the increase in the amount of DNA-PK might be related to the interaction between DNA-PK and gp70. Co-precipitation was not demonstrated between p53 and DNA-PK, p53 and ATM, or p53 and gp70 (data not shown). To determine whether the interaction occur in the cytoplasm or nucleus of FLV-infected cells, fractionated lysate was analyzed for co-precipitation of gp70 with DNA-PK. As shown in Fig. 6C, gp70 was mainly co-precipitated with DNA-PK in the cytoplasmic fraction of cell lysate from FLV + TBI treated mice.

4. Discussion

Ionizing irradiation induces a marked increase in cellular p53 protein followed by the consequent transmission of DNA damage signals [1]. The prominent apoptosis induced by FLV + TBI-treatment in the present study was *p53*-dependent, although the apoptosis observed in liquid-cultured FLV-induced primary erythroleukemic cells has been reported to be *p53*-independent [41]. As expected, expression of the *p53* target molecule, *bax*, was up-regulated, however, *p21* which has a role in cell cycle arrest signaling was also overexpressed. These findings suggested that FLV + TBI treatment induced p53 activation leading to not only apoptosis but also other DNA damage responses. Therefore, key molecules modifying the transcriptional activity of *p53* might be located upstream of p53 in the signaling pathway of this model.

In response to DNA-double strand breaks, candidates for the upstream activators of p53 would include two members of the PI3 kinase family, ATM and DNA-PK. ATM phosphorylates p53 in vivo [13], while DNA-PK has been proved to phosphorylate p53 in in vitro systems but not in in vivo systems [9]. Using mouse embryonic fibroblasts lacking DNA-PK, Jimenez et al. [15] have demonstrated that DNA-PK was not required for the p53-dependent response to DNA damage. However, our results demonstrated that not only ATM but also DNA-PK played an important role in inducing a lethal apoptosis in FLV + TBI-treated mice in vivo, whereas only ATM but not DNA-PK was required for the mild apoptotic response after low-dose TBI in bone marrow cells of C3H mice. The result suggested that FLV infection modifies the innate signaling pathway of C3H bone marrow cells to activate p53 after irradiation.

We report here that FLV-infection actually modifies the DNA-PK molecule after treatment with TBI. gp70, known as an env protein of F-MuLV, strongly interacted with DNA-PK. The interaction was mainly observed in the cytoplasmic fraction of FLV-infected cell lysate suggesting that DNA-PK with gp70 complex would be formed in the cytoplasm and then, function as a kinase and activate *p53* signaling. DNA-PK is known to associate with various proteins including Ku, which stimulates the catalytic subunit of DNA-PK (DNA-PKcs) leading to effective V(D)J recombination and DNA double-strand break repair in vivo [17,42]. Although other factors would be involved in the interaction between DNA-PK and gp70, gp70 might act as an enhancing factor for DNA-PK to be immediately accumulated and also in kinase activity to phosphorylate p53.

It remains unclear how DNA-PK or ATM works in DNA damage-induced signaling to activate p53 in FLV-infected C3H cells. Shangary et al. [37] demonstrated that ATM activated c-Abl kinase in response to ionizing irradiation and subsequently the activated c-Abl regulated DNA-PK activity in vivo. Thus, in the present experimental system also, DNA-PK and ATM might cooperate through other factors such as c-Abl kinase. Another possibility would be that DNA-PK and ATM separately activate p53 protein on FLV + TBI-treatment. The functional complementation of these two molecules was demonstrated by the fact that mice deficient in both DNA-PK (SCID mutation) and ATM show embryonic lethality [43]. In addition, the function of DNA-PK in non-homologous end joining (NHEJ) would partly be performed by ATM [38,44]. These results suggest that DNA-PK and ATM should have similar and sometimes complementary roles in various cellular pathways.

Recently, Woo et al. [45] have shown that DNA-PKcs forms a complex with latent p53 immediately following γ-irradiation, and latent murine p53 phosphorylated at Ser-18 by DNA-PK is required for DNA damage-induced apoptosis. In our experimental system, bone marrow cells from C3H·SCID mice exhibited positive signals for p53 activation and an apoptotic response when treated with TBI alone, but did not exhibit enhanced signals when treated with FLV + TBI. Thus, DNA-PK is not required for DNA damage-induced apoptosis, although signaling modulation by FLV infection would cause DNA-PK participation in apoptosis in response to DNA damage. Therefore, our present data would indicate the existence of some unidentified factor(s) amplifying mild DNA damage signals to induce severe cell death. Molecules involved in the DNA-PK-gp70 association or in the overexpression of DNA-PK might be the key to clarifying these mechanisms.

Controlling p53-mediated apoptosis would be one of the most attractive strategies of gene therapy for cancer [46]. The modifier of p53 for strong activation would be crucial to make the *p53*-gene therapy more effective. The mechanism of DNA-PK-associated p53 signaling modification leading to enhanced apoptosis should be clarified not only to understand the complexity of p53 signaling changed by retroviral infection but also to effectively use the p53 function in gene therapy aimed at cancer.

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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.
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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′-CAAGGACCACCG-CATCTCTACATTC-3′. For cIAP1 mRNA, sequences of the forward primer were 5′-CAGCCTGAGCAGCTTGCAA-3′ and the reverse primer, 5′-CAAGCCACCATCACAACAA-AA-3′; the TaqMan probe was 5′-TTTATTATGTGGGTCG-CAATGATGATGTCAAA-3′. For cIAP2 mRNA, sequences of the forward primer were 5′-TCCGTCAAGTTCAAGCC-AGTT-3′ and the reverse primer, 5′-TCTCCTGGGCTGTC-TGATGTG-3′; the sequence of the TaqMan probe was 5′-CCCTCATCTACTTGAACAGCTGCTAT-3′. Sequences of the forward primer for NAIP mRNA were 5′-GCTTCAC-AGCGCATCGAA-3′ and the reverse primer, 5′-GCTGGG-CGGATGCTTTC-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′-CACTGGCACGA-GCAGGGTTTCTTTATACTG-3′. Sequence of the forward primer for GAPDH mRNA were 5′-GAAGGTGAAGGTC-GGAGT-3′ and the reverse primer, 5′-GAAGATGGTGAT-GGGATTTC-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

