gawa et al., in press). We suggested that the splice patterns of this molecule might affect the subcellular localization, although the mechanisms controlling the splicing process remained unsolved. Thus, the significance of its nuclear-cytoplasmic localization in tumor cells is still controversial.

In the present study, we collected esophageal cancer cases with reference to normal mucosa to focus on the contribution of IAPs to the progression of esophageal cancer from normal squamous epithelia. The overall level of expression of survivin was relatively high in the cancer tissue compared with adjacent normal mucosa. Immunostaining revealed that survivin was mostly localized to the cytoplasm but in some cases to the nucleus. The expression of other IAPs including cIAP1, cIAP2, NAIP and XIAP, all of which appeared to suppress apoptosis by inhibiting caspase and procaspase (Duckett et al., 1996; Liston et al., 1996; Rothe et al., 1995; Roy et al., 1997), was also demonstrated in these samples and the significance of IAP family proteins in the pathogenesis as well as the biological character of esophageal cancer was discussed.

Materials and methods

Patients

Formalin-fixed paraffin-embedded samples from 34 patients with esophageal cancer were collected. In 10 patients, fresh frozen samples were also prepared for the mRNA analysis. Basically, central portions of tumor that consisted dominantly of carcinoma cells were taken for the analysis just after operation. At the same time, adjacent tissue samples from the normal esophageal mucosa were also collected. Age distribution and gender of the patients were as follows: for formalin-fixed subjects, median of age, 65; minimum 54, maximum 87; 32 male and 2 female patients, and for fresh frozen subjects, median of age, 66; minimum 51, maximum 74; 9 male and 1 female patients. All of the patients did not receive radiotherapy nor chemotherapy before the operation. The procedures followed were in accord with the ethical standards established by the ethics committee of Tokyo Medical and Dental University.

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

The RNA was extracted from frozen esophageal samples from normal mucosa and cancer tissue 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 (PerkinElmer Cetus, Norwalk, CT) were used with the ABI Prism 7900HT Sequence Detection System (PerkinElmer, Foster City, CA). Oligonucleotides as specific primers and TaqMan probes for the IAP family and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized at a commercial laboratory (PerkinElmer Cetus). The primers and TaqMan probes were as follows. The sequence of the forward primer for survivin mRNA was 5′-TGCCTGGCAGCCCTTTC-3′ and that of the reverse primer, 5′-CCTCCAAGAAGGGCCAGTTC-3′; the TaqMan probe was 5′-CAAGGACCACCGCATCTCTACATTC-3′. For cIAP1 mRNA, the forward primer was 5′-CAGCCTGAGCAGCTTGCAA-3′ and the reverse primer, 5′-CAAGCCACCATCACAACAAAA-3′; the TaqMan probe was 5′-TTTATTATGTGGGTCGCAATGATGATGTCAAA-3′. For cIAP2 mRNA, the forward primer was 5′-TCCGTCAAGTTCAAGCCAGTT-3′ and the reverse primer, 5′-TCTCCTGGGCTGTCTGATGTG-3′; the TaqMan probe was 5′-CCCTCATCTACTTGAACAGCTGCTAT-3′. The forward primer for NAIP mRNA was 5′-GCTTCACAGCGCATCGAA-3′ and the reverse primer, 5′-GCTGGGCGGATGCTTTC-3′; the TaqMan probe was 5′-CCATTTAAACCACAGCAGAGGCTTTAT-3′. The forward primer for XIAP mRNA was 5′-AGTGGTAGTCCTGTTTCAGCATCA-3′ and the reverse primer, 5′-CCGCACGGTATCTCCTTCA-3′; the TaqMan probe was 5′-CACTGGCACGAGCAGGGTTTCTTTATACTG-3′. The forward primer for GAPDH mRNA was 5′-GAAGGTGAAGGTCGGAGT-3′ and the reverse primer, 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 (Nakagawa et al., in press). 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.

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 (Kitagawa et al., 1998). Briefly, tissue sections were deparaffinized and incubated with proteinase K (prediluted, DAKO, Glostrup, Denmark) for 15 min at room temperature. After a 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, 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).

Immunohistochemistry for survivin

Tissue sections (4 μm thick) of formalin-fixed, paraffin-embedded esophageal samples from cancer tissue and normal mucosa 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. Primary antibody was applied to identify survivin using polyclonal rabbit antibody against human survivin (SURV 11-A, Alpha Diagnostic International, San Antonio, TX). 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.

In situ hybridization for mRNA of survivin

Tissue sections of paraffin-embedded samples from esophageal cancer were deparaffinized and then incubated with 1 ml of DEPC water containing 10 μl pepsin solution (Nichirei, Tokyo Japan) and 8.5 μl HCl. After incubation at 42 °C for 22 min followed by washing, samples were refixed with 4% paraformaldehyde. Then, the slides were treated with triethanolamine (TAE) solution (750 μl of TAE in 50 ml of DEPC water) for 1 min. After dehydration, slides were incubated with the hybridization solution containing 5 pM of digoxigenin-labeled probes at 42°C over night. Then, the slides were washed with wash buffer (DAKO) twice. The endogenous peroxidase was quenched with peroxidase-blocking solution (DAKO). After washing with PBS, slides were incubated with anti-digoxigenin antibody (DAKO), treated with biotinylated tyramide (DAKO) and mixed with peroxidase-conjugated streptavidin. All sections were developed using DAB as the chromogen. Negative control staining procedure was performed using the hybridization solution containing sense probes substituted for antisense probes. The antisense probes for survivin mRNA included the mixtureofthree oligonucleotides as follows: 5′-AGCAGAAGAAACACTGGGCCAAGTCTGGCTCGTTCTCAGT-3′, 5′-AGCTCCTTGAAGCAGAAGAAACACTGGGCCAAGTCTGGCT-3′, and 5′-CAACCGGACGAATGCTTTTTATGTTCCTCTATGGGGTCGT-3′.

