family members in AMLL cells, real-time quantitative RT-PCR was performed using bone marrow samples from control, AML, ALL, and AMLL cases.

TABLE III. Apoptotic Frequency and Proliferation Activity of Bone Marrow Cells From Control and Acute Leukemia Cases*

	$TUNEL^+$ cell ratio (%)	$Ki\text{-}67^+$ cell ratio (%)
Cases	Median (max-min)	Median (max-min)
Control	2.03 (2.81–0.848)[a,b,c]	19.2 (24.3–10.0)[d,e,f]
AML	0.769 (1.06–0.219)[a]	39.7 (47.8–32.4)[d,g]
ALL	0.543 (1.18–0.072)[b]	45.9 (71.9–34.2)[e,h]
AMLL	0.176 (1.69–0.021)[c]	26.7 (49.1–18.3)[f,g,h]

*Values indicate the median, maximum, and minimum. Differences were significant between the TUNEL-positive cell ratio for control and AML ([a]$P < 0.001$), control and ALL ([b]$P < 0.01$), and control and AMLL ([c]$P < 0.01$) as seen by the Mann–Whitney *U*-test. The Ki-67-positive cell ratio exhibited significant differences between control and AML ([d]$P < 0.0001$), control and ALL ([e]$P < 0.001$), control and AMLL ([f]$P < 0.01$), AML and AMLL ([g]$P < 0.05$), and ALL and AMLL ([h]$P < 0.05$) as seen by the Mann–Whitney *U*-test.

As shown in Fig. 2, the expression of survivin ($P < 0.05$), cIAP1 ($P < 0.05$), NAIP ($P < 0.01$), and XIAP ($P < 0.01$) exhibited significant up-regulation in AMLL compared with the controls. The mRNA for survivin ($P < 0.05$) showed significantly higher levels of expression in AMLL than AML, while the expression levels of survivin ($P < 0.05$), NAIP ($P < 0.05$), and XIAP ($P < 0.05$) in AMLL were significantly higher than those in ALL.

In summary, survivin expression in AMLL was significantly higher than the expression in control, AML, and ALL. The expression level of cIAP1 in AMLL was significantly higher than that in control, but similar with the expression in AML and ALL. Regarding cIAP2, the AMLL cases exhibited stronger expression than the control, AML, and ALL samples although the differences were not significant. NAIP expression in AMLL was significantly higher than control and ALL. The expression level of XIAP in AMLL was significantly higher than control and ALL but similar with AML. No remarkable differences were found between IAP protein expression and patients' age, sex, phenotype, or genotype for AMLL, although further analysis would be necessary because the number of cases was rather small.

These results indicate that the overall expression of IAP-family proteins in AMLL subjects tended to be higher than that for the control, AML, or ALL samples. Specifically, survivin expression in AMLL was significantly higher than that for the control, AML, and ALL samples.

Immunohistochemical Detection of Survivin in the Bone Marrow of AMLL Subjects

To investigate the distribution of survivin, immunohistochemical staining was performed on bone

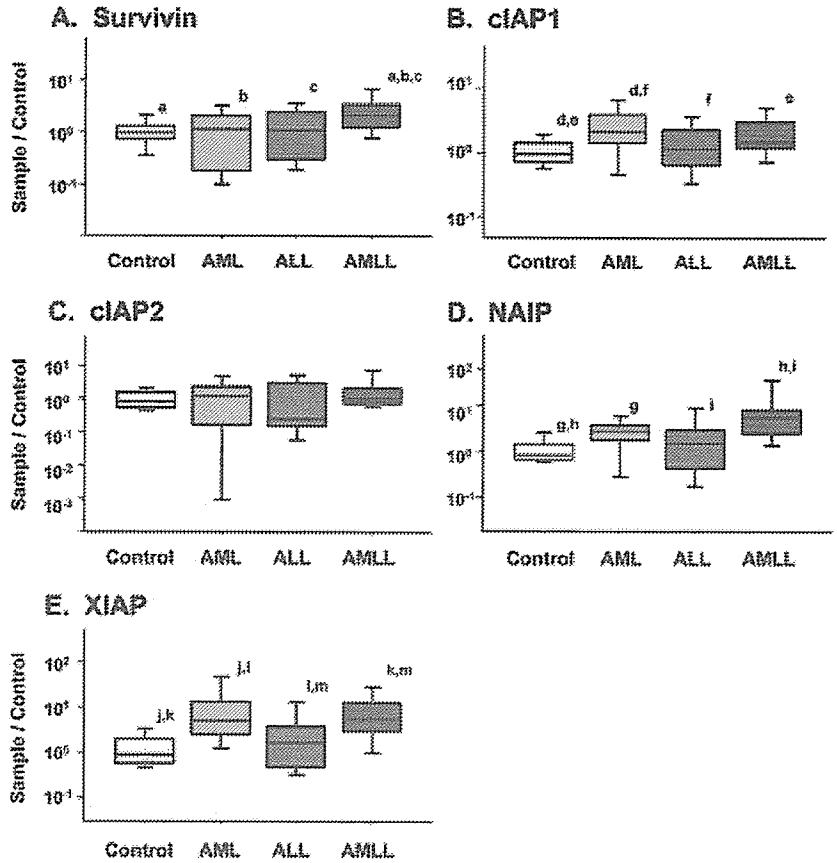

Fig. 2. Expression of IAP-family proteins in control bone marrow and acute leukemias determined by real-time quantitative RT-PCR. The relative intensity was calculated as (intensity of the reaction of IAP-family members [total Raji RNA, ng])/(intensity of the reaction of GAPDH [total Raji RNA, ng]). The intensities of the expressions from the AML, ALL, and AMLL samples are indicated as the ratios to the intensity of the control subjects. The box–bar graphs indicate the value of the control, AML, ALL, and AMLL cases: the bars indicate the 90th and 10th percentiles, and the box indicates the 75th to 25th percentiles. Differences were significant between samples as seen by the Mann–Whitney *U*-test as follows: (A) survivin—control and AMLL ($^{a}P < 0.01$), AML and AMLL ($^{b}P < 0.05$), and ALL and AMLL ($^{c}P < 0.05$). (B) cIAP1—control and AML ($^{d}P < 0.01$), control and AMLL ($^{e}P < 0.05$), and AML and ALL ($^{f}P < 0.05$). (C) cIAP2—differences were not significant. (D) NAIP—control and AML ($^{g}P < 0.05$), control and AMLL ($^{h}P < 0.01$), and ALL and AMLL ($^{i}P < 0.05$). (E) XIAP—control and AML ($^{j}P < 0.01$), control and AMLL ($^{k}P < 0.01$), AML and ALL ($^{l}P < 0.05$), and ALL and AMLL ($^{m}P < 0.05$).

marrow samples from AMLL subjects. As we previously showed [13], survivin was detected in only a few scattered myeloid cells in the control bone marrow samples and subcellular localization was mainly cytoplasmic but partly nuclear. The staining pattern and intensity in the control bone marrow was constant between different samples. All of the AMLL samples showed positive staining for survivin, although the staining intensity and frequency varied for each case. At the cellular level, survivin signals in AMLL cells were predominantly localized in the nucleus and also weakly in the cytoplasm (Fig. 3A). However, one case exhibited prominent cytoplasmic staining with mildly positive staining in the nucleus (Fig. 3B). The tissue sections that reacted with preimmune rabbit antibody of nonrelevant specificity showed no significant staining for any of the samples (not shown).