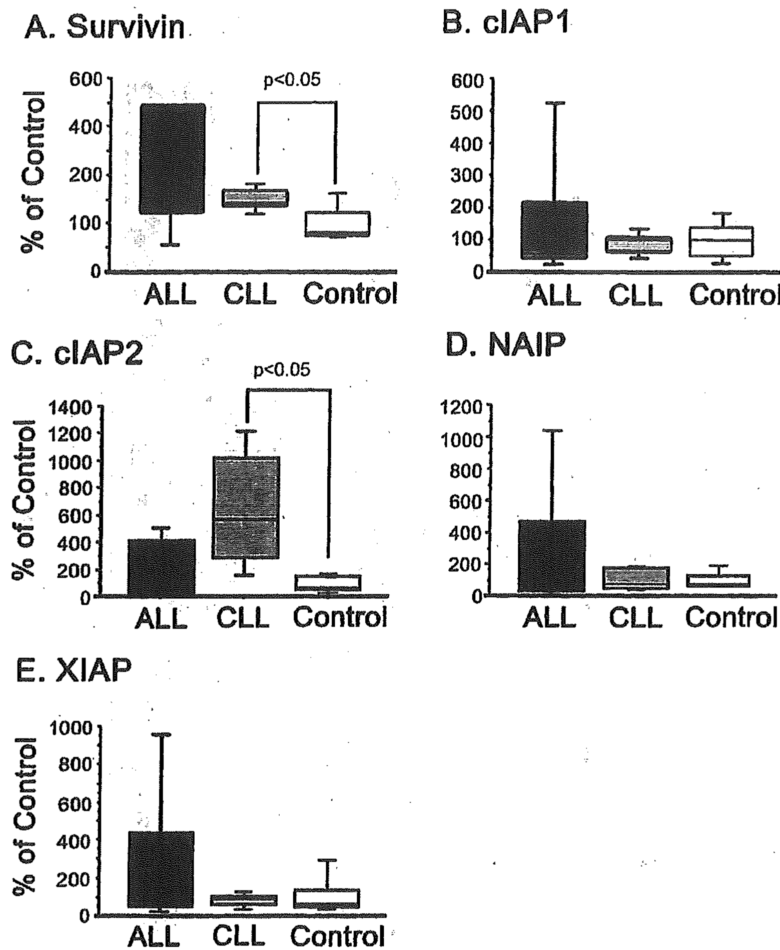


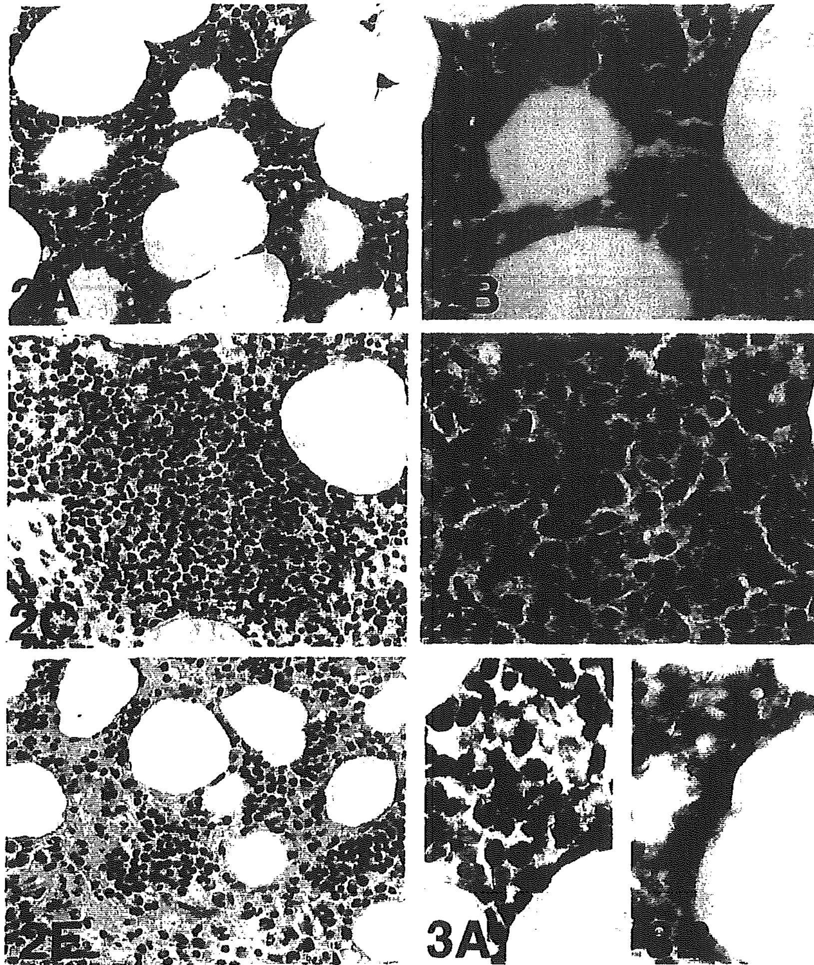
Fig. 1. ((A)–(E)) Quantitative RT-PCR analysis for IAP family proteins, survivin, cIAP1, cIAP2, NAIP and XIAP. Relative intensity was calculated as intensity of reaction of IAP family (total Raji RNA (ng))/intensity of reaction of GAPDH (total Raji RNA (ng)). The intensity of expression from ALL and 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.

