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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.

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′-CAAGGACCACCGCATCTCTACATTC-3′. For cIAP1 mRNA, the forward primer was 5′-CAGCCTGAGCAGCTTGCAA-3′ and the reverse primer was 5′-CAAGCCACCATCACAACAAAA-3′; the TaqMan probe was 5′-TTTATTATGTGGGTCGCAATGATGATGTCAAA-3′. For cIAP2 mRNA, the forward primer was 5′-TCCGTCAAGTTCAAGCCAGTT-3′ and the reverse primer was 5′-TCTCCTGGGCTGTCTGATGTG-3′; the TaqMan probe was 5′-CCCTCATCTACTTGAACAGCTGCTAT-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′-AGTGGTAGTCCTGTTTCAGCATCA-3′ and the reverse primer was 5′-CCGCACGGTATCTCCTTCA-3′; the TaqMan probe was 5′-CACTGGCACGAGCAGGGTTTCTTTATACTG-3′. The forward primer for GAPDH mRNA was 5′-GAAGGTGAAGGTCGGAGT-3′ and the reverse primer was 5′-GAAGATGGTGATGGGATTTC-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 ($P < 0.05$),

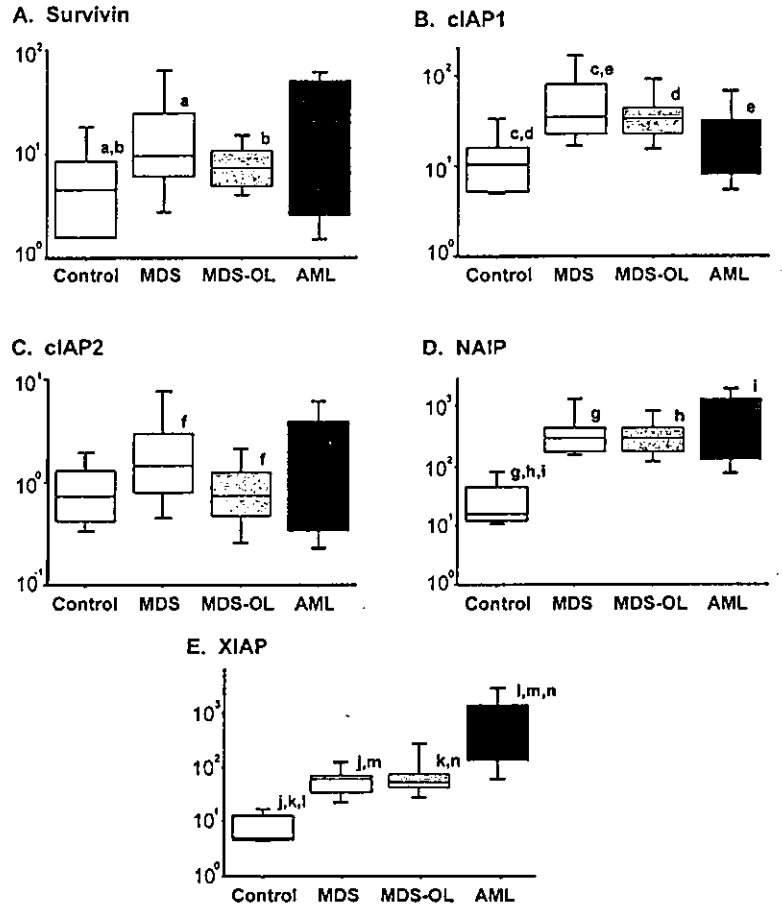

Fig. 1. Quantitative RT-PCR analysis of IAP family proteins, survivin, cIAP1, cIAP2, NAIP and XIAP in control ($n = 5$), MDS ($n = 11$), MDS → OL ($n = 11$) and de novo AML cases ($n = 5$). Relative intensity was calculated as (intensity of reaction of IAP family (total Raji RNA, ng))/(intensity of reaction 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 ($^aP < 0.0001$), control and MDS → OL ($^bP < 0.0001$) and control and de novo AML ($^cP < 0.0001$) with Mann–Whitney's *U*-test, MDS and MDS → OL ($^dP < 0.01$) using Wilcoxon's test, and MDS and de novo AML ($^eP < 0.05$) with Mann–Whitney's *U*-test. The Ki-67-positive cell ratio exhibited significant differences between control and MDS ($^fP < 0.0001$), control and MDS → OL ($^gP < 0.0001$), control and de novo AML ($^hP < 0.0001$), MDS and de novo AML ($^iP < 0.05$), and MDS → OL and de novo AML ($^jP < 0.01$) 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,

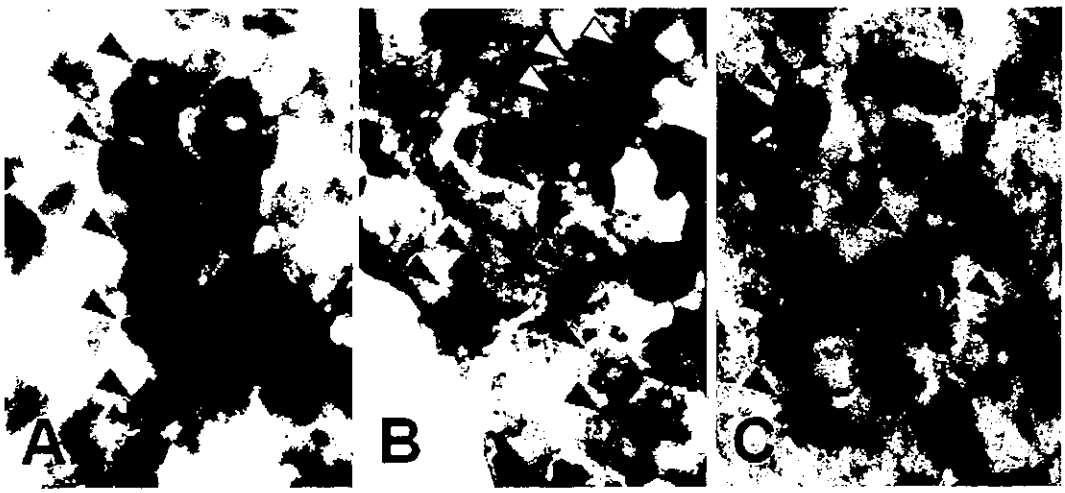

Fig. 2. Immunohistochemical detection of survivin in the bone marrow cells of MDS subjects (RAEB, 400× original magnification). Double staining for survivin (nuclear: brown) and cell markers (cytoplasmic/cell surface: blue) (A, neutrophil elastase; B, glycophorin A; C, CD34) demonstrated that survivin-positive 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.)