Statistical analysis

Statistically significant differences of quantitative analysis were determined using the Wilcoxon's test for the comparison between paired subjects from normal mucosa and cancer tissue. Differences between subjects with different histological types of esophageal cancer were determined using the Mann–Whitney's U test. The significance of correlation between the levels of survivin expression and apoptotic cell ratio was analyzed by calculating the correlation coefficient and the slope of standard curve.

Results

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

To quantitate the mRNA expression levels of the IAP family in esophageal cancer, real-time quantitative RT-PCR was performed using esophageal samples from normal mucosa and cancer tissue. As shown in Fig. 1A, the expression of survivin exhibited significant up-regulation in cancer tissue compared with normal mucosa ($P < 0.05$). Although the statistical differences were not significant, the mRNA for cIAP1, cIAP2, NAIP and XIAP also revealed higher levels of expression in cancer tissue than normal mucosa (Figs. 1B–E). When comparing the histological types of esophageal cancers, moderately/poorly differentiated squamous cell carcinomas showed significantly higher expression of survivin than well differentiated squamous cell carcinomas (Fig. 2) ($P < 0.05$).

Expression of survivin and apoptotic cell ratio of the esophageal cancer

To identify the correlation between the expression levels of survivin and the proportion of apoptotic cells in esophageal cancer, the TUNEL method was performed on paraffin-embedded tissue sections. As expected, the expression intensity of survivin showed reverse correlation with the proportion of apoptotic cells ($P < 0.05$) (Fig. 3). These results indicated that the expression of survivin might play a role in inhibiting the apoptosis in esophageal cancer, although pathways interacting with other IAP family proteins as well as other molecules should be clarified in the future study.

Immunohistochemical localization of survivin and in situ hybridization assay for survivin mRNA in esophageal cancer

To investigate the distribution of survivin, immunohistochemical staining was performed in esophageal subjects from normal mucosa and cancer tissue. Table 1 summarizes the staining results of survivin. As demonstrated previously (Grabowski et al., 2003), survivin was detected in the basal layer as well as middle layer of the squamous epithelia of

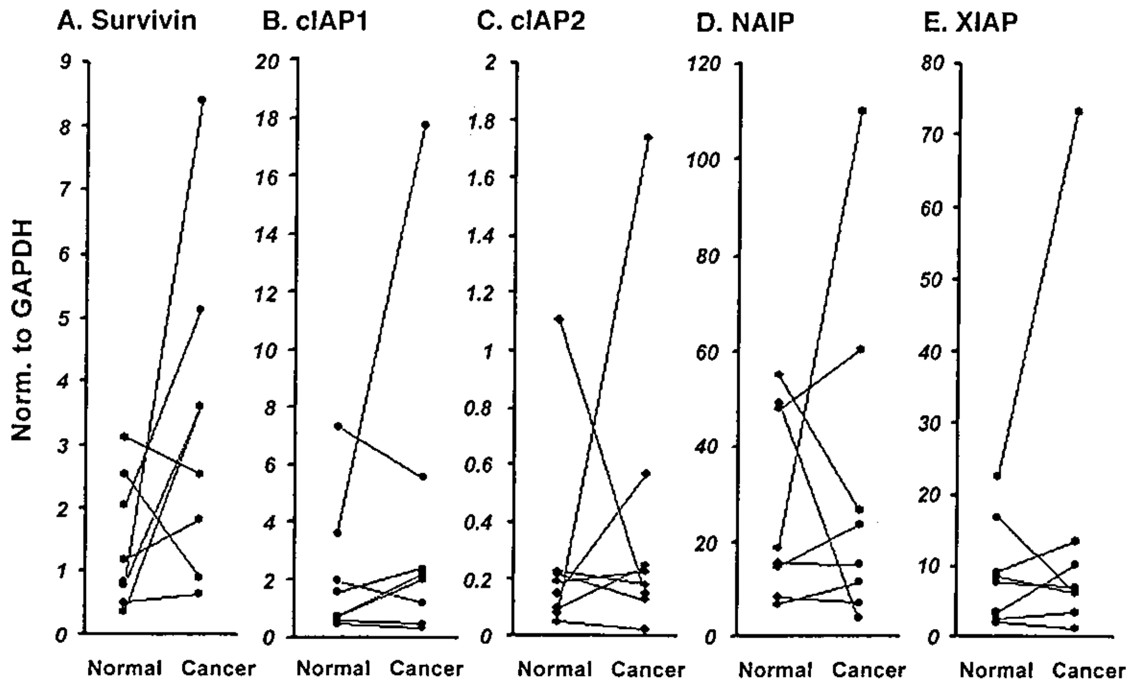

Fig. 1. Real-time quantitative RT-PCR analysis of IAP family proteins, (A) survivin, (B) cIAP1, (C) cIAP2, (D) NAIP and (E) XIAP, using esophageal samples from normal mucosa and cancer tissue. Relative intensity was calculated as [intensity of reaction of IAP family (total Raji RNA, ng)] / [intensity of reaction of GAPDH (total Raji RNA, ng)]. Difference was significant between the expression of survivin in samples from normal mucosa and cancer tissue ($P < 0.05$ by the Wilcoxon's test). Although the differences were not significant, cIAP1, cIAP2, NAIP and XIAP in cancer tissue tended to show higher expression as compared with normal mucosa.