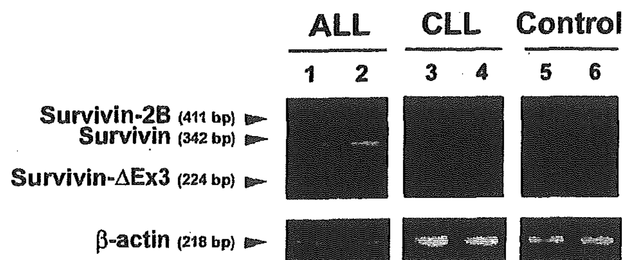


Fig. 4. RT-PCR analysis for the expression of wild-type survivin and the splice variant survivin-2B and survivin-ΔEx3 in ALL (lanes 1 and 2), CLL (lanes 3 and 4) and control bone marrow (lanes 5 and 6). In ALL and CLL samples, the predominant expression was observed in the 342 bp wild-type survivin. 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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Expression of IAP family proteins in myelodysplastic syndromes transforming to overt leukemia

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Abstract

Bone marrow cells of patients with myelodysplastic syndromes (MDS) frequently undergo apoptosis, though the apoptotic cell ratio decreases when overt leukemia (OL) develops. Thus, we compared the expression of the inhibitor of apoptosis protein (IAP) gene family proteins in bone marrow samples from control, MDS, OL transformed from MDS (MDS → OL), and de novo acute myelogenous leukemia (AML) subjects by the quantitative real-time RT-PCR method and an immunohistochemical approach. Overexpression of mRNA for survivin, cIAP1, NAIP and XIAP was significant in MDS bone marrow cells compared with control samples. However, the expression of mRNA for survivin, cIAP1 and cIAP2 exhibited a remarkable decrease after the development of OL (MDS → OL). By immunohistochemistry, survivin was found to localize to the nucleus of myeloid cells in the majority of MDS cases. Next, the chronological changes in the expression of IAPs were determined in cases of MDS with evolution of OL. Although the expression of cIAP1 and cIAP2 revealed a sudden or gradual decrease as OL developed, survivin in many cases and XIAP in the majority of cases exhibited a peak of expression before a decline, indicating that these IAPs could be associated with the early events in the development of OL.
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Keywords: Survivin; IAP; Apoptosis; MDS; Overt leukemia; Bone marrow

1. Introduction

The regulation of apoptotic cell death may have a profound effect on the pathogenesis and progression of hematological malignancies. Myelodysplastic syndromes (MDS) are a heterogenous group of hematological malignancies exhibiting peripheral blood cytopenias despite bone marrow hypercellularity [1–3]. Therefore, one characteristic feature of MDS is ineffective hematopoiesis mainly caused by frequent apoptosis of bone marrow hematopoietic cells [4,5]. Regarding the complicated regulating mechanisms regulating apoptosis in MDS bone marrow, we demonstrated previously that a variety of cytokines and their receptors, such as tumor necrosis factor (TNF)-α/TNFRI and II, Fas-ligand/Fas, play an important role in hematopoietic cells [6–10]. However, another distinctive feature of MDS is the potential to develop into overt leukemia (OL) [11]. Leukemic cells derived from MDS usually show marked proliferation in the bone marrow [12], thus these cells have gained the ability to grow rapidly and/or avoid apoptosis.

Inhibitor of apoptosis protein (IAP) family proteins, including survivin, block apoptosis induced by a variety of triggers [13,14]. Although the exact biochemical mechanism by which the IAP family 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 [14,15]. The expression of survivin is ubiquitous in fetal tissues, but becomes restricted during development, and appears to be negligible in the majority of terminally differentiated adult tissues [16,17]. However, an analysis of the differences in gene expression between normal and tumor cells has revealed that survivin is one of the proteins whose gene is most consistently overexpressed in tumor cells relative to normal tissue [18]. In fact, survivin

Abbreviations: IAP, inhibitor of apoptosis proteins; MDS, myelodysplastic syndromes; OL, overt leukemia; AML, acute myelogenous leukemia

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is prominently expressed in transformed cell lines and in many human cancers including hematopoietic cell tumors [19]. Survivin is usually detected in the cytoplasm of tumor cells, and is therefore widely regarded as a cytoplasmic protein [16,20,21]. However, several studies have shown a nuclear accumulation of survivin in gastric cancer cells [22] and lung cancer cells [23]. We have reported recently that ALL cells principally exhibit a nuclear localization of survivin, while CLL cells have a cytoplasmic distribution [24]. Although the significance of this nuclear–cytoplasmic expression in tumor cells is still controversial, the subcellular localization of survivin should also be clarified in MDS, OL and de novo acute myelogenous leukemia (AML) subjects.

To focus on the contribution of IAPs to the progression from MDS to OL, we collected cases of MDS that developed into OL in the present study. The overall level of expression of survivin was relatively high in the bone marrow cells from these MDS samples compared with control bone marrow, however, the level of expression appeared lower when OL developed. The expression of other IAPs including cIAP1, cIAP2, NAIP and XIAP, all of which appeared to suppress apoptosis by inhibiting caspase and procaspase [25–28] was also demonstrated in these samples and the significance of IAP family proteins in the transformation of MDS to OL was discussed.

2. Materials and methods

2.1. Patients

Formalin-fixed paraffin-embedded bone marrow aspiration samples from 13 individuals with no hematological disorders as normal controls (male:female, 13:0; age, median 63 years, maximum 76 years, minimum 51 years), 13 patients with MDS (3 with refractory anemia (RA), 4 with RA with excess of blasts (RAEB) and 6 with RAEB in transformation (RAEB-t); male:female, 10:3; age, median 67 years, maximum 77 years, minimum 55 years) who developed overt leukemia later in the course (duration, 3 months to 3 years), and 10 patients with de novo AML (9 with M2 and 1 with M1 according to the FAB classification, male:female, 4:6; age, median 55 years, maximum 76 years, minimum 23 years) were analyzed. To rule out the influence of aging on bone marrow cells, age-matched control cases were analyzed. Diagnoses were based on standard clinical and laboratory criteria, including cell morphology [1–3]. All samples were collected at the time of the initial aspiration biopsy and the samples from de novo AML exhibited a proliferation of blastic cells accounting for more than 80% of all 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. Further, bone marrow samples were taken from MDS cases when the patients developed OL and analyzed.

In addition, fresh frozen bone marrow samples from control (5 cases, age, median 55 years, maximum 74 years, minimum 43 years; male:female, 3:2), MDS (11 cases, 3 with RA, 5 with RAEB and 3 with RAEB-t; male:female, 8:3; age, median 66 years, maximum 77 years, minimum 55 years) with transformation to OL, and de novo AML (5 cases of M2 by FAB classification, male:female, 3:2; age, median 62 years, maximum 76 years, minimum 49 years) cases were used for the PCR experiments. In MDS, we could follow the bone marrow changes before and after the transformation to OL and further, chronological changes determined at more than three points could be analyzed in six cases.