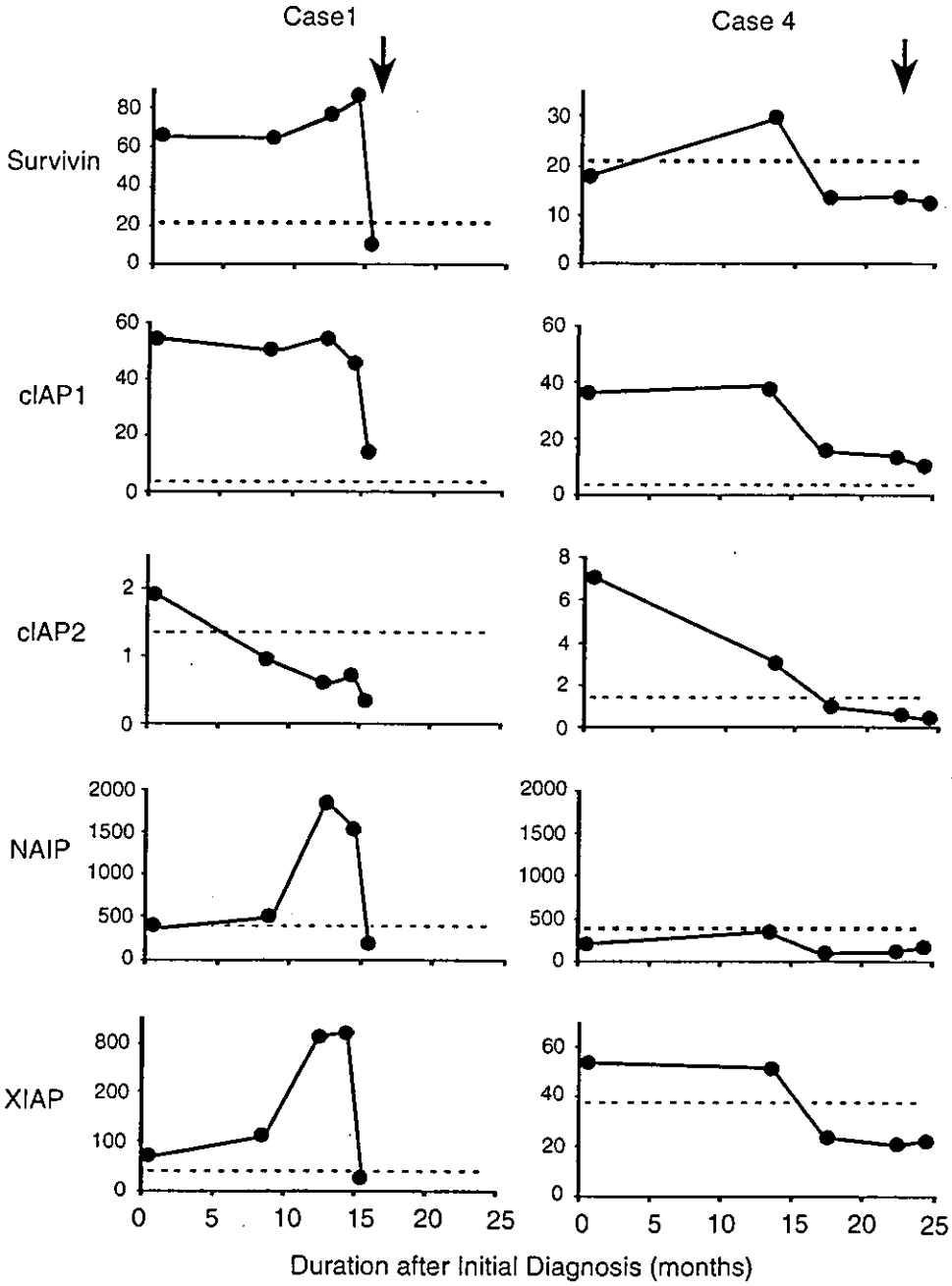

Fig. 3. Chronological changes of IAP family protein expression in MDS → OL cases (cases 1 and 4) determined by quantitative RT-PCR. Arrows indicate the time of OL evolution and dotted lines indicate median values of the control samples. Note that the dynamics of survivin and XIAP expression tended to 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.

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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protein complex, preventing the degradation of beta-catenin. Stable beta-catenin accumulates in the cytoplasm and is available to translocation to the nuclei, where it coactivates the transcription of Wnt target genes (Behrens et al., 1996).

The APC gene mutation is the most common cause of Wnt signaling dysfunction in CRC, being observed 58 in 60–80% of cases. (Jass et al., 2002; Miyoshi et al., 1992; Morin et al., 1997; Powell et al., 1992), while beta-catenin is mutated in some cases (3%) (Morin et al., 1997; Kitaeva et al., 1997). In either case, the mutation prevents the down-regulation of beta-catenin, resulting in stabilization and accumulation of large quantities of the protein in the cytoplasm (Morin et al., 1997). The stable beta-catenin enters the nuclei and promotes constitutive transcription of Wnt target genes (Korinek et al., 1997).

The pattern of target gene activation is not uniform and the resulting expression pattern apparently influences the phenotype and behavior of CRCs (Brabletz et al., 2002). Higher nuclear accumulation of beta-catenin is observed in cancer than in adenomas (Fodde et al., 2001), and in the same tumor it tends to accumulate in the nuclei of dedifferentiated cells at the invasive front, while in differentiated areas of the tumor it is preferentially located in the membrane, with low nuclear expression (Brabletz et al., 2001). The infiltrative phenotype associated with nuclear expression may facilitate dissemination and metastasis of the neoplastic tissue. Widespread nuclear accumulation of beta-catenin has been correlated with the progress of the lesion, and poor prognosis of the patient (Cheah et al., 2002).