DISCUSSION

AMLL blasts are expected to possess more immature or intermediate characters of AML and ALL blasts because they express both myeloid and lymphoid phenotypes. Regarding the expression of survivin in myeloid neoplasms, previous studies have revealed the significant expression of survivin in AML [34,35]. Adida et al. [35] reported that survivin expression frequently occurs in AML, detecting it in 60% of a series of 125 patients analyzed, and survivin expression was found to be an unfavorable prognostic factor. In contrast, in lymphoid neoplasms, several

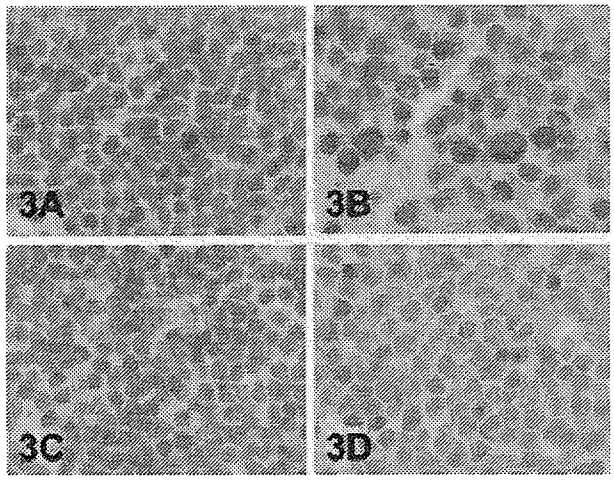

Fig. 3. Immunostaining for survivin in the bone marrow of AMLL (A, case 5; and B, case 8) in comparison with AML (C) and ALL (D) (original magnification 400×). Development was performed using the peroxidase–DAB system (brown) with hematoxylin counterstaining. Note the positive signals in the nucleus as well as the cytoplasm of AMLL cells (A) in contrast to the cytoplasmic staining (B). AML (C) and ALL (D) cases exhibited nuclear and partial cytoplasmic staining. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

studies investigated the dynamics of survivin expression in association with cell proliferation. The in vitro data on mononuclear cells from peripheral blood or bone marrow indicated that B-CLL cells expressed survivin in concert with CD40 and that survivin was the only IAP whose expression was induced by the CD40 ligand (CD40L) [36]. CD40 belongs to the TNF receptor superfamily [37], and its stimulation rescues B-CLL cells from apoptosis and induces proliferation [38]. We recently found that ALL as well as CLL cells exhibited significant expression of survivin and cIAP2 [13]. Thus, both in myeloid and lymphoid neoplasms, IAPs are expressed and seemed to influence the prognosis of patients. Therefore, we can imagine that IAPs would have functions also in AMLL blasts; however, little is known about the potential roles of survivin and other IAPs in the pathogenesis of AMLL.

A major problem with leukemia treatment is drug resistance to chemotherapeutic agents, which may already be present upon diagnosis or after chemotherapy for minimal residual blasts. Resistance originates from genetic or epigenetic mutations during growth of the leukemic clone. Anti-apoptosis mechanisms, alterations of tumor suppressor genes, altered immunogenicity, and drug-resistance mechanisms act in combination [39]. AMLL exhibits strong resistance against chemotherapy, resulting in poor patient prognosis [40,41]. In the present study, expression levels of IAPs in AMLL blasts were higher than those in control samples. Furthermore, several IAPs, such as survivin, NAIP, and XIAP, exhibited stronger expression in AMLL compared with conventional acute leukemias. Thus, the IAP expression level is one criterion that can be used to explain the strong drug resistance in this category of leukemia. The IAP might function probably via the inhibition of caspase-dependent apoptotic signaling. Although we have yet to clarify the caspase-independent pathway of apoptosis in AMLL, the findings of the present study suggest that the regulation of IAPs may become a possible target of AMLL therapy in the future.

In addition to its anti-apoptotic function, survivin also helps regulate cell-cycle progression during mitosis [20]. The highly proliferative activity of AMLL bone marrow cells as well as AML/ALL cells might be associated with survivin expression. As for the expression of IAPs in AML/ALL, the present study found strong expression in some cases and control levels in others, suggesting that AML/ALL cases are heterogeneous in terms of IAP expression.

The human *MLL* gene is involved in about 50 different chromosomal translocations associated with the acute leukemia phenotype [42]. Although chromosomal rearrangement involving chromosome 11q23 was not identified, the cases in the present study were not examined for the presence of *MLL* gene rearrangement by PCR analysis at the DNA level. Further studies are necessary to clarify the interaction of the *MLL* gene and IAP-family genes in association with apoptotic signaling in AMLL blasts.

In conclusion, we showed that strong expression of IAPs, especially survivin and NAIP, occurs in AMLL. Further studies are warranted to clarify the regulatory mechanisms of IAP expression in AMLL in association with drug resistance in this leukemia.

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Differential expression of survivin in bone marrow cells from patients with acute lymphocytic leukemia and chronic lymphocytic leukemia

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Abstract

Survivin, a member of the inhibitor of apoptosis protein (IAP) gene family, has been detected widely in fetal tissue and in a variety of human malignancies. In the current study, we investigated the expression of IAP family proteins in bone marrow samples from acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL) and control cases by quantitative real-time RT-PCR method and an immunohistochemical approach. Overexpression of survivin and cIAP2 mRNA was significant in CLL bone marrow cells ($P < 0.05$, respectively) compared with control samples. By immunohistochemistry, survivin was detected in a few scattered myeloid cells in all cases of control bone marrow. Concerning the ALL bone marrow, more than half the cases demonstrated positive expression of survivin (8 out of 13), while the majority of CLL cases (20 out of 21) exhibited intense expression of survivin. The differential subcellular localization of survivin was distinct between ALL and CLL cases. ALL cells essentially revealed nuclear localization of survivin as well as cytoplasmic signals in some cases, while CLL cells from the majority of cases predominantly showed cytoplasmic expression. Next, RT-PCR was performed for the expression of survivin and its splicing variant, survivin-2B and survivin-ΔEx3 in ALL and CLL cells, as the distribution of these variants would be regulated by nuclear/cytoplasmic transport system. In both ALL and CLL bone marrow samples, the expression of wild-type survivin was more predominant than that of survivin-2B or survivin-ΔEx3, although the expression of survivin-ΔEx3 was prominent in samples from survivin-expressing ALL cases. Thus, the splicing of survivin mRNA may be differently regulated in ALL and CLL cells, causing distinct manners of nuclear/cytoplasmic transport of survivin protein. In conclusion, our observations indicate a differential regulatory mechanism for the expression of IAP family proteins in ALL and CLL cells, although the functions of IAP families and the mechanisms of nuclear/cytoplasmic transport of survivin should be clarified in future studies.

Keywords: Survivin; IAP; Bone marrow; ALL; CLL

1. Introduction

The regulation of apoptotic cell death may have a profound effect on the pathogenesis and progression of hematological malignancies. Chronic lymphocytic leukemia (CLL) is characterized by clonal expansion of relatively mature B cells with a high percentage of cells arrested in the non-proliferative G0/G1 cell cycle phase [1,2]. The progressive rise of lymphocytes, despite the very low proportion of proliferating cells, has led to the notion that the pathogenesis of CLL is primarily related to defective apoptosis. In contrast, acute lymphocytic leukemia (ALL) cells exhibit highly proliferative character with a very low percentage of apoptotic cells [1,3,4]. Thus, ALL and CLL cells may be regulated by different types of cell-proliferation/cell-death signaling pathway. To begin to clarify the antiapoptotic pathways in lymphocytic leukemias, the expression and modulation of the family of inhibitor of apoptosis proteins (IAPs), especially survivin, were investigated and compared in control, ALL and CLL bone marrow samples.

Survivin is expressed widely in fetal tissues, but becomes restricted during development, and appears to be negligibly expressed in the majority of terminally differentiated adult tissues [5,6]. However, analysis of the differences in gene expression between normal and tumor cells has revealed that survivin is one of the genes most consistently overexpressed

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in tumor cells relative to normal tissue [7]. In fact, survivin is prominently expressed in transformed cell lines and in many of the human cancers including hematopoietic cell tumors [8].

As with other IAP family proteins, survivin blocks apoptosis induced by a variety of apoptotic triggers [9,10]. Although the exact biochemical mechanism by which survivin suppresses apoptosis has been debated, survivin is known to bind directly to and inhibit caspase-3 and -7, which act as terminal effectors in apoptotic protease cascades [10,11]. Survivin is usually detected in the cytoplasm of tumor cells, and is therefore widely regarded as a cytoplasmic protein [5,12,13]. However, several studies have shown nuclear accumulation of survivin in gastric cancer cells [14] and lung cancer cells [15]. Thus, the mechanisms that control its nuclear-cytoplasmic localizations in tumor cells are still controversial.

Many cellular proteins either reside in the nucleus or shuttle between the nucleus and the cytoplasm across the nuclear envelope. In a recent study, survivin was shown to be a nuclear shuttling protein that was actively exported from the nucleus via the chromosome region maintenance 1 (CRM1)-dependent pathway [15]. CRM1 was shown to be a receptor for the nuclear export signal that bound to the nuclear export sequences of the proteins. Thus, the molecular export sequences are very important in determining the subcellular localization of proteins. Differences in the amino acid sequence of the carboxy-terminal domain of survivin determine the dramatically different localization of survivin and its splice variant, survivin-ΔEx3. Survivin-ΔEx3 lacks exon 3 but has additional sequences that could mediate its strong nuclear accumulation. Therefore, wild-type survivin localizes to the cytoplasm, while survivin-ΔEx3 accumulates in the nucleus.