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 identify apoptotic cells, the terminal deoxy-transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method was used as described previously [29]. Briefly, tissue sections were deparaffinized and incubated with proteinase K (prediluted, DAKO, Glostrup, Denmark) for 15 min at room temperature. After a wash, 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, and color development was achieved with DAB containing 0.3% hydrogen peroxide solution. 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 (counting more than 1000 cells).

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

The RNA was extracted from frozen bone marrow samples of control subjects with no hematological disorders, MDS patients and de novo AML patients using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. For quantitative RT-PCR, fluorescent hybridization probes and the 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 the IAP family and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized at a commercial laboratory (Perkin-Elmer Cetus). The primers and TaqMan probes 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′-CAAGGACCACCGCATCTCTACA-TTC-3′. For cIAP1 mRNA, the forward primer was 5′-CAGCCTGAGCAGCTTGCAA-3′ and the reverse primer was 5′-CAAGCCACCATCACAACAAAA-3′; the TaqMan probe was 5′-TTTATTATGTGGGTCGCAATGATGA-TGTCAAA-3′. For cIAP2 mRNA, the forward primer was 5′-TCCGTCAAGTTCAAGCCAGTT-3′ and the reverse primer was 5′-TCTCCTGGGCTGTCTGATGTG-3′; the TaqMan probe was 5′-CCCTCATCTACTTGAACAGC-TGCTAT-3′. The forward primer for NAIP mRNA was 5′-GCTTCACAGCGCATCGAA-3′ and the reverse primer was 5′-GCTGGGCGGATGCTTTC-3′; the TaqMan probe was 5′-CCATTTAAACCACAGCAGAGGCTTTAT-3′. The forward primer for XIAP mRNA was 5′-AGTGGTAGTCC-TGTTTCAGCATCA-3′ and the reverse primer was 5′-CCGCACGGTATCTCCTTCA-3′; the TaqMan probe was 5′-CACTGGCACGAGCAGGGTTTCTTTATACTG-3′. The forward primer for GAPDH mRNA was 5′-GAAGGTGAA-GGTCGGAGT-3′ and the reverse primer was 5′-GAAGA-TGGTGATGGGATTTC-3′; the TaqMan probe was 5′-CAAGCTTCCCGTTCTCAGCC-3′. Conditions for the 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). The expression of survivin and other IAP family proteins was quantitated according to a method described elsewhere [24]. 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 were normalized using the data for GAPDH in each sample.

2.4. Immunohistochemistry for survivin and proliferative cells

Formalin-fixed tissue sections (4 μm thick) of bone marrow from control (13 cases), MDS (13 cases) and de novo AML cases (10 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, and to characterize proliferative cells. Primary antibodies included polyclonal rabbit antibody against human survivin (SURV 11-A, Alpha Diagnostic International, 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.

2.5. Double staining for survivin and cell markers

The phenotype of survivin-expressing cells was identified by double immunostaining using a polyclonal antibody against survivin and a monoclonal antibody against glycophorin A (DAKO), anti-neutrophil elastase (DAKO) or anti-CD34 antibody (Nichirei, Tokyo, Japan) followed by the anti-rabbit IgG 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).

2.6. Statistical analysis

Statistically significant differences in the quantitative analysis were determined using Mann–Whitney's *U*-test for comparisons between control, MDS, MDS → OL and de novo AML samples except for the comparison of paired samples from MDS and MDS → OL, for which Wilcoxon's test was used.

3. Results

3.1. Apoptotic and proliferative cell ratio of the bone marrow cells from control, MDS, overt leukemia derived from MDS (MDS → OL), and de novo AML subjects

To identify the apoptotic and proliferative cells 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 [12,29], the frequency of apoptosis was significantly higher in MDS bone marrow cells than control cells or de novo AML cells and the proliferative cell ratio in MDS bone marrow was as high as that in AML cases and significantly higher than that of control bone marrow ($P < 0.01$ by the Mann–Whitney's *U*-test). When OL developed (MDS → OL), the apoptotic cell ratio was reduced in MDS bone marrow, in spite that the proliferative cell ratio was similar to that in MDS before transformation to OL (Table 1). These findings suggested that the proliferation of blasts of MDS-derived overt leukemia in the bone marrow occurred in association with reduced apoptosis but not with the increased activity for cell proliferation.

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

To quantitate the mRNA expression levels of the IAP family in MDS bone marrow cells, real-time quantitative RT-PCR was performed using bone marrow samples from control, MDS, MDS → OL and de novo AML cases. As shown in Fig. 1, the expression of survivin ($P < 0.05$),