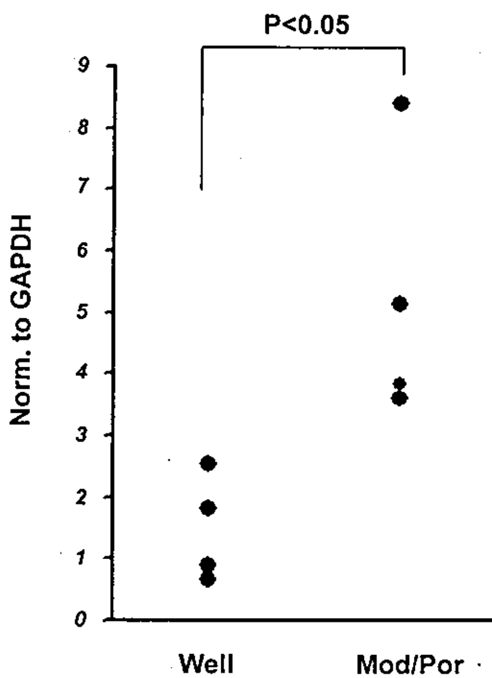

Fig. 2. Comparison of the levels of survivin expression between well (Well) and moderately/poorly differentiated squamous cell carcinoma (Mod/Por) of the esophagus determined by the real-time quantitative RT-PCR. The plots indicate the values for each histological type of esophageal cancer. Note the significantly higher expression of survivin in moderately/poorly differentiated carcinoma ($P < 0.05$ by the Mann–Whitney's U test).

the normal mucosa in most cases (Fig. 4A). The subcellular localization was mainly cytoplasmic but partly nuclear. The staining pattern and intensity in the normal mucosa varied between different samples. The majority of the samples from esophageal cancer revealed positive staining for survivin, although the staining intensity and frequency varied from case to case (Table 1). At the cellular level, survivin signals in esophageal cancer cells were predominantly

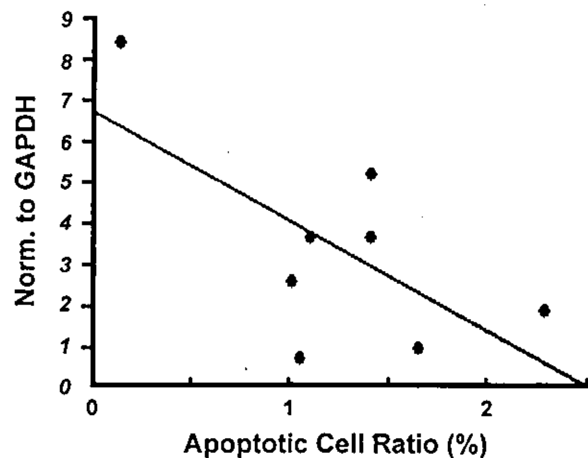

Fig. 3. Correlation between the expression intensity of mRNA for survivin and the apoptotic cell ratio of esophageal cancer. Note the negative slope of the standard line indicating the inverse correlation between these parameters ($P < 0.05$).

Table 1
Subcellular localization of survivin in normal esophageal mucosa and esophageal cancer tissue determined by immunohistochemistry

Samples	Cytoplasmic			Nuclear		
	−	+	++	−	+	++
Normal mucosa	14[a] (41.2)[b]	19 (55.9)	1 (2.9)	20 (58.8)	10 (29.4)	4 (11.8)
Cancer tissue	3 (9.4)	13 (40.6)	16 (50.0)	6 (18.8)	14 (43.7)	12 (37.5)

Staining intensity; −, less than 5%;+, 5–20%; ++, more than 20% of normal epithelial cells or cancer cells were positive. Note the higher frequency of positive cells in cancer tissues.
[a] Values indicate the number of cases.
[b] Numbers in parenthesis indicate percentage of total cases examined.

localized to the cytoplasm (Fig. 4B), although in many cases, prominent reaction was also observed in the nucleus (Fig. 4C). Patterns of subcellular localization and the expression levels of mRNA for survivin or apoptotic cell ratio did not show significant correlation. In addition, histological type (well, moderately or poorly differentiated squamous cell carcinoma) did not influence the localization of survivin in cancer cells. Tissue sections that were reacted with preimmune rabbit antibody with irrelevant specificity showed no significant staining in all of the samples (not shown).

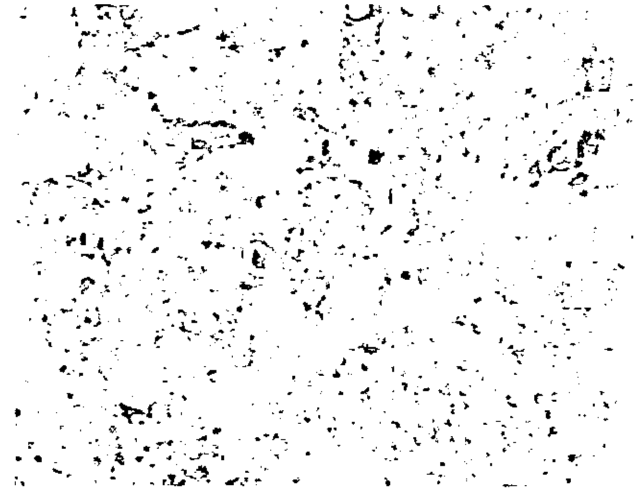

Fig. 5. In situ hybridization for mRNA of survivin in esophageal cancer. Note the positive signals in the cytoplasm of the majority of cancer cells (original magnification × 400).

To further confirm the overexpression of survivin in esophageal cancer at the mRNA level, in situ hybridization analysis was performed. As shown in Fig. 5, positive signals for survivin mRNA were diffusely observed in the majority of esophageal cancer samples. The signals were in the cytoplasm of cancer tissue.