The influence of nuclear beta-catenin on tumor characteristics possibly results from the expression pattern of Wnt target genes. c-Myc was one of the first described Wnt target genes (He et al., 1998) and is overexpressed in most colorectal cancers (Sikora et al., 1987; Smith et al., 1993). c-Myc controls tumor growth by its effect on both cell proliferation and apoptosis, and has a role in the repression of genes responsible for cell arrest and differentiation (van de Wetering et al., 2002). Overexpression of c-Myc was observed to correlate with higher incidence of hematogenous and lymphatic metastasis and lower disease-free survival of the patient (Kakisako et al., 1998; Sato et al., 1994; Yang et al., 1996).

The age of the patient is considered the most important single risk factor for CRC development (DePinho, 2000). Moreover, age also seems to influence the prognosis of the patient, resultant from the basal condition of the patient and possibly from a distinct behavior of the tumor in this age group (CCCG, 2000; Nagaoka et al., 2003). The tumorigenesis process of colorectal carcinomas is associated with constitutive activation of Wnt target genes (Korinek et al., 1997). The activation and expression of these target genes, however, are not uniform, and the expression pattern apparently determines the variable phenotype and behavior observed in CRCs. This study evaluates the nuclear localization of beta-catenin protein and the expression of beta-catenin and c-Myc mRNAs, and correlates their expression pattern with the age of the patient and pathological characteristics of the CRC.

Materials and methods

Tissue specimens

This study included 101 samples of colorectal adenocarcinomas obtained from surgical specimens. The samples were fresh material placed in an embedding medium (Tissue-Tek OCT compound, Sakura, Tokyo, Japan), frozen in liquid nitrogen, and stored at −80°C. The specimens were obtained from the Japanese Red Cross Medical Center, Tokyo Metropolitan Institute of Gerontology, and Tokyo Medical and Dental University. Information about the age and gender of the patient as well as location, size, macroscopic type, depth of invasion, and stage of the lesion was obtained from the pathological report. The histological type of the lesion was classified using hematoxylin and eosin (H&E)-stained slides according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus (Japanese Society for Cancer of the Colon and Rectum). No patients had familial adenomatous polyposis (FAP) or inflammatory bowel disease. The subjects were divided into three age groups; <69 years, 70–84 years, and 85+ years. This study obtained permission from the hospital ethical board to obtain samples and carry out the experiments.

Immunohistochemistry

Formalin-fixed, paraffin-embedded material used to perform immunohistochemistry was available in 35 cases. Immunohistochemistry was performed using the routine protocols of this laboratory. Sections 4-μm-thick were mounted in APS-coated glass (Matsunami, Osaka, Japan), deparaffinized in xylene, and rehydrated in graded ethanol following routine protocols. Subsequently, the sections were incubated with 0.3% hydrogen peroxide (H_2O_2) for 30 min to block endogenous peroxidase activity. Antigen retrieval was performed by autoclave treatment for 20 min in 0.01 M citrate buffer (pH 6.0). After rinsing in phosphate-buffered saline (PBS), background staining was blocked using normal goat serum (1: 20, DAKO, Carpinteria, US) for 10 min. The sections were then incubated with the primary antibody overnight at 4°C. Anti-beta-catenin (1:500, Novocastra, Newcastle, UK) and Ki-67/MIB-1 (1:5000, ABL, Tokyo, Japan) mouse monoclonal antibody were used. After washing with PBS, the sections were incubated with biotinylated goat anti-mouse Ig (Vector, Burlingame, US) for 30 min, washed with PBS, and then samples reacting with Ki-67 were incubated with streptavidin-peroxidase conjugate (1: 500, Vector), and samples reacting with beta-catenin were incubated with envision (DAKO, Carpinteria, US) for 30 min. Tissue staining was developed with 3,3-diaminobenzidine-tetrahydrochloride solution (DAB; Sigma-Aldrich, Dorset, UK) and 0.1% H_2O_2 and counterstained with hematoxylin.

For negative controls, duplicate sections were used in which the primary antibodies were omitted and replaced with PBS.

Evaluation of Ki-67 labeling index

Ki-67 antigen expression is expressed in all phases of the cell cycle except G0 and is thus a generally accepted marker of cell proliferation (Gerdes et al., 1984). The distribution of Ki-67-positive cells in each lesion was considerably heterogeneous. Therefore, the labeling indices were determined using the average percentage of cells showing nuclear staining counted in five high-power fields.

Evaluation of beta-catenin staining

The immunostaining of beta-catenin was assessed with respect to its localization; nuclear, cytoplasmic, or membranous, as previously reported (Cheah et al., 2002). Adjacent normal colonic epithelium was used as an internal control of staining efficiency and as a parameter for comparison. Nuclear expression of beta-catenin was evaluated using a score system that included both the staining intensity and the percentage of cells with nuclear staining. The average staining intensity was graded by the subsequent criteria: 0, no staining; 1, weak staining; 2, moderate staining; 3, intense staining. The percentage of tumor cells with nuclear staining was obtained by counting the cells in five high power fields and scored as follows: 1, 10–30%; 2, 30–50%; 3, 50–70%; 4, >70%. Carcinoma samples containing 70% or more of cells with nuclear expression with strong staining intensity were classified as high beta-catenin expressors. The remaining samples were classified as low expressors. Cytoplasm staining was evaluated by comparing the intensity of staining in neoplastic cells with the cytoplasm of nonneoplastic epithelium. The samples were classified as having normal or increased cytoplasm staining. The evaluation of membrane staining followed similar criteria, comparing the staining of cancer cells with nonneoplastic cells. The samples were classified as normal or decreased membrane expression of beta-catenin.

The staining findings were correlated to the age and other clinicopathological characteristics of the subjects. Because the number of samples from patients over 85 years available for immunohistochemistry was very limited, only two age groups were separated for staining comparison; younger than 69 years and older than 70 years.

RNA extraction and reverse transcription

Cancer tissues of the 101 samples were dissected from the frozen block. Total RNA was obtained from the cancer tissue using an extraction kit (Isogen, Nippon Gene, Kanazawa, Japan) according to manufacturer's protocol. Fifty nanograms of RNA of each sample was used for subsequent reverse transcription (RT). RT was run for 60 min, at 42°C, using MMLV reverse transcription (Takara, Kyoto, Japan) and random nucleotide primers (GIBCO, Grand Island, NY) (Nagaoka et al., 2003).