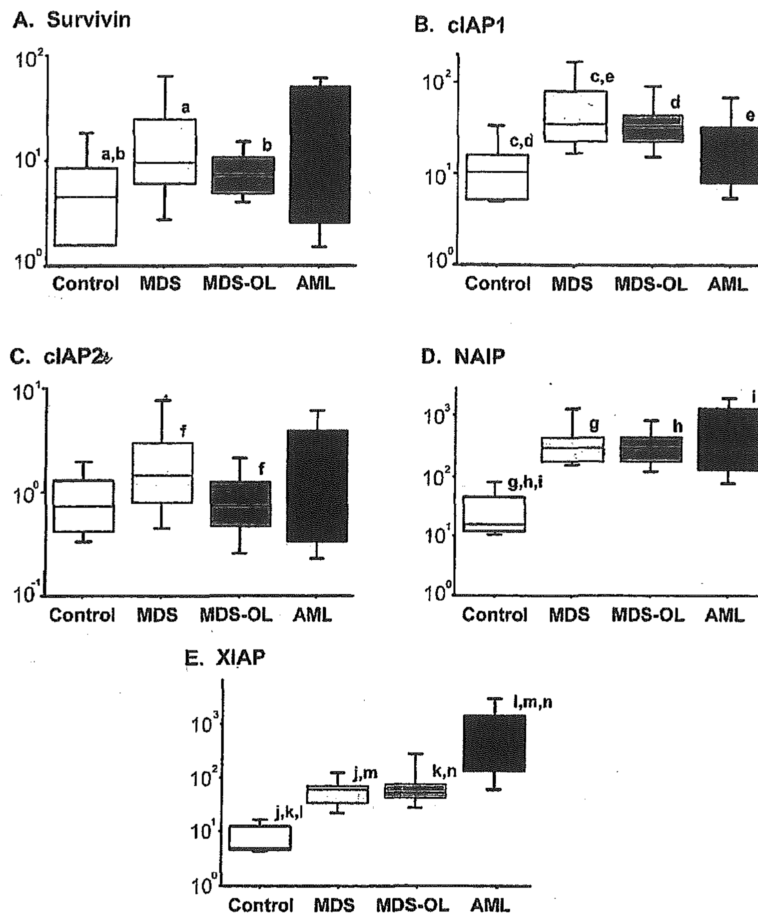

Fig. 1. Quantitative RT-PCR analysis of IAP family proteins, survivin, cIAP1, cIAP2, NAIP and XIAP in control ($n = 5$), MDS ($n = 11$), MDS → OL ($n = 11$) and de novo AML cases ($n = 5$). Relative intensity was calculated as (intensity of reaction of IAP family (total Raji RNA, ng))/(intensity of reaction of GAPDH (total Raji RNA, ng)). The box plot graphs indicate the values for MDS and AML cases. Bars indicate 90 and 10 percentile and boxes indicate 25–75 percentile. Differences were significant between the samples as follows using Mann–Whitney's U-test or Wilcoxon's test: survivin, control and MDS ($^{a}P < 0.05$), and control and MDS → OL ($^{b}P < 0.01$); cIAP1, control and MDS ($^{c}P < 0.01$), control and MDS → OL ($^{d}P < 0.01$), and MDS and de novo AML ($^{e}P < 0.01$); cIAP2, MDS and MDS → OL ($^{f}P < 0.05$); NAIP, control and MDS ($^{g}P < 0.01$), control and MDS → OL ($^{h}P < 0.001$), and control and de novo AML ($^{i}P < 0.01$); XIAP, control and MDS ($^{j}P < 0.01$), control and MDS → OL ($^{k}P < 0.01$), control and de novo AML ($^{l}P < 0.05$), MDS and de novo AML ($^{m}P < 0.01$), and MDS → OL and de novo AML ($^{n}P < 0.01$).

cIAP1 ($P < 0.01$), NAIP ($P < 0.01$) and XIAP ($P < 0.01$) exhibited a significant up-regulation in MDS compared with controls. The mRNA for survivin, cIAP1, NAIP and XIAP and cIAP2 also revealed higher level of expression in de novo AML bone marrow than control marrow, although the difference was significant only for NAIP ($P < 0.01$) and XIAP ($P < 0.05$). The expression of cIAP1 was significantly higher ($P < 0.01$) and that of XIAP significantly lower ($P < 0.01$) in MDS samples than de novo AML samples. The MDS → OL samples had lower levels of XIAP than de novo AML samples ($P < 0.01$). No significant differences were found between IAP protein expression and the patients' age, sex, or subtype of MDS or AML. Regarding the IAPs in MDS → OL bone marrow, survivin, cIAP1, and cIAP2 tended to exhibit a low level of expression compared with that in the initial MDS samples, although statistical significance was demonstrated only in the case of cIAP2 ($P < 0.05$).

These results indicated that the expression of IAP family proteins might not correlate with the frequency of apoptosis in bone marrow cells, because highly apoptotic MDS bone marrow cells exhibited higher levels of IAPs. Further, MDS → OL samples tended to have lower expression levels of IAPs and thus, the less frequent apoptosis in fully-developed OL would not to be associated with the overexpression of IAPs.

3.3. *Immunohistochemical detection of survivin in the bone marrow of control, MDS, and overt leukemia derived from MDS (MDS → OL) subjects*

To investigate the distribution of survivin, immunohistochemical staining was performed in bone marrow samples from control, MDS, and MDS → OL subjects. As we have demonstrated previously [24], survivin was detected in only a few scattered myeloid cells of the control bone marrow

Table 1
Apoptotic and proliferative cell ratio of bone marrow from control, MDS, MDS → OL and AML subjects

Cases	n	TUNEL⁺ cell ratio (%)[a]	Ki-67⁺ cell ratio (%)[a]
Control	13	2.3 (1.3–3.9)[a,b,c]	4.7 (3.2–6.7)[f,g,h]
MDS	13	15.6 (7.5–24.3)[a,d,e]	30.6 (13.9–36.4)[f,i]
MDS → OL	13	8.6 (4.4–14.8)[b,d]	29.1 (12.0–47.5)[g,j]
De novo AML	10	9.4 (4.0–20.0)[c,e]	20.4 (9.1–31.6)[h,i,j]

[a] Values indicate median (minimum–maximum). Differences were significant between the TUNEL-positive cell ratio of control and MDS ([a]$P < 0.0001$), control and MDS → OL ([b]$P \leq 0.0001$) and control and de novo AML ([c]$P < 0.0001$) with Mann–Whitney's U-test, MDS and MDS → OL ([d]$P < 0.01$) using Wilcoxon's test, and MDS and de novo AML ([e]$P < 0.05$) with Mann–Whitney's U-test. The Ki-67-positive cell ratio exhibited significant differences between control and MDS ([f]$P < 0.0001$), control and MDS → OL ([g]$P < 0.0001$), control and de novo AML ([h]$P < 0.0001$), MDS and de novo AML ([i]$P < 0.05$), and MDS → OL and de novo AML ([j]$P < 0.01$) with Mann–Whitney's U-test.

samples. The 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 MDS samples revealed positive staining for survivin, although the staining intensity and frequency varied among cases. At the cellular level, survivin signals in MDS bone marrow cells were predominantly localized to the nucleus, although in some cases, a strong reaction was also observed in the cytoplasm. Survivin-positive cells basically resembled myeloid cells in morphology. Double immunostaining confirmed that the majority of survivin-positive cells also expressed neutrophil elastase, a myeloid cell marker, but not the erythroid cell marker glycophorin A (Fig. 2A and B). The CD34 antigen, stem cell marker, was expressed in some of the survivin positive cells (Fig. 2C). The differences in staining intensity and patterns for survivin were not evident between MDS samples and MDS → OL samples from the same cases. Tissue sections reacted with preimmune rabbit antibody of irrelevant specificity showed no significant staining in any of the samples (not shown).