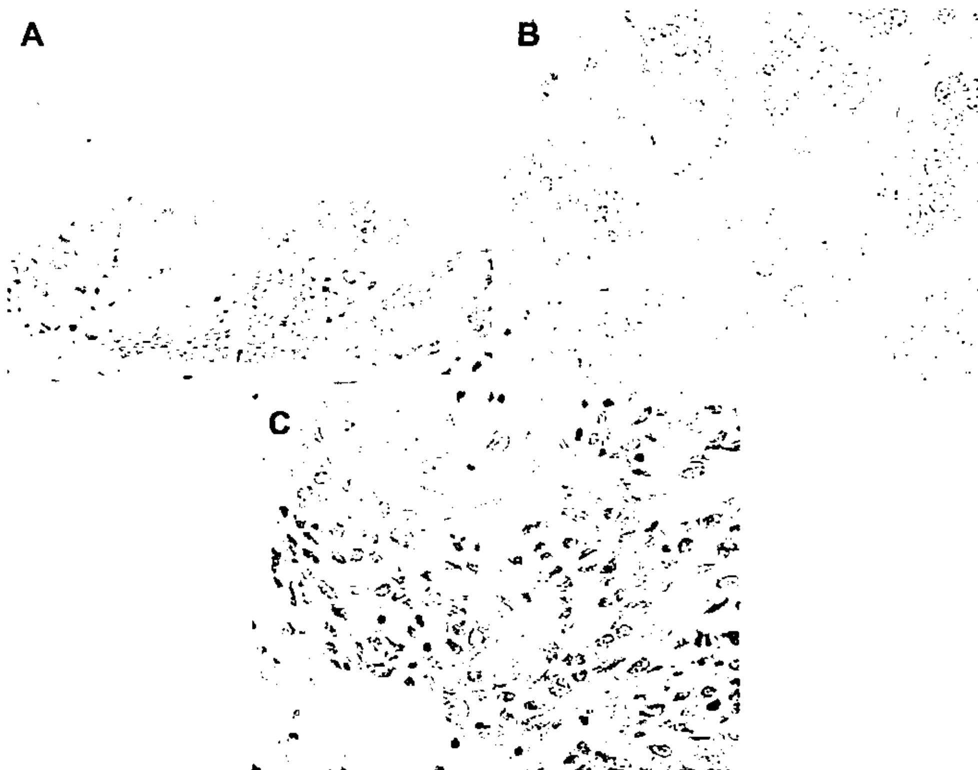

Fig. 4. Immunohistochemical localization of survivin in the esophagus from normal mucosa (A) and cancer tissue (B, C) (original magnification ×400). The expression of survivin was observed in many of the cases of the normal squamous epithelia mainly in the basal layer (A). The subcellular distribution was dominantly cytoplasmic but in some cases nuclear. By contrast, the survivin-positive cells were distributed rather diffusely in cancer tissues and the staining was cytoplasmic (B) or sometimes nuclear (C) at the cellular level.

Discussion

Regarding the expression of survivin in esophageal cancer, previous studies revealed an overexpression in cancer tissue whereas normal squamous epithelia exhibited weak signals (Grabowski et al., 2003; Ikeguchi and Kaibara, 2002; Ikeguchi et al., 2003; Kato et al., 2001). However, little is known about the potential roles of IAP family proteins in the homeostasis of normal esophageal epithelia as well as the pathogenesis of esophageal cancer. In esophageal cancer samples in the present study, patterns of IAP expression were not uniform. Some cases exhibited very strong expression of particular IAPs, although others revealed an almost normal level of expression. Immunohistochemical staining revealed a very high frequency of survivin expression in many of the cancer cells in the majority of cases though in normal mucosa, positive cells were partial and in some cases the signals were negative. Thus, differences in the positive cell ratio would result in differences in intensity in the overall expression of mRNA for IAPs.

In association with the prognostic factors of esophageal cancer, the proliferative activity of cancer cells, p53 nuclear accumulation in cancer cells (Ikeguchi et al., 2003), overall survival of patients (Grabowski et al., 2003) and the response of patients to chemotherapy (Kato et al., 2001) have been reported to correlate with survivin expression. However, lymph node metastasis, depth of tumor invasion, apoptotic frequency in cancer cells, tumor angiogenesis (Ikeguchi et al., 2003), histologic type of cancer and stage of tumors (Ikeguchi and Kaibara, 2002) did not correlate with the intensity of survivin expression in cancer cells. This study demonstrated the significant correlation of survivin expression with the apoptotic frequency of cancer cells and histological type of tumor. Further study may be necessary to determine the significance of survivin expression upon the biological characters of esophageal cancer. Studies including the comparison of early stage and advanced stage cancers would help defining the role of survivin in tumorigenesis and progression of esophageal cancer.

In addition to its anti-apoptotic function, survivin also plays a role in the regulation of cell cycle progression during mitosis (Gianani et al., 2001). The highly proliferative activity of esophageal cancer cells might be associated with the significant expression of survivin. Kato et al. (2001) reported that survivin expression was a frequent event in esophageal cancer and was identified as an unfavorable prognostic factor. Here, we could find the significant difference in the expression of survivin between the different histological types of esophageal cancer that may be related to the prognosis of the patients.

Wild-type p53, but not mutant p53, represses survivin expression at both the mRNA and protein levels (Hoffman et al., 2002). The modification of chromatin within the survivin promoter would explain the silencing of survivin gene transcription by p53 (Mirza et al., 2002). 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 (Mirza et al., 2002). As the high frequency of p53 mutations in many of the solid cancers, p53 mutation would play a role in controlling the overexpression of survivin in the esophageal cancer cases.

Recently, target gene within an amplicon at 11q22 in esophageal squamous cell carcinoma has been suggested as cIAP1 (Imoto et al., 2001). The expression of cIAP1 was consistently up-regulated in esophageal squamous cell carcinoma cell lines that showed amplification at 11q21-q23. The present study revealed overexpression of cIAP1 in the majority of esophageal cancer samples. Thus, these findings would suggest that a part of primary esophageal cancers might overexpress the cIAP1 protein in association with the amplification of chromosomal DNA at 11q21-q23.

In conclusion, we demonstrated the overexpression of several IAPs in esophageal cancer samples. Further study is warranted to clarify the regulatory mechanisms of IAP expression in esophageal cancer in association with the apoptotic signaling pathways.