Real-time semiquantitative PCR

Expression of beta-catenin and c-Myc transcripts was determined by real-time semiquantitative PCR assay using an ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, US). The PCR reaction was carried out according to the manufacturer's protocol. The standard curve was constructed with serial dilutions of a cDNA sample of human normal skeletal muscle. The obtained quantity of mRNA for each sample was normalized by the corresponding quantity of the internal housekeeping gene, GAPDH. The final quantity was calculated as previously described (Saito et al., 2002). The following primers and probes were used: beta-catenin forward primer, 5-CCGCAAATCATGCACCTTT-3; reverse primer, 5-ATGTGCACGAACAAGCAACTG-3; and probe, 5-TGAGCAGGGTGCCATTCCACGACT-3. c-Myc forward primer, 5-CGTCTCCACACATCAGCACAA-3; reverse primer, 5-TCTTGGCAGCAGGATAGTCCTT-3; and probe, 5-TACGCAGCGCCTCCCTCCA-3.

Statistical analysis

The chi-square test was used to evaluate the relationship of clinicopathological characteristics of the subjects with age group and to evaluate the relationship of beta-catenin staining expression with age group and clinicopathological characteristics of the subjects. ANOVA was used to evaluate the relationship of c-Myc and beta-catenin mRNA expression with age (two-way factorial) and clinicopathological characteristics of the subjects (one-way factorial). The Spearman rank correlation test was used to correlate beta-catenin nuclear staining with c-Myc and beta-catenin mRNA quantity and Ki-67 LI. Pearson correlation test was used to correlate the Ki-67 LI with age and quantity of mRNA, and to correlate quantity of c-Myc mRNA with beta-catenin mRNA. All statistics analyses were performed using StatView software. Statistically significant was defined as $P < 0.05$.

Results

Clinicopathological characteristics of the subjects and age

The mean age of the patients at the time of diagnosis was 70.82 years, ranging from 36 to 97 years. They were fairly similarly distributed between age groups of less than 69

Table 1
Clinicopathological characteristics of 101 subjects

	Age group in year			*P* value
	<69 (n = 50)	70–84 (n = 43)	>85 (n = 8)	
*Gender**				
Female	13	22	3	0.04
Male	37	21	5	
Site[a]				
Proximal	18	20	2	0.53
Distal	32	23	6	
Size[b]				
<4.9 cm	17	15	3	0.31
>5.0 cm	13	24	3	
Macroscopy[c]				
1	7	3	1	0.18
2	22	30	7	
3	12	8	0	
4	0	1	0	
*Histology**				
Well	25	19	3	<0.0001
Mod	21	14	5	
Por	3	4	0	
Muc	1	5	0	
Sig	0	1	0	
*Depth of invasion**				
Early cancer				
m	6	2	0	0.03
sm	6	1	0	
Advanced cancer				
pm	7	0	2	
ss	17	22	5	
se/a	11	17	1	
si/ai	3	1	0	
Dukes				
A	13	3	2	0.13
B	13	19	4	
C	24	21	2	

well, well-differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma; muc, mucinous adenocarcinoma; sig, signet ring cell carcinoma;
m, mucosa; sm, submucosa; pm, proper muscle; ss, subserosa; se, serosa; a, adventitis si/ai, invasion into a contiguous organ.
[a] Proximal large intestine includes cecum, ascending colon, and transverse colon.
[b] Some information is not available for all samples.
[c] Gross type according to Borrmann classification.
* $P < 0.05$; chi-square test.

years (n = 50, 49%) and 70–84 years (n = 43, 42%). The 85+ years age group consisted of 8% (n = 8) of all patients. Table 1 presents a summary of the clinicopathological characteristics of the subjects and shows the comparison among the different age groups.

In total, there were samples from 38 females and 63 males in this study. The proportion of women was significantly higher in patients older than 70 years (P = 0.04) than in younger patients. The group aged 70–84 years consisted of more women than men, and although the relative number of female patients was less in the 85+ group, the proportion of female patients was higher than in the <69 years group (<69 years, 26%; 70–84 years, 56%; and years 85+, 37%).

Patients aged 70–84 years had a significantly higher proportion of poorly differentiated adenocarcinoma and mucinous carcinoma than younger and older patients (P < 0.01). Poorly differentiated and mucinous carcinomas composed 9% (n = 4) and 11% (n = 5) of the lesions in the 70–84 years group, respectively, while in the group younger than 69 years, 6% (n = 3) of lesions was poorly differentiated adenocarcinoma and 2% was mucinous carcinoma (n = 2). In the oldest old group, all lesions were well or moderately differentiated adenocarcinoma.

Patients older than 70 years included in this study were more likely to present advanced lesions (invading proper muscle or beyond, P = 0.03). The percentage of advanced lesions was 76% in <69 years, 94% in 70–84 years, and 100% in 85+ years. In addition, patients aged 70–84 years tended to have more locally advanced lesions than the younger and older groups. Although all lesions in the 85+ patients were advanced, they had proportionally fewer lesions reaching the serosa (n = 1, 12%) than the 70–84 aged group (n = 18, 42%) and the <69 aged group (n = 14, 28%).

Patients from the 70–84 aged group were less likely to be at stage A than younger patients (P = 0.03), but these two age groups had a rather similar distribution of Dukes' C stage. Though not significant, only 25% of lesions from the 85+ aged group was at Dukes' C compared to 48% in the <69 aged group and 46% in the 70–84 aged group.

No significant differences were observed concerning site distribution, size of the lesions, and macroscopic type.

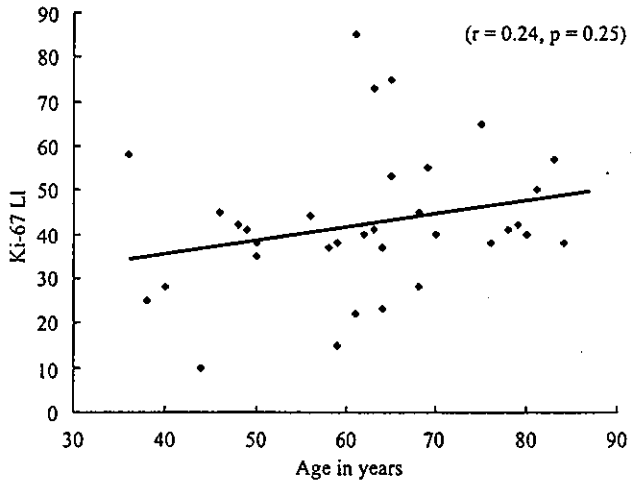

Fig. 1. The proliferative activity of the tumor is visualized by Ki-67 labeling indices (LI). The diamond-shaped dots represent the Ki-67 LI corresponding to the age of the patient. The Ki-67 LI ranged from 10% to 85%. Some trend of increasing proliferative activity with advancing of age can be observed, although not statistically significant (r = 0.24; P = 0.25). Pearson correlation test.