Here, in the present study, overall survivin expression was significantly up-regulated in the bone marrow cells from ALL and CLL compared with the control bone marrow. However, different localization of survivin was shown by the nuclear expression in ALL and the cytoplasmic expression in CLL. Expression of other IAPs including NAIP, cIAP1, cIAP2 and XIAP, all of which appeared to suppress apoptosis by caspase and procaspase inhibition [16–19] was also determined in these samples and the significance of IAP family protein expression in lymphocytic leukemias was discussed.

2. Materials and methods

2.1. Patients

Formalin-fixed paraffin-embedded bone marrow aspiration samples from 13 patients with adult-onset ALL (7 with B-ALL and 6 with T-ALL; male:female = 5:8; age: median 48, maximum 78, minimum 19), 21 patients with B-CLL (male:female = 11:10; age: median 57, maximum 87, minimum 49) and 13 cases with no hematological disorders as age-matched normal controls (male:female = 13:0; age: median 63, maximum 76, minimum 51) were analyzed. To rule out the influence of aging effect on bone marrow cells, ALL cases with adult-onset were analyzed and cases with childhood ALL were excluded from the study. Diagnosis was based on standard clinical and laboratory criteria, including cell morphology [20–22]. All samples were collected at the time of the initial aspiration biopsy and the samples from ALL and CLL exhibited proliferation of the blastic cells accounting for more than 80% of the total bone marrow cells. The patients were not infected with specific viruses including HTLV-1 and had not been treated with therapeutic drugs prior to the study. The procedures followed were in accord with the ethical standards established by the ethics committee of Tokyo Medical and Dental University.

2.2. Identification of apoptotic cells

To determine apoptotic cells, the terminal deoxy-transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method was used for the assay as described previously [23]. Briefly, tissue sections were deparaffinized and incubated with proteinase K (prediluted, DAKO, Glostrup, Denmark) for 15 m at room temperature. After washing, TdT, fluorescein isothiocyanate (FITC)-dUTP and dATP (Boehringer Mannheim, Mannheim, Germany) were applied to the sections, which were then incubated in a moist chamber for 60 min at 37 °C. Anti-FITC-conjugated antibody-peroxidase (POD converter, Boehringer Mannheim) was employed for detecting FITC-dUTP labeling, followed by color development with DAB containing 0.3% hydrogen peroxide solution. Sections were then observed under microscopy and the TUNEL-positive cell ratio was determined by dividing the cell number of positively stained cells by the total cell number (counting more than 1,000 cells).

2.3. RNA preparation and quantitative assay for IAP family proteins using TaqMan RT-PCR

The RNA was extracted from the frozen bone marrow samples from seven cases with ALL (four with B-ALL and three with T-ALL), seven cases with B-CLL and eight cases with no hematological disorders using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. For quantitative RT-PCR, fluorescent hybridization probes, TaqMan PCR Core Reagents Kit with AmpliTaq Gold (Perkin-Elmer Cetus, Norwalk, CT) were used with the ABI Prism 7900HT Sequence Detection System (Perkin-Elmer, Foster City, CA). Oligonucleotides as specific primers and TaqMan probes for IAP family and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized by a commercial laboratory (Perkin-Elmer Cetus). The primers and TaqMan probes were as follows. Sequences of the forward primer for survivin

mRNA were 5′-TGCCTGGCAGCCCTTTC-3′ and the reverse primer, 5′-CCTCCAAGAAGGGCCAGTTC-3′; the sequence of the TaqMan probe was 5′-CAAGGACCACCGCATCTCTACATTC-3′. For cIAP1 mRNA, sequences of the forward primer were 5′-CAGCCTGAGCAGCTTGCAA-3′ and the reverse primer, 5′-CAAGCCACCATCACAACAAAA-3′; the TaqMan probe was 5′-TTTATTATGTGGGTCGCAATGATGATGTCAAA-3′. For cIAP2 mRNA, sequences of the forward primer were 5′-TCCGTCAAGTTCAAGCCAGTT-3′ and the reverse primer, 5′-TCTCCTGGGCTGTCTGATGTG-3′; the sequence of the TaqMan probe was 5′-CCCTCATCTACTTGAACAGCTGCTAT-3′. Sequences of the forward primer for NAIP mRNA were 5′-GCTTCACAGCGCATCGAA-3′ and the reverse primer, 5′-GCTGGGCGGATGCTTTC-3′; the sequence of the TaqMan probe was 5′-CCATTTAAACCACAGCAGAGGCTTTAT-3′. Sequences of the forward primer for XIAP mRNA were 5′-AGTGGTAGTCCTGTTTCAGCATCA-3′ and the reverse primer, 5′-CCGCACGGTATCTCCTTCA-3′; the sequence of the TaqMan probe was 5′-CACTGGCACGAGCAGGGTTTCTTTATACTG-3′. Sequence of the forward primer for GAPDH mRNA were 5′-GAAGGTGAAGGTCGGAGT-3′ and the reverse primer, 5′-GAAGATGGTGATGGGATTTC-3′; the sequence of the TaqMan probe was 5′-CAAGCTTCCCGTTCTCAGCC-3′. Conditions of one-step RT-PCR were as follows: 30 min at 48 °C (stage 1, reverse transcription), 10 min at 95 °C (stage 2, RT inactivation and AmpliTaq Gold activation) and then 40 cycles of amplification for 15 s at 95 °C and 1 min at 60 °C (stage 3, PCR). Expression of survivin and other IAP family proteins was quantitated according to the method described elsewhere [24]. Briefly, the intensity of reaction was evaluated by the quantity of total RNA of Raji cells (ng) corresponding to the initial PCR cycle numbers to reveal the linear increase of reaction intensity (threshold cycle) in each sample on the logarithmic scale standard curve. Data of the Raji RNA quantity (ng) for IAP family were normalized by the data for GAPDH in each sample.

2.4. Immunohistochemistry for survivin, p53 and cell markers

Four micrometer-thick tissue sections of bone marrow from control, ALL and CLL cases were cut on slides covered with adhesive. Sections were deparaffinized, and endogenous peroxidase was quenched with 1.5% hydrogen peroxide in methanol for 10 min. Antibodies were applied to identify survivin, to characterize B cells (CD20) and T cells (CD45RO), and to identify accumulation of p53 protein. Primary antibodies included polyclonal rabbit antibody against human survivin (SURV 11-A, Alpha Diagnostic International, San Antonio, TX), monoclonal antibodies against CD20 (DAKO), CD45RO (DAKO) and p53 (Novocastra Laboratories Ltd., Newcastle, UK). All sections were developed using biotin-conjugated secondary antibodies against rabbit IgG or mouse IgG followed by a sensitive peroxidase-conjugated streptavidin system (DAKO) with DAB as the chromogen. Negative control staining procedure was performed using rabbit or mouse immunoglobulin of irrelevant specificity substituted with the primary antibody in each staining.

Phenotype determination of survivin-expressing cells was performed by double immunostaining using polyclonal antibody against survivin and monoclonal antibody against CD20 or CD45RO followed by the peroxidase–DAB development system and then, alkaline phosphatase-conjugated anti-mouse IgG (DAKO) followed by development with the alkaline phosphatase-nitroblue tetrazolium chloride-5-bromo-4-chloro-3-indolyl phosphatase development system (DAKO).