3.4. Chronological changes of IAP expression in bone marrow samples from cases of MDS leading to overt leukemia

The apoptotic frequency of bone marrow cells exhibited a significant decrease during the transformation of MDS into OL as described earlier (Table 1). Thus, to determine whether the IAP family proteins play a role in inhibiting apoptotic signals during the development of OL, real-time quantitative PCR was performed in specimens sampled chronologically from the time of the initial diagnosis of MDS to the time of the transformation to OL. Representative patterns of IAP expression in two cases of MDS (cases 1 and 4) with progression to OL are shown in Fig. 3. The patterns of change in IAP expression could be classified into five types; (1) a gradual decrease in expression from MDS to MDS → OL (e.g., Fig. 3, cIAP2 in cases 1 and 4) (decrease: D); (2) a high expression level but a sudden fall before the development of OL (e.g., Fig. 3, cIAP1 in cases 1 and 4, XIAP in case 4) (increase followed by a decrease: ID); (3) a high level of expression that increases further (not shown in Fig. 3) (increase: I); (4) low or high level expression, then, an increase beyond the initial level followed by a drop below the control level (e.g., Fig. 3,

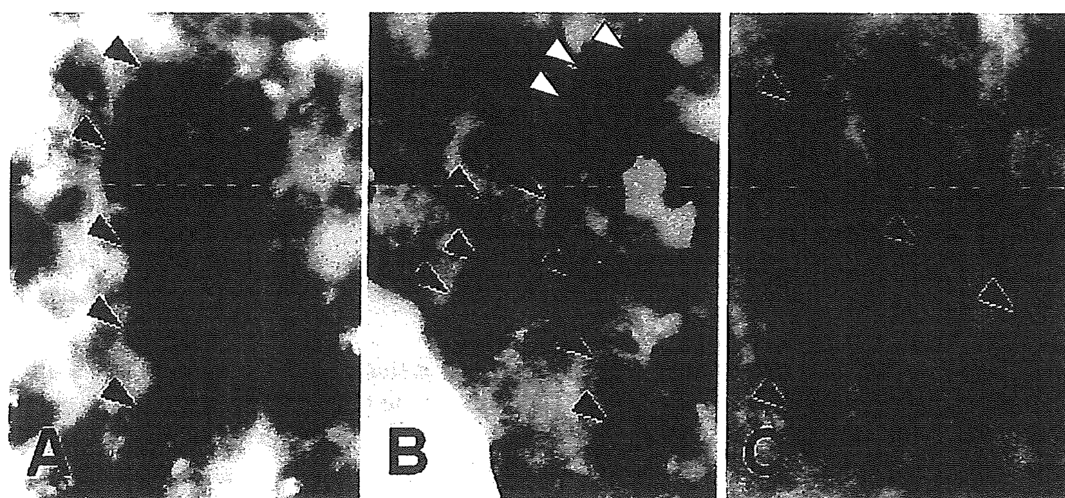

Fig. 2. Immunohistochemical detection of survivin in the bone marrow cells of MDS subjects (RAEB, 400× original magnification). Double staining for survivin (nuclear: brown) and cell markers (cytoplasmic/cell surface: blue) (A, neutrophil elastase; B, glycophorin A; C, CD34) demonstrated that survivin-positive cells were basically the myeloid series (A, neutrophil elastase-positive, arrowheads) and not the erythroid series of cells (B, black arrowheads indicate survivin-positive cells and white ones glycophorin A-positive cells). A part of survivin-positive cells were also positive for CD34 antigen (C, arrowheads). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