Age and Ki-67 labeling index

The Ki-67 labeling indices ranged from 10% to 85%. Fig. 1 illustrates some trend in increase of proliferative activity with advancing age, but this was not statistically significant ($r = 0.24$, $P = 0.25$). The average Ki-67 LI of patients older than 70 years was slightly higher than of younger patients (45.66% and 41.32%, respectively), but this difference was not significant ($P = 0.49$).

Beta-catenin immunostaining expression

Beta-catenin is a key element in the neoplastic process of CRC (Morin et al., 1997). One pool of it participates in the cell adhesion mechanism with E-cadherin to configure the epithelial phenotype of intestinal cells (Conacci-Sorrell et al., 2002; El-Bahrawy et al., 2001). Another pool participates in the Wnt signaling pathway, activating gene transcription when located in the nuclei (Behrens et al., 1996). Therefore, to exert its function and consequently influence tumor characteristics, the intracellular localization of beta-catenin is important (Brabletz et al., 2002).

The intracellular expression of beta-catenin protein was evaluated through immunohistochemistry. Fig. 2 exemplifies the staining from nonneoplastic and neoplastic tissue. The nonneoplastic tissue was used as a normal standard for comparison. Typically, it displays a distinct staining of the membrane, corresponding to the pool of beta-catenin that participates in the cadherin-catenin cell adhesion mechanism. The neoplastic tissue presented a more heterogeneous staining pattern. Membrane staining was decreased in 55% of the lesions while cytoplasm staining was present in all lesions, though in quite variable intensity. The nuclear localization of beta-catenin was much more heterogeneous. Nuclear staining was observed in 80% of the lesions. However, the percentage of cells showing nuclear localization as well as intense staining was rather variable between samples and in different areas of the same tumor. Thirty-one percent of the lesions displayed areas with 70% or more nuclear staining, and 20% of the lesions had widespread staining (>70%) in cells with intense nuclear staining. No correlation was found either between nuclear and membranous localization, or between nuclear and cytoplasmic expression of beta-catenin.

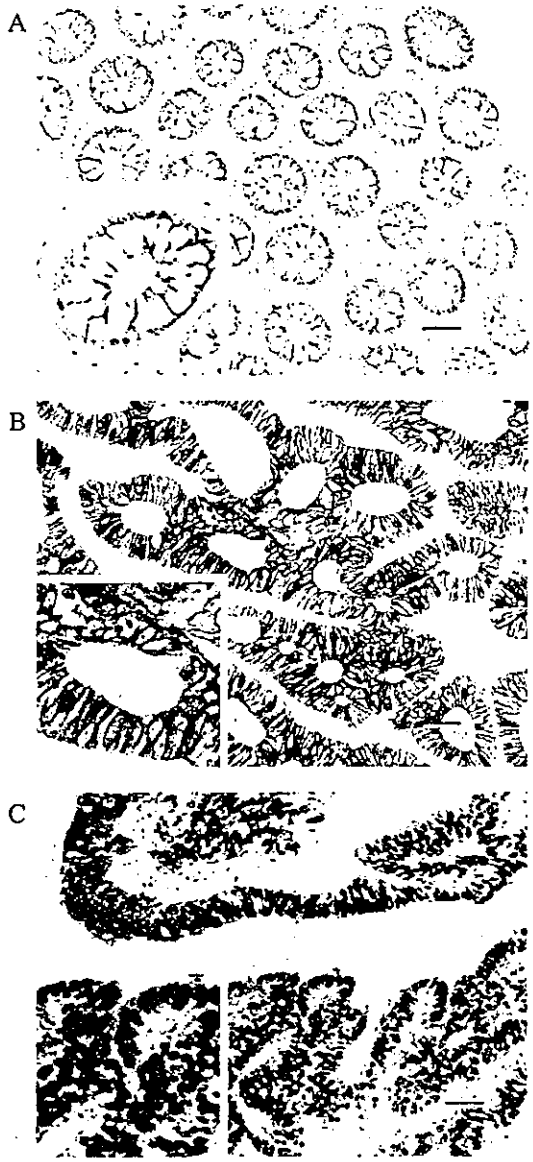

Fig. 2. Typical beta-catenin staining patterns of normal epithelium and cancer tissue of the colon and rectum. Dark staining indicates antibody staining. (A) Normal colonic mucosa used for control. Characteristically, the membrane of the crypt cells is stained, while the nuclei are not. (B) Cancer tissue showing typical membrane staining and increase of cytoplasm staining of beta-catenin. Most of the cells have no nuclear staining. (C) Cancer tissue showing decreased membrane staining but increased cytoplasm and strong nuclear staining. All pictures are in the same magnification. Scale bar represents 100 μm. The inset picture is a four times magnification of the main picture.

Age and beta-catenin immunostaining expression

Table 2 displays the comparison of nuclear localization of beta-catenin between the <69 aged group (36–69 years) and the >70 aged (70–87 years) group. High nuclear expression was observed in 50% of samples from patients older than 70 years, while only 10% of young patients had cancer with similar characteristic, which was significantly different ($P = 0.01$). It is important to note that this difference existed when nuclear staining was widespread (>70% of the cells) and included cells with intense nuclear staining. Another important point is that a significant difference of expression among different age groups was found only when the age groups were divided at 70 years, but not at a younger age. Cytoplasm staining and membranous localization had no correlation with age.

Table 2
Comparison between nuclear stain of beta-catenin and age

Age group	n	Nuclear stain of beta-catenin	
		High	Low
<69 years	24	2	22
>70 years	10	5	5

$P = 0.015$, chi-square test.