2.5. RT-PCR analysis for survivin and splice variants, survivin-2B and survivin-ΔEx3

To determine the pattern for the splicing of survivin, RT-PCR analysis was performed using specific primers that could distinguish each type of splicing variant, survivn-2B and survivin-ΔEx3, by product size [25]. The PCR reaction was performed as described elsewhere [26,27]. Briefly, 100 ng of the RNA was used for RT-PCR. For complementary (c)DNA synthesis, 100 ng in 4 μl of sample RNA solution was heated at 65 °C for 5 min and cooled rapidly. After adding 20 U of ribonuclease inhibitor (Takara, Japan), 1 μl of 1.25 mM dNTP (dATP, dCTP, dGTP, dTTP, Pharmacia, Uppsala, Sweden) and 20 U of Rous-associated virus reverse transcriptase (Takara Biomedicals, Kyoto, Japan), the mixture was incubated at 40 °C for 30 min, then heated at 94 °C for 5 min and cooled rapidly. Oligonucleotides as specific primers for survivin were synthesized by a commercial laboratory (Invitrogen Life Technologies, Tokyo, Japan). The sequences of primers were as follows: forward primer, 5′-ACCGCATCTCTACATTCAAG-3′ and the reverse 5′-CTTTCTTCGCAGTTTCCTC-3′. In the control reaction β-actin was also determined using the forward primer 5′-AAGAGAGGCATCCTCACCCT-3′, and the reverse 5′-TACATGGCTGGGGTGTTGAA-3′. The PCR reaction mixture contained 10 μl of cDNA, 10 μl of 10 × PCR buffer, 11 μl of 20 mM $MgCl_2$, 16 μl of 1.25 M dNTP, 42.5 μl of DEPC-water, 100 pM forward and reverse primers, and 2.5 U of thermostable Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA). The amplification was achieved with a DNA thermal cycler (Perkin-Elmer Cetus). After denaturing at 94 °C for 10 min, the amplification was conducted for 45 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s. This was followed by re-extension for 10 min at 72 °C. Ten microliters aliquots of the product samples were analyzed by electrophoresis on a 1.8% agarose gel and visualized by UV fluorescence after staining with ethidium bromide. The expected sizes of the PCR product were 342 bp for wild-type survivin, 411 bp for survivin-2B, 224 bp for survivin-ΔEx3 and 218 bp for β-actin. φX174/Hae III-cut DNA was run in parallel as a molecular size marker.

2.6. Statistical analysis

Statistically significant differences were determined using the Mann–Whitney's *U*-test.

3. Results

3.1. Apoptotic cell ratio of the bone marrow cells from ALL, CLL, and control cases

To identify the apoptotic cells in the bone marrow samples, the TUNEL method was performed on paraffin-embedded sections. We compared the overall TUNEL-positive cell ratio of control bone marrow with the ratio of ALL or CLL samples, although the apoptotic cells of control bone marrow were not necessarily the lymphoid cells. The apoptotic cell ratio was rather low even in the control bone marrow samples as shown in Table 1, however, the ratio was lower in ALL and CLL cases than in control cases. Differences were significant between ALL and control ($P < 0.01$ by the Mann–Whitney's *U*-test) and CLL and control cases ($P < 0.0001$). ALL cells exhibited relatively lower frequency of TUNEL-positive signals than CLL cells ($P < 0.0001$). These findings suggested that apoptosis was actually infrequent in ALL cells as well as in CLL cells.

3.2. Expression of mRNA for IAP family proteins determined by real-time quantitative PCR

To quantitate the mRNA expression levels of IAP family proteins in lymphocytic leukemia cells, real-time quantitative RT-PCR was performed using bone marrow samples from ALL, CLL and control cases. The expression of mRNA for survivin, cIAP1, cIAP2, NAIP and XIAP was found in all of the control samples although the expression levels varied. Thus, the expression intensity of IAP family proteins was demonstrated as the percentage of control in each group. Differences were significant between survivin expression of CLL and control ($P < 0.05$) and cIAP2 expression of CLL and control ($P < 0.05$) (Fig. 1). The intensities of mRNA expression of cIAP1, cIAP2, NAIP and XIAP proteins in ALL cases tended to be higher than the intensity of control cases, although the differences were not significant. This is caused by the fact that some of the ALL cases revealed very high expression, while other ALL cases had as low expression as control cases. No significant differences were found between survivin expression and patients' age, sex or phenotypic character of leukemic cells (B cell-lineage or T cell-lineage). These results indicated that the expression of survivin and cIAP2 would be significant in CLL bone marrow, whereas survivin as well as other IAP family proteins might possibly have a role only in some ALL cases.

Table 1
Apoptotic cell ratio of the bone marrow from ALL, CLL and control cases

Cases	TUNEL-positive cell ratio (%)[a]	
	Median	Maximum–minimum
ALL	0.044	0.38–0.0032[b,c]
CLL	0.13	0.98–0.011[c,d]
Control	1.08	3.65–0.58[b,d]

[a] Values indicate the median value, the maximum and the minimum values.
[b] Differences were significant between the TUNEL-positive cell ratio of ALL and control cases ($P < 0.01$) by the Mann–Whitney's *U*-test.
[c] Differences were significant between the TUNEL-positive cell ratio of ALL and CLL cases ($P < 0.0001$) by the Mann–Whitney's *U*-test.
[d] Differences were significant between the TUNEL-positive cell ratio of CLL and control cases ($P < 0.0001$) by the Mann–Whitney's *U*-test.

3.3. Immunohistochemical localization of survivin in the bone marrow of control, ALL and CLL cases

To investigate the localization of survivin, immunohistochemical staining was performed in bone marrow samples from ALL, CLL and control cases. ALL cells exhibited various degrees of survivin expression from case to case. In one case, the majority of cells stained positively, while in seven cases, staining was partial (Table 2). In five cases of ALL, survivin was not detected immunohistochemically. At the cellular level, survivin signals in ALL cells were predominantly localized to the nucleus (Fig. 2A and B), although in some cases, prominent reaction was also observed in the cytoplasm of ALL cases. Moreover, survivin was detected in most of the bone marrow samples from CLL cases by immunohistochemical staining (20 out of 21 cases). Positive staining was observed in the majority of CLL cells in half the cases (10 out of 20 survivin-positive cases), while other cases exhibited positive signals in some CLL cells (Table 2). In contrast to the subcellular localization of survivin in ALL cases, survivin in CLL cells was predominantly localized to the cytoplasm with minimal nuclear staining (Fig. 2C and D). By contrast, survivin was detected in only a few scattered myeloid cells of the control bone marrow samples (Fig. 2E). The subcellular localization was mainly cytoplasmic but partly nuclear. The staining pattern and intensity of the control bone marrow was constant between different samples. Tissue sections that were reacted with preimmune rabbit antibody with irrelevant specificity showed no significant staining in all of the samples (not shown). Double staining procedure revealed survivin-expressing cells were CD20-positive cells both in B-ALL and B-CLL (Fig. 3A and B) suggesting that these cells were actually leukemic cells.

3.4. Expression of survivin and splice variants, survivin-2B and survivin-ΔEx3 in ALL, CLL and control cases

To examine whether the differential subcellular localization of survivin between ALL and CLL cases was due to the difference in nuclear/cytoplasmic transport state, RT-PCR analysis was performed to distinguish the wild-type and