Acknowledgments

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References

Adida, C., Crotty, P.L., McGrath, J., Berrebi, D., Diebold, J., Altieri, D.C., 1998. Developmentally regulated expression of the novel cancer anti-apoptotic gene survivin in human and mouse differentiation. Am. J. Pathol. 152, 43–49.

Ambrosini, G., Adida, C., Altieri, D.C., 1997. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat. Med. 3, 917–921.

Duckett, C.S., Nava, V.E., Gedrich, R.W., Clem, R.J., Van Dongen, J.L., Gilfillan, M.C., Shiels, H., Hardwick, J.M., Thompson, C.B., 1996. A conserved family of cellular genes related to the baculovirus IAP gene and encoding apoptosis inhibitors. EMBO J. 15, 2685–2694.

Gianani, R., Jarboe, E., Orlicky, D., Frost, M., Bobak, J., Lehner, R., 2001. Expression of survivin in normal, hyperplastic, and neoplastic colonic mucosa. Hum. Pathol. 32, 119–125.

Grabowski, P., Kühnel, T., Mürh-Wilkenshoff, F., Heine, B., Stein, H., Höpfner, M., Germer, C.T., Scherübl, H., 2003. Prognostic value of nuclear survivin expression in oesophageal squamous cell carcinoma. Br. J. Cancer 88, 115–119.

Hoffman, W.H., Biade, S., Zilfou, J.T., Chen, J., Murphy, M., 2002. Transcriptional repression of the anti-apoptotic *survivin* gene by wild type p53. J. Biol. Chem. 277, 3247–3257.

Ikeguchi, M., Kaibara, N., 2002. Survivin messenger RNA expression is a

good prognostic biomarker for oesophageal carcinoma. Br. J. Cancer 87, 883–887.

Ikeguchi, M., Yamaguchi, K., Kaibara, N., 2003. Survivin gene expression positively correlates with proliferative activity of cancer cells in esophageal cancer. Tumour Biol. 24, 40–45.

Imoto, I., Yang, Z.-Q., Pimkhaokham, A., Tsuda, H., Shimada, Y., Imamura, M., Ohki, M., Inazawa, J., 2001. Identification of cIAP1 as a candidate target gene within an amplicon at 11q22 in esophageal squamous cell carcinomas. Cancer Res. 61, 6629–6634.

Kato, J., Kuwabara, Y., Mitani, M., Shinoda, N., Sato, A., Toyama, T., Mitsui, A., Nishiwaki, T., Moriyama, S., Kudo, J., Fujii, Y., 2001. Expression of survivin in esophageal cancer: correlation with the prognosis and response to chemotherapy. Int. J. Cancer 95, 92–95.

Kawasaki, H., Toyoda, M., Shinohara, H., Okuda, J., Watanabe, I., Yamamoto, T., Tanaka, K., Tenjo, T., Tanigawa, N., 2001. Expression of survivin correlates with apoptosis, proliferation, and angiogenesis during human colorectal tumorigenesis. Cancer 91, 2026–2032.

Kitagawa, M., Yamaguchi, S., Takahashi, M., Tanizawa, T., Hirokawa, K., Kamiyama, R., 1998. Localization of Fas and Fas ligand in bone marrow cells demonstrating myelodysplasia. Leukemia 12, 486–492.

LaCasse, E.C., Baird, S., Korneluk, R.G., MacKenzie, A.E., 1998. The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene 17, 3247–3259.

Liston, P., Roy, N., Tamai, K., Lefebvre, C., Baird, S., Cherton-Horvat, G., Farahani, R., McLean, M., Ikeda, J.E., MacKenzie, A., Korneluk, R.G., 1996. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature 379, 349–353.

Mirza, A., McGuirk, M., Hockenberry, T.N., Wu, Q., Ashar, H., Black, S., Wen, S.F., Wang, L., Kirsehmeier, P., Bishop, W.R., Nielsen, L.L., Pickett, C.B., Liu, S., 2002. Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. Oncogene 21, 2613–2622.

Nakagawa, Y., Yamaguchi, S., Hasegawa, M., Nemoto, T., Inoue, M., Suzuki, K., Hirokawa, K., Kitagawa, M., 2004. Differential expression of survivin in bone marrow cells from patients with acute lymphocytic leukemia and chronic lymphocytic leukemia. Leuk. Res. (in press).

Okada, E., Murai, Y., Matsui, K., Ishizawa, S., Cheng, C., Masuda, M., Takano, Y., 2001. Survivin expression in tumor cell nuclei is predictive of a favorable prognosis in gastric cancer patients. Cancer Lett. 163, 109–116.

Rodriguez, J.A., Span, S.W., Ferreira, C.G.M., Kruyt, F.A.E., Giaccone, G., 2002. CRM1-mediated nuclear export determines the cytoplasmic localization of the antiapoptotic protein survivin. Exp. Cell Res. 275, 44–53.

Rothe, M., Pan, M.G., Henzei, W.J., Ayres, T.M., Goeddel, D.V., 1995. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell 83, 1152–1243.

Roy, N., Dereraux, Q.L., Takahashi, R., Salvesen, G.S., Reed, J.C., 1997. The cIAP-1 and cIAP-2 proteins are distinct inhibitors of specific caspases. EMBO J. 16, 6914–6925.

Shin, S., Sung, B.-J., Cho, Y.-S., Kim, H.-J., Ha, N.-C., Hwang, J.-I., Chung, C.W., Jung, Y.K., Oh, B.H., 2001. An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7. Biochemistry 40, 1117–1123.

Tamm, I., Wang, Y., Sausville, E., Scudiero, D.A., Vigna, N., Oltersdorf, T., Reed, J.C., 1998. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), bax, caspases, and anticancer drugs. Cancer Res. 58, 5315–5320.