Age and mRNA quantification by real-time PCR

c-Myc is one of the target genes of the beta-catenin protein (He et al., 1998), and its overexpression is observed in most colorectal cancers (Sikora et al., 1987; Smith et al., 1993). The expression pattern of c-Myc has also been observed to correlate with the behavior of the tumor and the prognosis of the patient (Kakisako et al., 1998; Sato et al., 1994; Yang et al., 1996).

Fig. 3 demonstrates a comparison between the expression of c-Myc and beta-catenin genes according to the age group. c-Myc mRNA quantity was significantly variable among the three age groups ($P = 0.04$). Samples from patients aged 70–84 years had substantially higher mRNA expression than samples from younger patients, with an average increase of 58% ($P = 0.01$). Interestingly, the average c-Myc mRNA quantity was 33% lower in samples from the 85+ patients when compared to the 70–84 years group, but this change did not reached statistic significance, possibly due to the small number of patients in the older group.

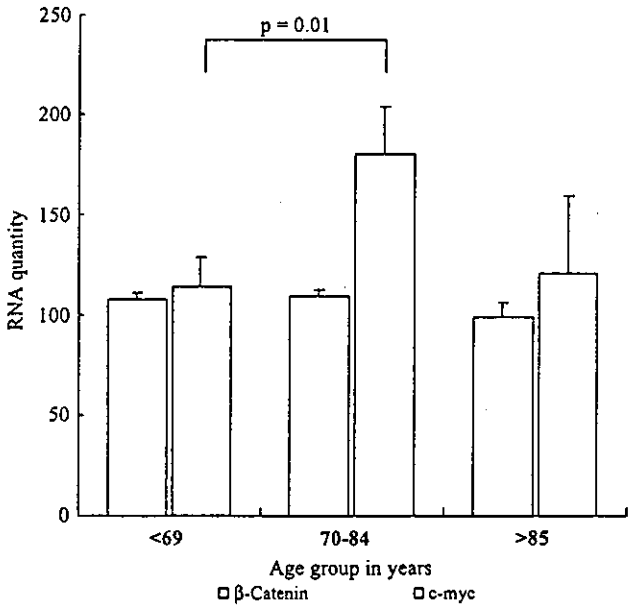

Fig. 3. Variable expression of c-Myc mRNA between different age groups ($P = 0.04$) and constant beta-catenin mRNA with the advancing of age ($P = 0.49$). c-Myc expression increases significantly in the 70–84-year-old group ($P = 0.01$) when compared to the younger group. In the 85+ group, c-Myc expression tended to decline, though this change is not significant. The numeric quantity of beta-catenin mRNA was elevated 75-fold in this graph to allow comparison with c-Myc quantity. Mean (±SE). ANOVA two-way factorial.

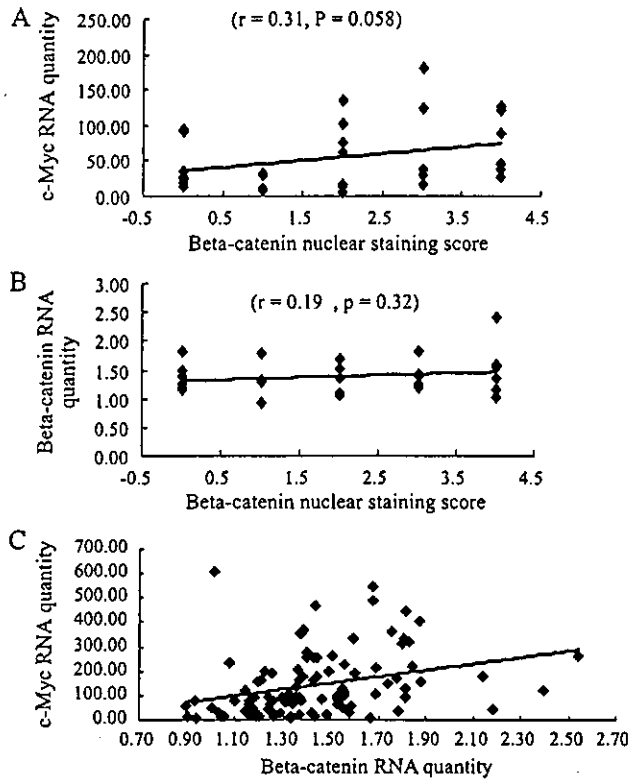

Fig. 4. (A) c-Myc mRNA expression was closely correlated with the nuclear localization of the beta-catenin protein in CRC ($r = 0.31$, $P = 0.05$). (B) Nuclear expression of beta-catenin protein was not dependent on the expression of beta-catenin mRNA ($r = 0.19$, $P = 0.32$). (C) The basal expression of beta-catenin mRNA apparently influenced the final c-Myc mRNA expression ($r = 0.29$, $P = 0.0025$). Spearman's rank correlation test was used to correlate mRNA quantity with nuclear staining. Pearson correlation test was used to correlate the quantity of c-Myc and beta-catenin mRNA.

Beta-catenin mRNA expression is not necessarily related to the tumorigenesis of CRCs because the basic problem supposedly consists in a failure of beta-catenin protein degradation process (Kinzler and Vogelstein, 1996). However, the quantity of RNA available for transcription may

Table 3
Correlation between nuclear stain of beta-catenin and clinicopathologic characteristics of the subjects

	n	Nuclear stain of beta-catenin		P value
		High	Low	
Depth of invasion				
m/sm/pm	7	0	7	0.13
ss/se/a	28	7	21	
*Dukes' stage**				
A	5	0	5	0.03
B	15	6	9	
C	15	1	14	

m, mucosa; sm, submucosa; pm, proper muscles; ss, subserosa; se, serosa; a, adventitia.
*$P < 0.05$; chi-square test.

influence the amount of protein produced, and therefore influence some tumor features (Roh et al., 2001).

Contrary to c-Myc mRNA expression and nuclear localization of beta-catenin protein, beta-catenin mRNA expression was rather uniform in the different age groups ($P = 0.49$).

Correlation of nuclear localization of beta-catenin and gene expression with other clinicopathological characteristics

Beta-catenin nuclear expression and c-Myc mRNA expression had a relevant association with age. These molecular findings were also compared with other clinicopathological characteristics.