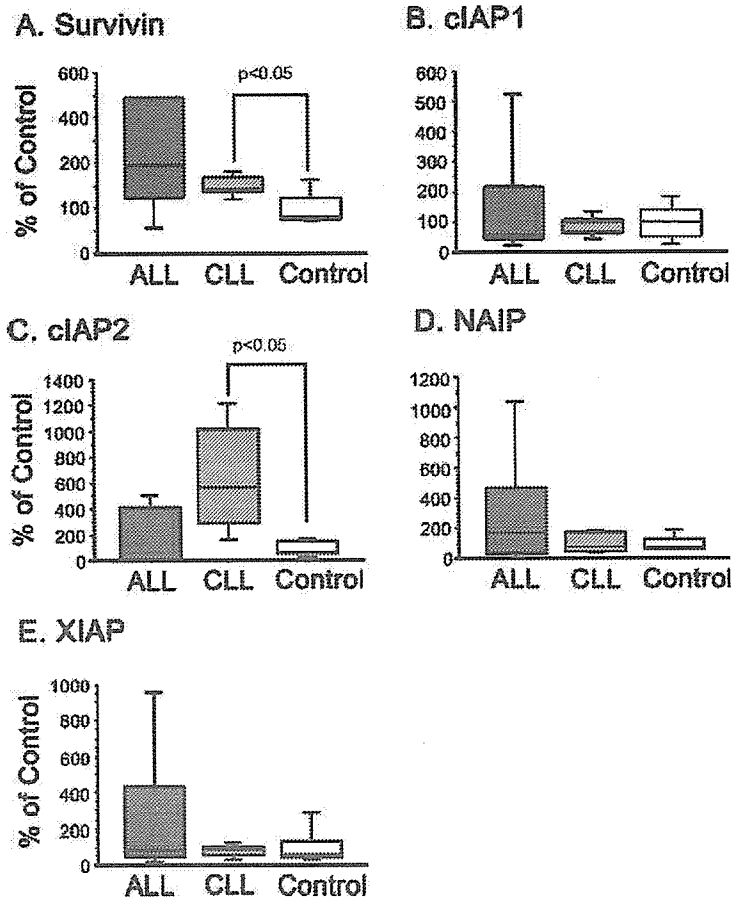


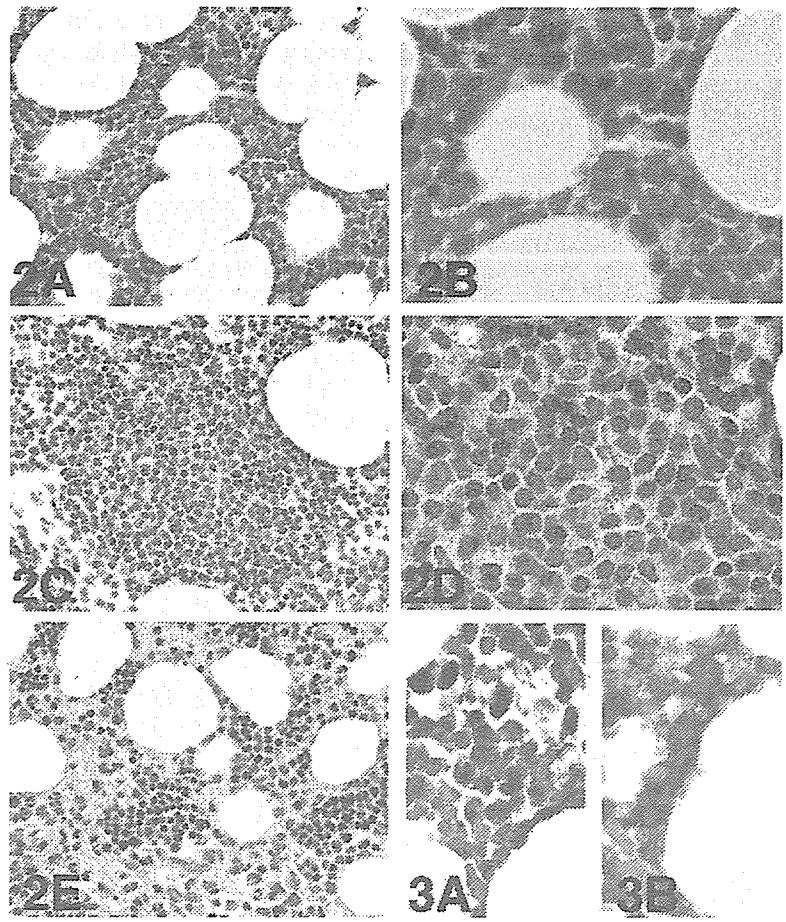
Fig. 1. ((A)–(E)) Quantitative RT-PCR analysis for IAP family proteins, survivin, cIAP1, cIAP2, NAIP and XIAP. Relative intensity was calculated as intensity of reaction of IAP family (total Raji RNA (ng))/intensity of reaction of GAPDH (total Raji RNA (ng)). The intensity of expression from ALL and CLL samples is indicated as the percentage of the intensity of control samples. The box plot graphs indicate the value of ALL, CLL and control cases. Bars indicate 90% tile and 10% tile and box indicates 75% tile to 25% tile. Differences were significant between survivin expression in CLL and control cases ($P < 0.05$) and cIAP2 expression in CLL and control cases ($P < 0.05$) by the Mann–Whitney's U-test.

splice variants of survivin. In an in vitro transfection study, the wild-type survivin and the splice variant survivin-2B have been shown to localize to the cytoplasm. In contrast, another variant, survivin-ΔEx3, has a carboxy-terminal end that is different from other types of survivin and mediates strong nuclear accumulation [15]. Thus, the difference in the splicing patterns of survivin would indicate the difference in the state of nuclear/cytoplasmic transport system. In ALL and CLL bone marrow samples, predominant expression was confined to the wild-type survivin, although a weak expression of survivin-2B and survivin-ΔEx3 was also identified (Fig. 4). The splicing for survivin-ΔEx3, appeared more frequent in ALL cases compared with CLL cases, although the expression of wild-type survivin was the strongest even in ALL cases. Therefore, splicing patterns seemed different between ALL and CLL cells. These findings suggested that

Table 2
Immunohistochemical localization of survivin in the bone marrow from ALL, CLL and control cases

Cases	Number of cases					Positive cases (%)	Subcellular localization
	Total	+++	++	+	−		
ALL	13	1	4	3	5	62	Nuclear > cytoplasmic
CLL	21	10	5	5	1	95	Cytoplasmic
Control	13	0	0	0	13	0	Nuclear/cytoplasmic, scattered myeloid cells

+++: the majority of cells exhibited intense expression; ++: more than 50% of cells revealed positive signal; +: positive staining was observed in 10–50% of cells; −: positive cells were less than 10%.



Figs. 2–3. (2) Immunohistochemical localization of survivin in the bone marrow from ALL ((A) and (B)), CLL ((C) and (D)) and control (E) cases. Development procedures were performed using the peroxidase–DAB system (brown). Note that the majority of cells were positively stained in ALL and CLL cases, while only a few myeloid cells exhibited positive signals in control bone marrow ((A), (C) and (E), original magnification 200×). ALL cells showed striking signals in the nucleus as well as in the cytoplasm, whereas positive signals in CLL cells were mainly cytoplasmic and not nuclear ((B) and (D), original magnification 400×). (3) Double immunostaining for survivin and CD20 in the bone marrow from B-ALL (A) and B-CLL cases (B) (original magnification 200×). For double immunostaining, development procedures were performed using the peroxidase–DAB system for survivin (brown) and the alkaline phosphatase-nitroblue tetrazolium system for CD20 (blue). In both ALL (A) and CLL (B) cases, survivin-positive cells (brown) were also positive for CD20 antigen (blue) suggesting that the leukemic cell expressed survivin.

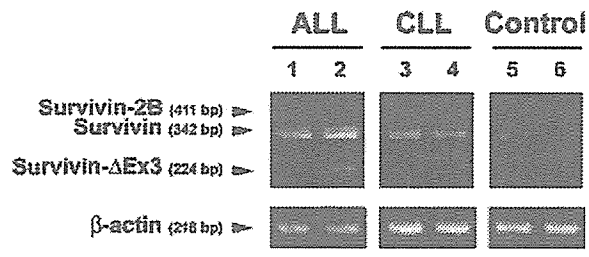


Fig. 4. RT-PCR analysis for the expression of wild-type survivin and the splice variant survivin-2B and survivin-ΔEx3 in ALL (lanes 1 and 2), CLL (lanes 3 and 4) and control bone marrow (lanes 5 and 6). In ALL and CLL samples, the predominant expression was observed in the 342 bp wild-type survivin. However, note that ALL cases have distinct 224 bp signals for survivin-ΔEx3, while CLL cases exhibit very weak 411 bp survivin-2B signals other than wild-type survivin. In some ALL cases, the expression of survivin-ΔEx3 was prominent as shown in lane 2.

the mechanism of the nuclear/cytoplasmic transport system of survivin such as the CRM1 system or the distribution of survivin-binding proteins such as caspases might be differently regulated in ALL cells compared with CLL cells.

3.5. p53 expression in the bone marrow cells of ALL and CLL cases

To determine whether p53-dependent apoptotic pathways were associated with the expression of survivin in ALL and CLL cases, immunohistochemical staining was performed for detecting p53 accumulation in bone marrow samples. As expected from the previous studies [2,4], accumulation of p53 protein was not frequent in ALL and CLL cells in the present study. A positive reaction was observed only in 8% of ALL (1 out of 13 cases) and 10% of CLL cases (2 out of 21 cases). Because the overexpression of survivin was

observed in more than half the ALL cases and the majority of CLL cases, these results suggested that survivin expression in ALL and CLL cases would not be associated with p53 mutation.