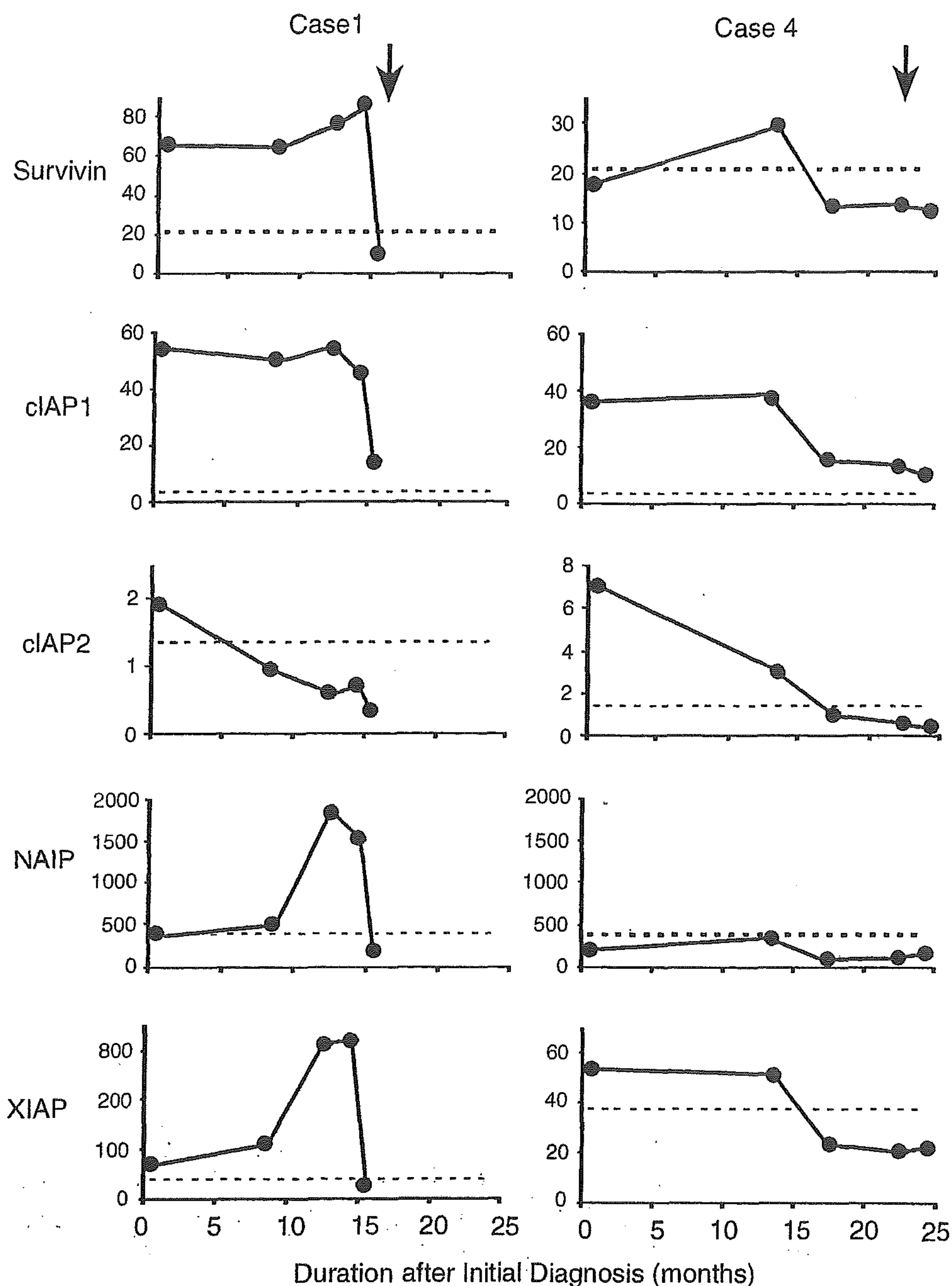

Fig. 3. Chronological changes of IAP family protein expression in MDS → OL cases (cases 1 and 4) determined by quantitative RT-PCR. Arrows indicate the time of OL evolution and dotted lines indicate median values of the control samples. Note that the dynamics of survivin and XIAP expression tended to form a peak between the time of initial diagnosis and OL development, while cIAP1 and cIAP2 expression decreased during the evolution of OL from MDS.

survivin in cases 1 and 4, NAIP in case 1) (peak formation: P); (5) stable low-level expression than an increase to the control level with no remarkable change (e.g., Fig. 3, NAIP in case 4) (not significant: N). The pattern ID and P would have the similar significance in the sense that the expression level once increased before the evolution of overt leukemia. And the changes forming peak expression (such as P and ID patterns) might be associated with the functional aspects of IAPs in the development of overt leukemia. As shown in Table 2, cIAP1 and cIAP2 expression basically revealed a sudden or gradual decrease from the initial diagnosis of MDS to the time that OL developed suggesting that these proteins are not associated with the suppression of apoptosis during the evolution of OL, although cIAP1 may have made a partial contribution in cases 5 and 6. By contrast, survivin in a half of the cases and XIAP in the majority of cases exhibited an increase in expression reaching a peak during the course (pattern 4, P). In cases with a decrease in the expression of IAP family proteins (patterns 1, D and 2, ID), the point of any peak might have been missed as

Table 2
Patterns of changes in expression of IAP family proteins during the course of evolution of OL in MDS bone marrows

	IAPs					Duration MDS → OL (months)
	Survivin	cIAP1	cIAP2	NAIP	XIAP	
Case 1	P[a]	ID	D	P	P	15
Case 2	ID	ID	D	D	P	3
Case 3	P	ID	D	ID	P	24
Case 4	P	ID	D	N	ID	24
Case 5	N	ID	ID	ID	P	35
Case 6	N	P	N	I	I	5

[a] Pattern 1, gradual decrease of expression from MDS to MDS → OL (decrease: D); pattern 2, high level of expression but a sudden decrease at the time of OL development (increase followed by a decrease: ID); pattern 3, an initially high level of expression that increases further (increase: I); pattern 4, an initially low or high level of expression, an increase beyond the initial level and then a reduction below the control level forming a peak during the course (peak formation: P); pattern 5, stable expression below the control level with no remarkable change (not significant: N).

seen in pattern 4, P. Thus, in most cases, survivin as well as XIAP could show a peak in expression during the development of OL. The expression of NAIP showed various patterns of change and did not exhibit specific changes during the transformation to OL.

In any event, MDS → OL bone marrow did not exhibit an increase in the expression of cIAP1 and cIAP2 proteins in most cases, although these proteins were highly expressed at the time of the initial diagnosis of MDS. Thus, the expression of these proteins would not be associated with the reduction in apoptosis in MDS → OL and would be secondarily regulated by other mechanisms. In contrast, survivin and XIAP might be associated with the mechanisms for avoiding apoptotic signals because they showed a peak of expression (pattern 4) or maintained a high level of expression (pattern 1 or 3) during the course of OL evolution in most cases.

4. Discussion

Regarding the expression of survivin in myeloid neoplasms, previous studies revealed overexpression in MDS samples including RA and RAEB [30] and a significant level of expression in de novo AML [31,32]. However, little is known about the potential roles of IAPs in the pathogenesis of MDS, MDS → OL and de novo AML. In lymphoid neoplasms by contrast, several studies have demonstrated 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 on stimulation with CD40 and that survivin was the only IAP whose expression was induced by the CD40 ligand (CD40L) [33]. CD40 belongs to the TNF receptor superfamily [34] and its stimulation appears to rescue B-CLL cells from apoptosis and induce proliferation [35]. We have demonstrated recently that ALL as well as CLL cells exhibited significant expression of survivin and cIAP2 [24].