Velculescu, V.E., Madden, S., Zhang, L., Lash, A.E., Yu, J., Rago, C., Lal, A., Wang, C.J., Beaudry, G.A., Ciriello, K.M., Cook, B.P., Dufault, M.R., Ferguson, A.T., Gao, Y., He, T.C., Hermeking, H., Hiraldo, S.K., Hwang, P.M., Lopez, M.A., Laderer, H.F., Mathews, B., Petroziello, J.M., Polyak, K., Zawel, L., Kinzler, K.W., et al., 1999. Analysis of human transcriptomes. Nat. Genet. 23, 387–388.

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ORIGINAL ARTICLE

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Expression of IAP family proteins in colon cancers from patients with different age groups

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Abstract Members of the inhibitor of apoptosis protein (IAP) family including survivin, are expressed in many tumors. However, age-related changes in their expression in cancer have not been clarified. Thus, we investigated the expression of mRNA-coding for IAP family proteins in colon cancer samples from young (< 70 years of age) and elderly (> 70 years) patients by real-time quantitative RT-PCR. Samples were collected from cases with well-differentiated adenocarcinoma or moderately differentiated adenocarcinoma and their adjacent normal epithelial tissue. Well-differentiated adenocarcinoma tended to express higher levels of survivin than normal mucosa, and expression in moderately differentiated adenocarcinoma was significantly greater than in normal mucosa in samples from both groups of patients ($p < 0.05$, respectively). When samples were compared between the different age groups, the normal mucosa exhibited similar levels of survivin expression. However, samples from older patients showed a significantly higher level of expression than those from younger patients in well and moderately differentiated adenocarcinomas ($p < 0.05$, respectively). In contrast, the levels of expression of cIAP1, cIAP2, and NAIP in the cancerous tissues were lower than those found in normal mucosa regardless of age. As for age-related changes, the expression of cIAP2 in normal mucosa and moderately differentiated adenocarcinoma was stronger in the elderly group than the young group ($p < 0.05$, respectively), and NAIP expression in well-differentiated adenocarcinoma was higher in the young group than the elderly group ($p < 0.05$). XIAP expression was similar in normal and cancerous tissues in both the young and elderly groups. These results suggest that the expression of IAP family proteins, especially survivin, is associated with the age-related biological characteristics of colon cancer.

Keywords Colon cancer · IAP · Immunohistochemistry · Real-time PCR · Survivin

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Introduction

The regulation of apoptotic cell death has a profound effect on the pathogenesis and progression of colorectal malignancies. The inhibitor of apoptosis protein (IAP) family, including survivin, blocks apoptosis induced by a variety of triggers [1, 2], although the exact biochemical mechanism by which members of the IAP family suppress apoptosis is under debate. Survivin directly binds to and inhibits caspase-3 and caspase-7, which act as terminal effectors in apoptotic protease cascades [2, 3]. The expression of survivin is ubiquitous in fetal tissues, but is restricted during development, and is negligible in the majority of terminally differentiated adult tissues [4, 5]. However, analysis of the differences in gene expression between normal and tumor cells revealed that survivin was one of the proteins most consistently overexpressed in tumor cells relative to normal tissue [6]. In fact, survivin is prominently expressed in transformed cell lines and in many human cancers including colorectal cancer [7].

Survivin is usually found in the cytoplasm of tumor cells and is therefore widely regarded as a cytoplasmic protein [5, 8]. However, several studies showed a nuclear accumulation of survivin in gastric cancer cells [9] and lung cancer cells [10]. In colon cancer, Kawasaki et al. [7]

revealed the cytoplasmic expression of survivin using immunohistochemistry. We recently reported that ALL cells principally exhibit nuclear localization of survivin, while CLL cells exhibit cytoplasmic distribution [11]. We suggested that the splice patterns of this molecule might affect its subcellular localization, although the mechanisms controlling the splicing process remained unresolved. Thus, the significance of its nuclear-cytoplasmic localization in tumor cells remains controversial.

In the present study, we collected colon cancer tissues from young and elderly patients to focus on the contribution of IAPs to the progression of colon cancer in association with aging. The expression of survivin was higher in cancerous tissue compared with adjacent normal mucosa. Immunostaining revealed that survivin was primarily localized in the cytoplasm. The expression of other IAPs, including cIAP1, cIAP2, NAIP and XIAP, all of which suppressed apoptosis by inhibiting caspase and procaspase [12–15], was also demonstrated in these samples. The IAPs are characterized by one or more 70–80 amino acid baculoviral IAP repeat (BIR) domains. They function as an endogenous caspase inhibitor, as well as participate in cell cycle regulation and in the modulation of receptor-mediated signal transduction [16]. The significances of IAP family proteins with regard to the aging effect as well as the biological characteristics of colon cancer are discussed.

Materials and methods

Patients

Fresh frozen samples from 32 patients with adenocarcinoma of the ascending, transverse, and descending colon were collected and prepared for mRNA analysis. Central portions of tumors that dominantly consisted of carcinoma cells were collected for analysis just after operation. At the same time, adjacent tissue samples from normal colonic mucosa were also collected. To eliminate the effects of factors other than the aging effect as much as possible, we collected well-differentiated or moderately differentiated adenocarcinoma with the same tumor histology from younger (< 70 years old) and elderly (> 70 years old) patients. The age distribution and gender of the patients were as follows: for well-differentiated adenocarcinoma from younger patients, the median of age was 58 years, the minimum was 38, and the maximum was 65, among six male and two female patients; for well-differentiated adenocarcinoma from elderly patients, the median of age was 81 years, the minimum was 75, and the maximum was 89, among five male and three female patients; for moderately differentiated adenocarcinoma from younger patients, the median of age was 56, the minimum was 45, and the maximum was 63, among six male and two female patients; and for moderately differentiated adenocarcinoma from elderly patients, the median of age was 81, the minimum was 77, and the maximum was 91, among three male and five female patients. Table 1 summarizes the Dukes' classification of cases indicating that the stages of tumors were not significantly different between young and old patients. All patients did not receive radiotherapy or chemotherapy prior to operation. The procedures followed were in accordance with the ethical standards established by the ethics committee of Tokyo Medical and Dental University.