4. Discussion

Regarding the survivin expression in lymphocytic leukemia cells, previous studies have revealed an overexpression in some ALL cases [28,29] including adult T-cell leukemia [30] or a significant expression in B-CLL cases [31], while other group indicated that survivin was undetectable in the majority of B-CLL samples [32]. The in vitro data on mononuclear cells from the peripheral blood or bone marrow demonstrated that B-CLL cells expressed survivin on CD40 stimulation and that survivin was the only IAP whose expression was induced by the CD40 ligand (CD40L) [31]. CD40 belongs to the tumor necrosis factor (TNF) receptor superfamily [33] and its stimulation appears to rescue B-CLL cells from apoptosis and induce proliferation [34]. In CLL patients, CD40L would be provided as microenvironmental stimuli by activated $CD4^+$ T cells in the bone marrow. The present data indicated that not only survivin but also cIAP2 exhibited significant overexpression in the bone marrow from CLL patients in vivo. Thus, in CLL bone marrow, multiple microenvironmental factors other than the CD40–CD40L system may also influence the expression of IAP family proteins.

In ALL samples, IAP family expression patterns other than survivin were not uniform in the present study. Some cases exhibited very strong expression, while others revealed an almost normal level of expression. As a result, although the mean intensities of cIAP1, NAIP and XIAP in ALL were higher than those of control cases, the differences were not significant between ALL and control groups. Thus, several IAP family proteins other than survivin might also play a role in some ALL cases but may not be the general factors that regulate apoptotic pathways in ALL cells.

Immunohistochemical staining revealed a very high frequency of survivin expression in CLL cells and relatively high frequency in ALL cells in the present study. Thus, immunohistochemical analysis would be useful for detecting the few remaining leukemic cells after treatment and the very early stage of leukemic relapse of ALL/CLL cases on formalin-fixed routine bone marrow aspiration samples. We confirmed that the condition in leukemic relapse did not alter the state of survivin expression in several samples from ALL and CLL cases, however, further study should be made to clarify the influence of chemotherapeutic agents on the expression patterns of survivin.

Two splice variants of survivin, survivin-ΔEx3 and survivin-2B, have been identified [25]. Study on the regulation of alternative splicing is still a new and intriguing area. Thus, how different splice forms are turned on and off is still controversial except for several instances [35]. Genetic events in ALL/CLL pathogenesis might involve and alter the splicing mechanism of survivin, although a future study should clarify the details. By transfection experiments, survivin-ΔEx3 conserves antiapoptotic properties, while survivin-2B has a markedly reduced antiapoptotic potential. In the present study, ALL cases and CLL cases exhibited enhanced expression of wild-type survivin as well as survivin-2B, while survivin-ΔEx3 was more intensely expressed in ALL cases than in CLL cases. These variants of survivin might contribute to the suppression of the apoptotic process in the bone marrow cells as expected from the present TUNEL data.

It is difficult to explain the difference in apoptotic character of ALL and CLL only by the expression of survivin and cIAP2 at this moment. However, it is possible that the ability of survivin to counteract apoptosis is modulated by its localization to the nucleus or the cytoplasm of the cell [15]. In addition to its anti-apoptotic function, survivin also plays a role in the regulation of cell cycle progression during mitosis [8]. Highly proliferative activity of ALL cells but low proliferative activity of CLL cells might be associated with the differential expression pattern of survivin.

Wild-type p53, but not mutant p53, represses survivin expression at both the mRNA and protein levels [36]. The modification of chromatin within the survivin promoter would be a molecular explanation for the silencing of survivin gene transcription by p53 [37]. On the other hand, the over-expression of exogenous survivin protein rescues cells from p53-induced apoptosis in a dose-dependent manner, suggesting that loss of survivin mediates in part the p53-dependent apoptotic pathway [37]. In contrast to the high frequency of p53 mutations in many of the solid cancers, ALL (5–17%) and CLL (10–20%) cases have been shown to demonstrate a rather low frequency of p53 mutation [2,4]. We also observed that immunohistochemical accumulation of p53 was present only in 8% of cases with ALL and 10% of CLL cases. Therefore, p53 mutation would not appear to be the major factor controlling the overexpression of survivin in the bone marrow of ALL and CLL cases.

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Coincidental Outbreak of Methicillin-Resistant *Staphylococcus aureus* in a Hematopoietic Stem Cell Transplantation Unit

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Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common nosocomial pathogens among hospital-acquired infections, and immunocompromised patients are highly susceptive to infection. The molecular typing of isolated strains is a common method for tracing an outbreak of MRSA, but experience with this approach is still limited in the hematopoietic stem cell transplantation (HSCT) ward.

Methods: We experienced 6 cases of MRSA infection/colonization in our 26-bed HSCT ward during a 4-week period. This unusual outbreak strongly suggested that the same MRSA strain was involved despite strict isolation and aseptic patient care. Clarification of the transmission pattern was critical, and we applied pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP) assays for evaluation.

Results and conclusion: In four of the six cases, the pattern of bands examined by PFGE and AFLP analyses supported the idea that direct person-to-person transmission was very unlikely and the outbreak was coincidental. This experience highlights the clinical value of molecular typing methods for the clinical epidemiological assessment of MRSA outbreak. Am. J. Hematol. 81:664–669, 2006

Key words: outbreak; MRSA; stem cell transplantation

INTRODUCTION

The rapid increase in the incidence of hospital-acquired infection by methicillin-resistant *Staphylococcus aureus* (MRSA) is making infection control procedures very critical, particularly for immunocompromised patients [1]. Hospital-acquired infections also serve as a hallmark of the effectiveness and quality of infection control maneuvers [2]. Outbreaks of infection caused by MRSA have time-consuming and expensive consequences, and genetic analysis is useful, since it can be used to determine the route and origin of MRSA infection [3]. Currently available laboratory methods for determining DNA fragment sizes or sequences in MRSA isolates include Southern blotting [4], ribotyping [5], polymerase chain reaction (PCR) [6], and pulsed-field gel electrophoresis (PFGE) [4,7]. PFGE has become the most common tool for the rapid discrimination of MRSA strains due to its convenience, reliability, and cost-effectiveness [8,9]. However, the interpretation of PFGE bands still needs to be standardized [10]. Alternatively, the amplified fragment length

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TABLE I. Clinical Characteristics of 6 Cases*

UNP	Age	Sex	Disease	Day after transplantation	Cause of admission	Admission to hospital	First admission ward	Admission to SCT unit
1	53	M	MDS	109	GVHD	2002/11/13	Ward A	11/15/2002
2	35	M	NHL	219	GVHD	2002/11/20	Ward B	11/27/2002
3	35	M	GCT	43	GVHD	2002/11/29	Ward C	12/5/2002
4	58	M	MDS	−13	SCT	2002/10/31	SCT unit	10/31/2002
5	54	F	NHL	−22	SCT	2002/11/5	SCT unit	11/5/2002
6	63	M	CML	210	GVHD	2002/11/5	SCT unit	11/5/2002

* Cases 4 to 6 were admitted before case 1 showed severe intestinal symptoms induced by gut GVHD, complicated with continuous gastrointestinal bleeding. In 4 of these 6 cases, hospitalization was due to GVHD after transplantation (3 acute and 1 chronic), and 3 of these patients, including case 1, received corticosteroid therapy for the treatment of GVHD.

Note: MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; GCT, germ cell tumor; CML, chronic myelogeneous lymphoma; GVHD, graft versus host disease; PSL, prednisolone; SCT, stem cell transplantation.

polymorphism (AFLP) method is based on the selective amplification by PCR of a subset of restriction fragments from a digest of the whole bacterial genome [11,12]. AFLP has advantages over PFGE since it has more power for discriminating between different strains more quickly with higher specificity in the recognition of digestive fragments of whole bacterial genome [13].

The goal of these laboratory tests is to provide firm evidence that isolates, which are epidemiologically related during an outbreak of the infection, are also genetically related and thus represent the same strain. To enhance the reliability of such molecular laboratory results, the combined use of various genotyping methods appears to be effective [14,15]. An outbreak has been defined as infectious disease derived from the same pathogen, while an outbreak that originates from strains that are indistinguishable from each other by typing methods but for which no direct linkage can be demonstrated is called an "endemic outbreak" [16]. It has been suggested that in disease outbreak due to endemic strains, the common origin may be temporally distant from those in outbreak strains. From the perspective of infection control, this difference is critical, since different procedures are needed to prevent the spread of disease.

We experienced an outbreak of MRSA in our hematopoietic stem cell transplantation (SCT) ward that was initially suspected to be derived from a single origin. To address this serious problem, we tracked down the route of infection and obtained results that highlighted the clinical value of molecular typing using these methods.