In MDS samples, the expression of IAPs was stronger than that in control samples. Although the number of samples would not be enough for evaluating rather a heterogeneous entity of hematological malignancy, MDS, we could find the significant elevation of expression level in MDS samples. Thus, increased apoptosis in MDS would not be attributable to a lack of anti-apoptotic regulatory mechanisms by IAP family proteins. In other words, down-regulation of IAP family expression might not be a primary event in MDS pathogenesis. To the contrary, up-regulation of IAP family proteins in MDS bone marrow appears to be an attempt at counter-balancing the primary stimuli for apoptosis. The levels of IAPs basically dropped below those of the initial MDS samples after OL developed. These findings are inconsistent with reports that MDS bone marrow cells frequently undergo apoptosis, while OL cells are less prone to apoptosis. The decline in IAP protein expression may be attributable to gene silencing, for example, by DNA hypermethylation. Although this interpretation is speculative, overexpression of IAPs in MDS would be the reversible change whether the down-regulation in MDS → OL samples might be irreversible/reversible. The mechanisms for OL cells to avoid apoptosis might include the loss of critical components of the apoptotic signaling machinery. However, a chronological analysis of IAP expression dynamics demonstrated that some IAPs exhibited a transient overexpression during the development of OL. The peak expression might be associated with the initial signaling to avoid apoptosis, although the mechanisms for down-regulation of IAP expression in MDS → OL remained unexplained. However, inhibition of apoptosis in MDS → OL should not be explained solely by the dynamics of IAP expression. Although we could not find specific cytogenetic abnormalities in MDS → OL samples, interactions with various factors including many kinds of apoptosis-regulating molecules should be clarified in the future study.

Immunohistochemical staining revealed a very high frequency of survivin expression in many of the MDS cases though only a few cells were positive in some cases. Thus, differences in the positive cell ratio would influence the differences in intensity in the overall expression of mRNA for IAPs. As suggested by the double immunostaining in the present study, the survivin positivity in myeloid cells, but not erythroid cells, might reflect the growth advantage of apoptosis-resistant neoplastic stem cells compared to normal stem cells, and also the tendency of survivin-negative erythroid cells to undergo apoptosis. It would be important to correlate the genetic events/apoptotic signals and survivin-expressing cell clones in a future study.

In addition to its anti-apoptotic function, survivin also plays a role in the regulation of cell cycle progression during mitosis [19]. The highly proliferative activity of MDS bone marrow cells as well as AML cells might be associated with the expression of survivin. Adida et al. [32] reported that

survivin expression occurs frequently in de novo AML, detected in 60% of a series of 125 patients analyzed, and was an unfavorable prognostic factor. However, we could not find a significant difference in the expression of IAPs between the initial samples from MDS → OL patients who exhibited a short or long progression to OL (data not shown). As to the expression of IAPs in de novo AML, the present study demonstrated strong expression in some cases and control levels in others, suggesting that de novo AML cases are heterogeneous in terms of IAP expression. Overall, the expression of IAPs was relatively higher in de novo AML than MDS → OL samples, and a significant difference was observed in XIAP expression. Thus, the apoptosis/proliferation of bone marrow cells would be controlled via IAP family proteins differently between de novo AML and MDS → OL cases. Furthermore, it would be useful to determine XIAP expression to distinguish MDS → OL from de novo AML clinically.

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 explain the silencing of survivin gene transcription by p53 [37]. On the other hand, the overexpression 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, MDS (5–17%) and AML (10–20%) have been shown to demonstrate a rather low frequency of p53 mutation [38]. Therefore, p53 mutation would not appear to be the major factor controlling the overexpression of survivin in the bone marrow of patients with MDS and AML.

In conclusion, we demonstrated the transient overexpression of mRNA for several IAPs during the progression from MDS to OL, although the expression decreased below the initial level after the transformation was completed. Clinically, it would be useful to detect the increase in expression of mRNAs of IAPs for predicting the evolution of OL in MDS patients. However, the levels of mRNA could not reflect protein expression status directly. Further study is warranted to clarify the regulatory mechanisms of IAP expression in MDS → OL in association with the apoptotic signaling pathways.

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Expression level of Wnt signaling components possibly influences the biological behavior of colorectal cancer in different age groups

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Abstract

Advancing of age apparently influences the behavior of colorectal cancer (CRC). The pattern of activation and expression of Wnt target genes may influence the behavior of the cancer. In the present study, the level of activation of some elements of Wnt signaling was evaluated and correlated with the patient's age and clinicopathological characteristics of the tumor. Beta-catenin and c-Myc mRNA expressions were evaluated by semiquantitative real-time PCR, and subcellular localization of the beta-catenin protein was evaluated by immunohistochemistry. Patients aged 70–84 tended to have locally advanced disease more frequently than younger patients. The same group of patients also more frequently had high nuclear expression of beta-catenin protein and higher expression of c-Myc mRNA. Beta-catenin mRNA had a rather constant expression with advancing of age. High nuclear expression of beta-catenin and high expression of c-Myc were apparently also correlated with locally advanced disease. We concluded that the level of Wnt signaling activation might influence the behavior of the disease in different age groups.

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Keywords: Aging; Colorectal cancer; Cancer behavior; Wnt signaling; Beta-catenin; c-Myc

Introduction

Colorectal cancer (CRC) predominantly affects the elderly population, with more than 70% of cases occurring in those aged 65 years or older (CCCG, 2000; Yancik and Ries, 1994). Old age is also a sign of poor prognosis, possibly resulting from associated diseases, different received treatment, and from a presumed distinct behavior of the tumor in this age group (Arai et al., 2000; CCCG, 2000; Nagaoka et al., 2003). The incidence and pattern of CRC observed in the elderly population may result from the effect of senescence on various molecular mechanisms that underlie the disease (DePinho, 2000). The accumulation of somatic mutation over a lifetime combined with other genetic and environmental influences, such as epigenetic gene silencing, telomere dysfunction, and senescent stroma, may lead to the emergence of cancer and determine its phenotype in the elderly (DePinho, 2000).

Many sporadic CRCs have genetic dysfunction in the Wnt signaling pathway (Jass et al., 2002; Miyoshi et al., 1992; Morin et al., 1997; Powell et al., 1992). In the adult colon, Wnt signaling controls the proliferation and differentiation of epithelial cells, allowing a balanced replacement of the cells eventually lost (Polakis, 1999). The Wnt signal exerts its function by regulating the level of stable beta-catenin (Polakis, 1999). In absence of the Wnt signal, a multiprotein complex, which includes the adenomatous polyposis coli (APC) protein, targets beta-catenin for proteosomal destruction, keeping the levels of free beta-catenin low (Munemitsu et al., 1995). Activation of the pathway inhibits the multi-

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