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

RNA was extracted from frozen colonic samples of normal mucosa and cancer tissue using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's directions. For quantitative RT-PCR, fluorescent hybridization probes and a TaqMan PCR Core Reagents Kit with AmpliTaq Gold (PerkinElmer Cetus, Norwalk, CT, USA) were used with an ABI Prism 7,900HT Sequence Detection System (PerkinElmer, Foster City, CA, USA). Oligonucleotides as specific primers and TaqMan probes for the IAP family and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized at a commercial laboratory (PerkinElmer Cetus). The primers and TaqMan probes 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

Table 1 Dukes' classification of cases. Number of cases (percentage)

Cases	Dukes' classification		
	A	B	C
Well-differentiated adenocarcinoma			
Young	3 (38%)	2 (25%)	3 (38%)
Old	1 (13%)	3 (38%)	5 (63%)
Moderately differentiated adenocarcinoma			
Young	1 (13%)	2 (25%)	5 (63%)
Old	0 (0%)	3 (38%)	5 (63%)

TaqMan probe was 5′-CACTGGCACGAGCAGGG-TTTCTTTATACTG-3′. Finally the forward primer for GAPDH mRNA was 5′-GAAGGTGAAGGTCGGA-GT-3′ and the reverse primer was 5′-GAAGATGGT-GATGGGATTTC-3′; the TaqMan probe was 5′-CAAGCTTCCCGTTCTCAGCC-3′. The conditions for the one-step RT-PCR were as follows: 30 min at 48°C (stage 1, reverse transcription), 10 minutes 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 quantified according to a method described elsewhere [11]. 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 data for the GAPDH in each sample.

Immunohistochemistry for survivin

Tissue sections (4-µm thick) of formalin-fixed, paraffin-embedded samples from the same cases used in the RT-PCR study were sectioned and placed on slides covered with adhesive. Sections were deparaffinized, and endogenous peroxidase was quenched with 1.5% hydrogen peroxide in methanol for 10 min. Primary antibody was applied to identify survivin using polyclonal rabbit antibody against human survivin (SURV 11-A; Alpha Diagnostic International, San Antonio, TX, USA). 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, which was substituted for the primary antibody.

Statistical analysis

Statistically significant differences for the quantitative analysis were determined using the Mann–Whitney *U*-test.

▶

Fig. 1A–E Real-time quantitative RT-PCR analysis of IAP family proteins, **A** survivin, **B** cIAP1, **C** cIAP2, **D** NAIP, and **E** XIAP, using colonic samples from normal mucosa (*Normal*), well-differentiated adenocarcinoma (*Well*), and moderately differentiated adenocarcinoma (*Mod.*) of young and elderly patients. The relative intensity was calculated as [intensity of reaction of the IAP family (total Raji RNA, ng)] / [intensity of reaction of GAPDH (total Raji RNA, ng)]. Differences were significant between the expression of survivin (**A**) in samples from well-differentiated adenocarcinoma of young and elderly patients ($*p < 0.05$ by the Wilcoxon's test) and samples from moderately differentiated adenocarcinoma of young and elderly patients ($*p < 0.05$). The expression of survivin was also significantly different between normal mucosa and well-differentiated adenocarcinoma (${}^{a}p < 0.05$), normal mucosa and moderately differentiated adenocarcinoma (${}^{b}p < 0.05$), and well and moderately differentiated adenocarcinomas (${}^{c}p < 0.05$) in the elderly group. Between young and elderly groups, differences were significant in the expression of cIAP2 in normal mucosa ($*p < 0.05$) and moderately differentiated adenocarcinoma ($*p < 0.05$), and the expression of NAIP in well-differentiated adenocarcinoma ($*p < 0.05$). The expression of cIAP1, cIAP2, and NAIP in cancerous tissues tended to show a lower degree of expression compared with normal mucosa. Differences were significant for comparisons of the indicated groups (**B** cIAP1, ${}^{d}p < 0.05$, ${}^{e}p < 0.01$, ${}^{f}p < 0.05$, ${}^{g}p < 0.05$; **C** cIAP2, ${}^{h}p < 0.01$, ${}^{i}p < 0.01$, ${}^{j}p < 0.05$; **D** NAIP, ${}^{k}p < 0.05$, ${}^{l}p < 0.05$, ${}^{m}p < 0.05$)

Results

Age-related changes in the expression of IAP family protein mRNA in normal colonic mucosa