PATIENTS AND METHODS

Patients

The routine infection-monitoring procedure in the SCT ward includes surveillance cultures and identification for specific pathogens in the nasal swab, pharyngeal swab or sputum, urine, or stool, which are collected from patients who are suspected to have infection or colonization of the target pathogen including MRSA at the time of admission. In a 4-week period, we experienced six cases (UPN 1 to 6) of MRSA infection or colonization in the SCT ward, while the preceding incidence of MRSA detection in the SCT ward had been only one or two cases per month (mean 0.8/month, range 0–2/month, SD 0.61). Therefore, this was epidemiologically defined as an MRSA outbreak. The patient characteristics are summarized in Table I. We reviewed the medical records of the patients to collect the clinical information required to track down the transmission route. We documented the time course of MRSA identification in relation to patient characteristics, risks of nosocomial infection, and room assignment.

Samples

Isolates were grown from culturing sputum, urine, stool, pus, and blood, and a few were grown from culturing miscellaneous sites such as pharynx and nasal cavity. We examined the first sample isolated in each patient by molecular typing, PFGE, and AFLP analysis.

DNA Isolation and PFGE

Targeted bacterial strains were cultured at 37°C in Luria-Bertani broth. The cell component was lysed by proteinase K to extract DNA. Genomic DNA was digested with *Sma*I and resolved with the CHEF-DRII system (Bio-Rad Laboratories) as described by the manufacturer (traditional typing strategies) [17]. As a control strain, we used MRSA isolated from two groups: (1) two strains isolated from past patients in the same ward, which has no temporal relationship to our present cases (cases 7 and 8) and (2) five strains isolated from a different hospital

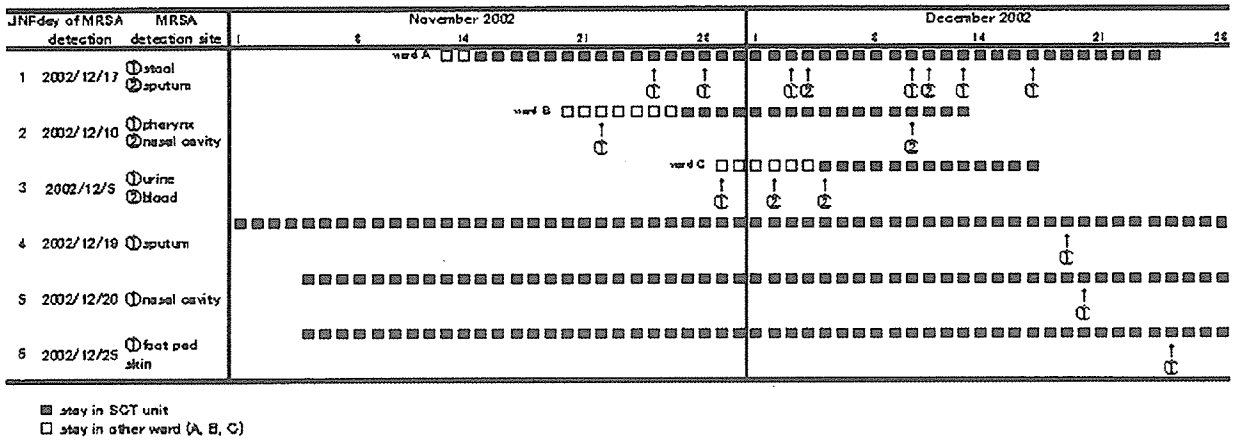

Fig. 1. Time course of MRSA detection in the SCT unit. MRSA was first isolated in the stool of case 1 on 25 November 2002. MRSA had been identified prior to admission to the SCT ward in cases 2 and 3. In contrast, in the other three cases (cases 4, 5, and 6), MRSA was detected after admission to the SCT ward.

(University of Tokyo Hospital, 1150 beds), which was not epidemiologically associated with our hospital (cases 9 to 13). PFGE banding was compared with that in case 1, who was thought to be the origin of this outbreak episode. The criteria described by Tenover et al. [16] were used for the molecular epidemiological interpretation of PFGE banding as follows:

(i) indistinguishable: outbreak was derived from the same isolate;
(ii) closely related: different isolates, closely related to the outbreak pattern;
(iii) possibly related: different isolates, possibly related to the outbreak pattern;
(iv) unrelated: different isolates, unrelated to the outbreak pattern.

One genetic event detected by PFGE was considered meaningful enough as different isolates.

AFLP

Bacterial DNA was prepared with a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's recommendations. DNA was then manipulated with an AFLP Microbial Fingerprinting kit (Applied Biosystems) according to the manufacturer's instructions based on a previous study [11]. Briefly, DNA was digested with *Eco*RI and *Mse*I and then ligated to the corresponding adapters. This was followed by preselective amplification and selective amplification, where *Eco*RI-A (FAM), *Eco*RI-C (NED), *Eco*RI-G (JOE), and *Mse*I-C primers were used. The AFLP reactions were evaluated by analyzing data from samples loaded and run on an ABI 310 Genetic Analyzer with GeneScan software. A dendrogram was constructed from a pairwise distance matrix with the Clustal W version 1.8 software package.

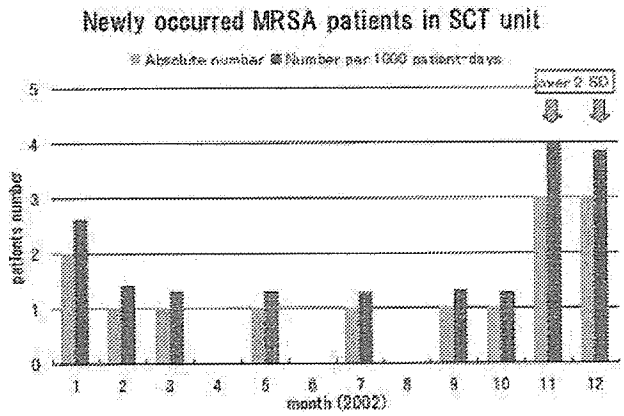

Fig. 2. The incidences of newly detected MRSA cases in SCT unit in 2002. Each bar indicates a number of patients clinically identified as MRSA in 2002.

Definitions [18]

"Methicillin-resistant" is defined according to NCCLS MIC criteria by dilution susceptibility tests. An "outbreak" of MRSA is defined as an increase in the rate of MRSA cases or a clustering of new cases in a specific place during a given period. In this report, we defined an unusual increase in MRSA cases as a multiply repeated isolation of MRSA from a physically independent ward (transplantation unit) with an incidence $\geq$ 2 SD over the baseline. The SCT unit is geographically separate from other wards and has an independent space that is managed to maintain sterilization. Patients from whom MRSA was isolated and who had any concomitant symptoms in the MRSA-detected part were referred to as "MRSA