Dukes' B tumors had a significantly higher nuclear expression of Beta-catenin than other cases ($P = 0.03$, Table 3). No tumor stage A had high nuclear expression, and quite surprisingly, only one sample of Dukes' C tumor had high nuclear expression. In addition, all cases showing high nuclear expression had already invaded within the subserosa or beyond (Table 3).

No significant correlation of nuclear expression with patient's gender or site, size, and histology of the tumor was found. Neither nuclear staining of Beta-catenin ($r = 0.18$, $P = 0.95$) nor c-Myc mRNA expression ($r = 0.2$, $P = 0.17$) was significantly correlated with Ki-67 LI.

c-Myc expression did not show a significant correlation with other clinicopathological characteristics than age (Table 4). Though not significant, the expression of c-Myc was apparently influenced by the shape and growth pattern of the lesion, the differentiation of the tissue, and the progress of the tumor. Ulcerative lesions and infiltrative growth pattern presented a higher average expression than lesions with polypoid shape and expansive growth ($P = 0.34$). Less differentiated adenocarcinomas also tended to express higher quantity of c-Myc than mucinous or well and moderately differentiated lesions ($P = 0.17$). Lesions invading deeper than the subserosa had in average 33% higher quantity of c-Myc ($P = 0.22$). A continuously increasing trend was also observed using Dukes' classification; the later the stage, the higher the c-Myc expression ($P = 0.21$).

Fig. 4A shows that while c-Myc expression was closely related to the nuclear staining of Beta-catenin ($r = 0.31$, $P = 0.05$), the nuclear expression was not necessarily dependent on the Beta-catenin gene expression ($r = 0.19$, $P = 0.32$; Fig. 4B). It was interesting to notice, however, that the expression of c-Myc was significantly correlated with Beta-catenin mRNA expression ($r = 0.29$, $P = 0.0025$; Fig. 4C).

Table 4
Correlation between c-Myc mRNA quantity and clinicopathologic characteristics of subjects

	n	c-Myc RNA		*P* value
		Average	SE	
Macroscopy				
1	11	92.94*	24.50	0.34
2	59	140.74*	16.36	
3	20	157.54*	26.67	
4	1	609.29	–	
Differentiation				
Well	47	124.36*	18.45	0.17
Moderate	40	161.05*	20.63	
Poorly	7	175.95*	21.67	
Mucinous	6	62.16*	15.49	
Depth of invasion				
m/sm/pm	24	114.67*	22.84	0.22
ss/se/a	77	152.58*	15.50	
Duke's stage				
A	13	106.32*	26.07	0.21
B	13	131.47*	17.83	
C	46	169.23*	21.96	

m, mucosa; sm, submucosa; pm, proper muscles; ss, subserosa; se, serosa; a, adventitia.
* ANOVA (one-way factorial).

Discussion

Age is considered the most important single risk factor for cancer development (DePinho, 2000). Moreover, the age of the patient can also affect the prognosis, which possibly results from the medical care received, higher incidence of associated diseases, and the distinct clinicopathological characteristics of the tumor in this age group (CCCG, 2000; Jessup et al., 1996; Nagaoka et al., 2003). The majority of CRCs presents a dysfunction of the Wnt signaling arising from a mutation in the APC or beta-catenin gene (Jass et al., 2002; Miyoshi et al., 1992; Morin et al., 1997; Powell et al., 1992). These mutations result in the accumulation of large quantities of beta-catenin, which translocates into the nuclei and promotes constitutive activation of Wnt target genes in the cytoplasm (Korinek et al., 1997). Although the majority of CRCs has a dysfunction in the same signaling pathway, CRCs do not develop uniform pathological features. Apparently, these differences result from the pattern of activation and expression of Wnt target genes (Brabletz et al., 2002). This study demonstrates an increase in expression of Wnt signaling components in CRC of patients aged 70–84 years, and this pattern of expression may influence the behavior of the cancer in this age group.

In this study, nuclear localization of beta-catenin and expression of c-Myc mRNA were apparently correlated with age, while expression of beta-catenin mRNA was not. This study demonstrates that high, widespread nuclear expression of beta-catenin is more frequent in tumors from patients aged 70 years or older (70–87 years) than younger patients. Previous studies did not demonstrate a significant correlation of nuclear expression of beta-catenin with age (Cheah et al., 2002; Hugh et al., 1999). It is important to emphasize, however, that the finding of

this study was significant when comparing age groups divided at 70 years of age and not when dividing the groups at a younger age. The patients involved in the previous studies were considerably younger than the patients in the present study, and they were grouped as younger or older population at a considerably lower age than in this study.

The same authors (Cheah et al., 2002; Hugh et al., 1999) highlighted the importance of intracellular localization of beta-catenin, associating its nuclear expression with the survival of the patient. Nuclear localization was associated with the progress of the lesion, and when paired for the same stage, patients harboring tumors with high nuclear expression had a worse prognosis. In this study, diffuse high nuclear expression was associated with locally advanced lesions, but not correlated with lymph node metastasis or final stage of the tumor. In a nuclear pool, beta-catenin functions as a gene co-transcriptor (Behrens et al., 1996), activating the expression of target genes involved in tumor initiation and progress (Brabletz et al., 2002). One of the first identified beta-catenin target genes was the protooncogene c-Myc (He et al., 1998).

Accordingly, in the present study, c-Myc mRNA expression was closely correlated with nuclear localization of beta-catenin, and similarly, the quantity c-Myc mRNA was significantly higher in tumors from patients aged 70–84 years. In the 85+ age group, the expression tended to decrease, though this finding was not significant, probably due to the small number of patients. An increased expression of c-Myc has been observed in senescent, non-neoplastic cells (fibroblasts) with the advancing of age (Nakamura et al., 1988). Higher expression of c-Myc protein was also described in colorectal cancer with advancing of age (Tanaka et al., 2002) and associated with related increasing Ki-67 LI and apoptosis indices in elderly patients.

c-Myc is a protooncogene that appears to regulate cell-cycle progression and cell growth, it promotes cellular proliferation while sensitizing cells to apoptotic stimuli and influencing the differentiation of cells (Hoffman and Liebermann, 1998; van de Wetering et al., 2002). Deregulated c-Myc seems to be able to promote tumorigenesis (Amati et al., 1998), and in fact, its overexpression is observed in most colorectal cancers (Sikora et al., 1987). The level of c-Myc expression apparently influences the growth and differentiation of tumor cells (Borre et al., 1996), and high expression has been correlated with higher incidence of metastasis and lower disease-free survival of the patient (Kakisako et al., 1998; Sato et al., 1994; Yang et al., 1996).