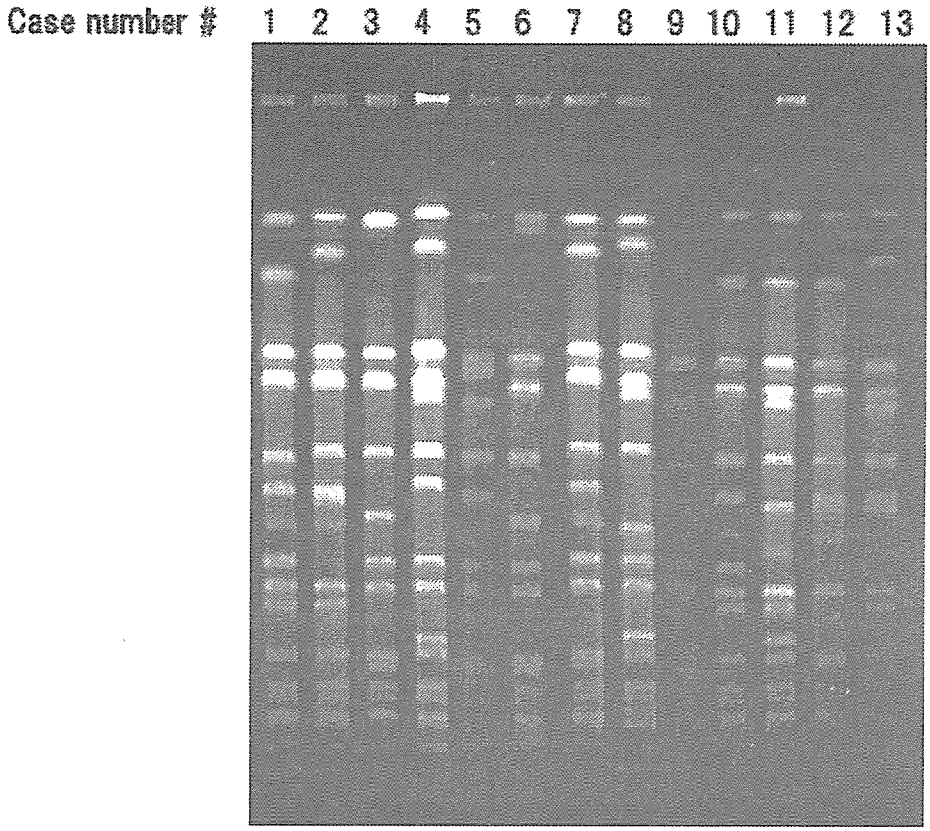

Fig. 3. PFGE analysis of isolated MRSA in the SCT unit. The PFGE pattern showed that there were no detectable differences in bands between cases 3 and 6, but more than two bands were identified in the other four cases (cases 1, 2, 4 and 5). Additionally, two strains that had been previously isolated in the SCT ward (cases 7 and 8) were distinguishable, and the five epidemiologically different isolates (cases 9 to 13) from the University of Tokyo Hospital (1,150 beds) were also distinguishable, with differences in more than two bands.

infection," while those without symptoms were considered "MRSA colonization."

RESULTS

Clinical Course of MRSA Outbreak

The clinical characteristics of six patients in whom MRSA was isolated are presented in Table I. The first patient (case 1) was admitted to the SCT ward because of severe intestinal symptoms induced by gut GVHD, chronic diarrhea, and continuous gastrointestinal bleeding, which occurred at 107 days after SCT. The patient received corticosteroid and intravenous antimicrobial therapy. For 4 weeks prior to his admission, there had been no case of MRSA infection or colonization in the ward. At 13 days after admission, the first isolation of MRSA in his stool was recorded (Table I). Subsequently, five other patients newly developed MRSA events over the next 4 weeks (Figure 1), while the incidence of MRSA detection of SCT ward had remained at one or two cases per month (mean 0.8 /month, range 0–2 /month, SD 0.61, Figure 2). Among these five cases, three (cases 4, 5, and 6) had been admitted to the SCT ward directly from the outpatient clinic without a past history of MRSA infection. The other two cases (cases 2 and 3) were transferred from other wards after the admission of case 1, and MRSA was isolated prior to transfer to the SCT ward (Figure 1). Since, in these two cases, MRSA was identified again in different site in SCT with different drug-sensitivity profile (data not shown) from a previous strain, we included these two cases in the analysis. There were no other patients who were previously identified with MRSA infection or colonization.

Tracing Procedure

The transmission, if any, appeared to take a random pattern, as illustrated in Figure 1. To better evaluate whether the transmission pattern was direct or indirect, we identified the layout of the patients' bed assignments. This revealed that there were nei-

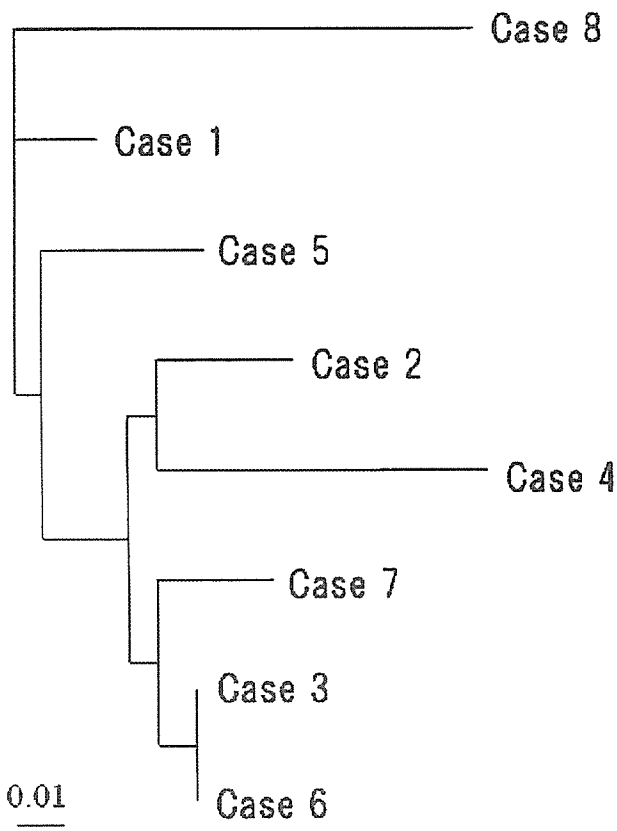

Fig. 4. AFLP pattern of isolated MRSA. AFLP analysis was performed for the same six isolates (case 1 to 6) described in Figure 4. Gene polymorphism showed same result of similarity as PFGE pattern had already indicated, i.e., cases 3 and 6 had the same polymorphism pattern and the others were different strains. The mutual relation of gene polymorphism is presented in the dendrogram and relatedness is indicated by the length of line. The scale bar drawn in the lower part indicated 1.0 % relatedness.

ther overlaps nor coexistence with preceding patients, except that cases 1 and 3 used the same room on different days without an overlap.

PFGE and AFLP Assays of MRSA Isolates

PFGE analysis of the six MRSA strains isolated (Figure 3) showed that two strains (cases 3 and 6) were indistinguishable and therefore considered to be derived from the same isolate, while the remaining four cases (cases 1, 2, 4, and 5) were considered to have different strains. Seven epidemiologically different isolates, i.e., two strains isolated from another ward at different times (cases 7 and 8) and five strains isolated from another hospital (cases 9 to 13) were used as in-hospital and extra-hospital controls, respectively. The results were confirmed by AFLP analyses as a dendrogram shown in Figure 4.

DISCUSSION

The spread of MRSA in highly protected care units, including ICU [19] and neonatal ICU [20], is a well-known major complication in compromised patients. Although few reports have been published on the outbreak of MRSA in a SCT unit, a continuous rise in the incidence of hospital-acquired MRSA infection [21] should influence the incidence of MRSA infection in SCT recipients [22]. Collin et al. reported that the incidence of multidrug resistant *S. aureus* was 15% in isolates from BMT patients with blood stream infection in 1991–1997 [23]. Prolonged neutropenia has been found to be a risk factor for the development of infectious complications in SCT recipients [24]. Since the outbreak of MRSA among immunocompromised patients can greatly affect their mortality, appropriate methods for infection control are strongly warranted. The Consensus Panel's guidelines for preventing the spread of MRSA recommend contact precautions and the isolation of infected or colonized patients in a single room or cohort, i.e., grouping them geographically with designated staff [18]. Also, since MRSA colonization precedes infection because of inpatient circumstances and rather strong treatments [25,26], a local control is very important for controlling MRSA outbreak in selected circumstances such as SCT ward in which many immunocompromised patients are taken care of.

In this report, we described an MRSA outbreak in the SCT ward during a limited period of 4 weeks. Initially, we suspected that all MRSA infections were caused by a single source, such as highly contaminated stool. However, unexpectedly, no direct contact was identified among patients and staff who were involved in their care. The transmission of MRSA mostly occurs through direct person-to-person contact, and transmission from the environment is extremely rare in places where strict precautions are taken and careful decontamination procedures are used. Hence, we undertook a molecular epidemiological analysis to critically examine the suspected break in our procedure. We found that four of the six isolates were genetically different, and our Infection Control Team concluded that horizontal transmission was unlikely. Nevertheless, the interest raised with this event resulted in further enforcement of essential precautions against droplets and contact,and the elimination of new MRSA cases for subsequent months.

Although our observation was well anticipated, in that molecular typing techniques are effective in the diagnosis and tracking of MRSA, the results are still unique, since they highlight the value of these methods over clinical judgment in a critical care situation

with highly immunocompromised patients. Since the molecular typing properties of MRSA are very similar in Japan, especially in the local areas [27], we focused on the genetic event detected by molecular typing and diagnosed those differences as different strain from outbreak. Thus, this report should be helpful for evaluating whether the routine application of these measures should be critically considered in the assessment of outbreak.

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