In this study, c-Myc expression was not significantly correlated with the clinicopathological characteristics of the subjects. However, some trend could be identified with increasing expression of the gene with the advance of the tumor because tumors with deeper invasion and at later stages tended to present higher expression of c-Myc. Differentiation of the tumor was another feature apparently influenced by the expression of c-Myc, with higher quantity of mRNA present in lesions less differentiated.

Contrary to other components of Wnt signaling, beta-catenin mRNA expression was rather constant in the different age groups. Studies in mice using antisense oligonucleotide against beta-catenin mRNA demonstrated the importance of basal production of beta-catenin RNA in protein expression (Green et al., 2001). They also suggested that beta-catenin mRNA expression plays a critical role in the tumorigenic growth of APC mutant colon cancer xenografts. The finding of rather uniform beta-catenin mRNA expression with advancing of age but variable nuclear expression of the protein may suggest an age-related difference in degradation, nuclear translocation, or retention of the beta-catenin protein.

The expression pattern of these Wnt components may influence the behavior of the tumor in each age group. The most prominent association of beta-catenin nuclear localization and c-Myc expression was the local advance of the tumor for both and the histological differentiation for c-Myc expression. In fact, those were the distinctive pathological characteristics of the tumors among the different age groups; patients aged 70–84 years tended to present with more locally advanced tumors and had a higher incidence of poorly differentiated adenocarcinomas and mucinous carcinomas than their younger or older counterparts.

Patients aged 70–84 years in this study more frequently presented locally advanced lesions but had similar lymph node invasion status when compared to younger patients. These findings were reflected in the stage classification, although there was no major difference in the later-stage distribution, they were less frequent at an early stage than in younger patients. Recently, a large review of data on colorectal cancer identified that older patients were more likely to present with more advanced disease than younger patients (CCCG, 2000). Moreover, a former study identified an increase in the emergency presentation of patients over 70 years due solely to the progression of the primary tumor (Waldron et al., 1986). Coincident with the findings of the present study, Arai et al. (2000) observed that older elderly patients had a lower frequency of lymph node invasion than younger patients and suggested that cancer in that age group was biologically distinct, with less aggressive behavior.

Histological type assesses the state of differentiation of a neoplasm, the type of adenocarcinoma, and the configuration of glands. The morphologic features of a tumor have been associated with the behavior of the tumor, while well-differentiated lesions are associated with early stage disease, and poor differentiation and excess mucin production are associated with more advanced-stage disease and poorer prognosis (Jessup et al., 1996). In this study, patients aged 70–84 presented a higher incidence of poorly differentiated and mucinous carcinomas than the younger and oldest patients. Those tumors represented 9% and 11%, respectively, of the carcinomas in patients over

70 years. Although these figures are similar to the incidence reported by former studies (Arai et al., 2000), the subset of cancers with these features may be too small to represent a characteristic of elderly patients. A high incidence of CRC with poor or mucinous differentiation has been reported in very young patients (Liang et al., 2003; Parramore et al., 1998). In the present study, only 2% of the patients were less than 40 years old, which may explain why there were few cases with that kind of lesion in the younger group.

In conclusion, this study recognizes different clinicopathologic characteristics of colorectal carcinoma according to the age group of the patient. The obtained data suggest that there is a variable expression pattern of nuclear beta-catenin and c-Myc mRNA with advancing of age (increased expression at 70–85 years), and the pattern of expression of these components may influence the behavior of the colorectal carcinoma in each age group.

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Expression of IAP family proteins in esophageal cancer

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Abstract

Members of the inhibitor of apoptosis protein (IAP) family, including survivin, have been reported to be expressed in many tumors. However, their expression in esophageal cancer has not been clarified completely. We investigated the expression of mRNA for IAP family proteins in samples from esophageal cancers and their adjacent normal mucosa tissues by real-time quantitative RT-PCR. The survivin expression in esophageal cancer was significantly higher than that in normal mucosa ($P < 0.05$). Other IAP family proteins including cIAP1, cIAP2, NAIP and XIAP tended to show stronger expression in cancer tissue than normal mucosa, although the differences were not significant. As to the histological type of tumor, poorly differentiated squamous cell carcinomas exhibited significantly higher level of expression than well-differentiated carcinomas ($P < 0.05$). The proportion of apoptotic cells of cancer tissue inversely correlated with the intensity of survivin expression ($P < 0.05$). Immunohistochemical staining demonstrated cytoplasmic as well as nuclear expression of survivin in esophageal cancer, and further, in situ hybridization analysis demonstrated cytoplasmic expression of mRNA for survivin. The results suggest that the expression of IAP family proteins, especially survivin, may be associated with the biological character of esophageal cancer, such as apoptosis.

Keywords: Survivin; IAP; Esophageal cancer

Introduction

The regulation of apoptotic cell death may have a profound effect on the pathogenesis and progression of esophageal malignancies. IAP (inhibitor of apoptosis protein) family proteins, including survivin, block apoptosis induced by a variety of triggers (LaCasse et al., 1998; Tamm et al., 1998). 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 (Shin et al., 2001; Tamm et al., 1998). 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 (Adida et al., 1998; Ambrosini et al., 1997). 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 (Velculescu et al., 1999). In fact, survivin is prominently expressed in transformed cell lines and in many human cancers including esophageal cancer (Grabowski et al., 2003; Ikeguchi and Kaibara, 2002; Ikeguchi et al., 2003; Kato et al., 2001).

Survivin is usually detected in the cytoplasm of tumor cells and is therefore widely regarded as a cytoplasmic protein (Ambrosini et al., 1997; Gianani et al., 2001; Kawasaki et al., 2001). However, several studies have shown a nuclear accumulation of survivin in gastric cancer cells (Okada et al., 2001) and lung cancer cells (Rodriguez et al., 2002). Also, in esophageal cancer, nuclear localization of survivin has been reported to correlate with the prognosis (Grabowski et al., 2003). We have reported recently that ALL cells principally exhibit a nuclear localization of survivin, whereas CLL cells have a cytoplasmic distribution (Naka-

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