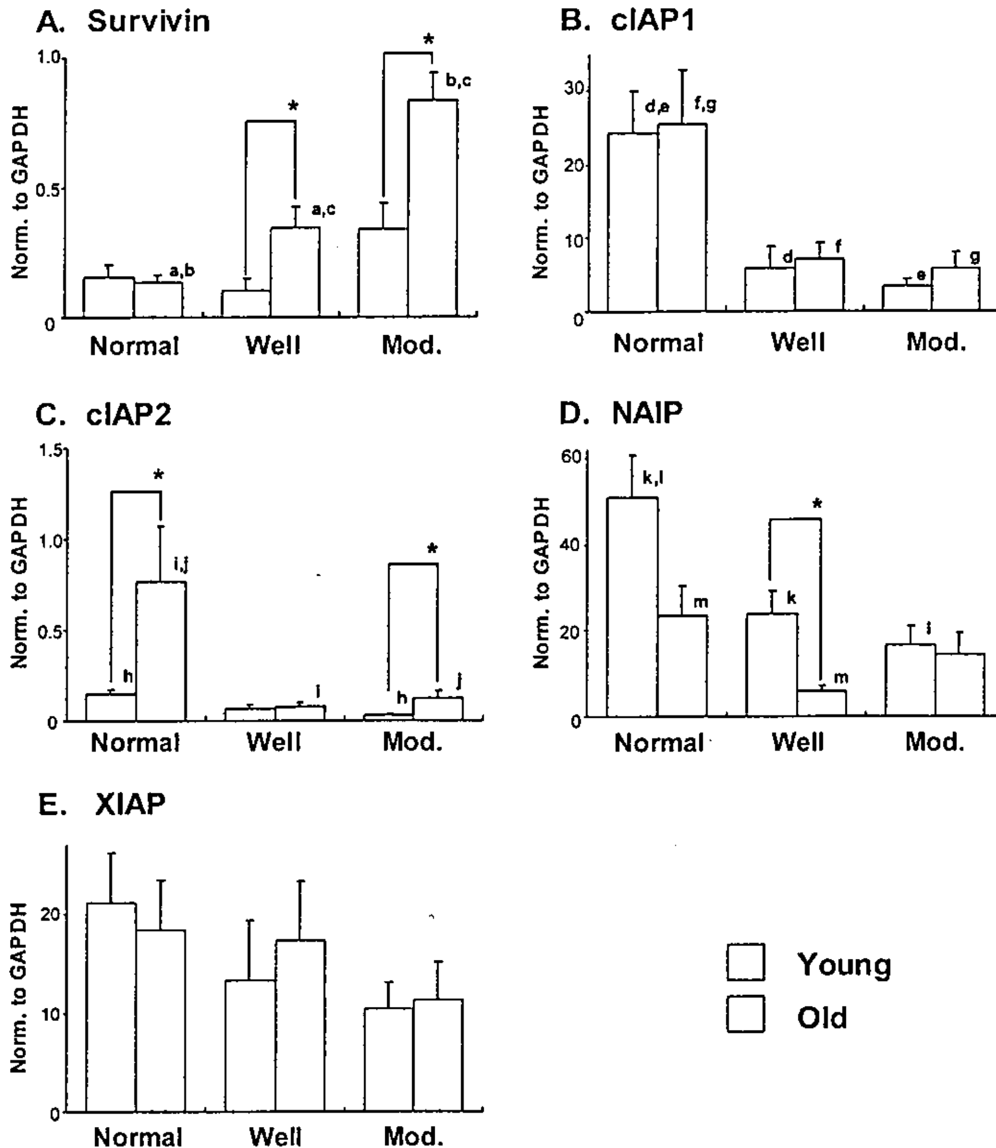
To quantitate age-related changes in mRNA expression of the IAP family in normal colonic mucosa, real-time quantitative RT-PCR was performed using samples from the normal mucosa of different age groups. As shown in Fig. 1A, B, D, and E, the expression levels of survivin, cIAP1, NAIP, and XIAP were similar in young and elderly individuals. However, cIAP2 expression of the normal mucosa was significantly higher in elderly individuals than young individuals (Fig. 1C; $p < 0.05$).

Age-related changes in the expression of IAP family protein mRNA in well-differentiated adenarcinomas of the colon

To determine age-related changes in the expression of IAP family proteins in colon cancer, samples were collected from different age groups with similar histological features. First, well-differentiated adenocarcinomas from young and elderly patients were compared. As shown in Fig. 1A, the expression of survivin was significantly higher in elderly patients than in young patients ($p < 0.05$). Conversely, NAIP expression was significantly higher in younger patients than in elderly patients (Fig. 1D; $p < 0.05$). Expression levels of other IAPs including cIAP1, cIAP2, and XIAP were almost similar between young and elderly patients.

Age-related changes in the expression of IAP family protein mRNA in moderately differentiated adenocarcinomas of the colon

Next, the expression levels of IAP family proteins in moderately differentiated adenocarcnomas were compared. As shown in Fig. 1A, the expression of survivin was significantly higher in elderly patients than young patients ($p < 0.05$). Similarly, cIAP2 expression was sig-



nificantly higher in elderly patients than younger patients (Fig. 1C; $p < 0.05$). Other IAPs including cIAP1, NAIP, and XIAP did not exhibit significant difference between young and elderly patients.

Comparison of the expression of IAP family protein mRNA in normal and cancerous tissues in young and elderly patients

As shown in Fig. 1A, the cancer tissues tended to express higher levels of survivin than that found in normal mucosa. Significant differences were observed between normal mucosa and well-differentiated adenocarcinoma ($p < 0.05$), normal mucosa and moderately differentiated adenocarcinoma ($p < 0.01$), and well and moderately differentiated adenocarcinoma ($p < 0.05$) in the elderly group. By contrast, the expression of cIAP1, cIAP2, and NAIP in cancerous tissues was lower than that found in normal mucosa. For cIAP1 expression (Fig. 1B), differences were significant between the normal mucosa and the well-differentiated adenocarcinoma (young group, $p < 0.05$; elderly group, $p < 0.05$) and normal mucosa and moderately differentiated adenocarcinoma (young group, $p < 0.01$; elderly group, $p < 0.05$). For the expression of cIAP2 (Fig. 1C), significant differences were observed between normal mucosa and well-differentiated adenocarcinoma (elderly group, $p < 0.01$) and normal mucosa and moderately differentiated adenocarcinoma (young group, $p < 0.01$; elderly group, $p < 0.05$). The difference in the expression of NAIP (Fig. 1D was significant between normal mucosa and well-differentiated adenocarcinoma (young group, $p < 0.05$; elderly group, $p < 0.05$) and normal mucosa and moderately differentiated adenocarcinoma (young group, $p < 0.05$). The expression of XIAP did not exhibit any specific tendency between normal and cancerous tissues, as well as between the young and elderly groups (Fig. 1E).

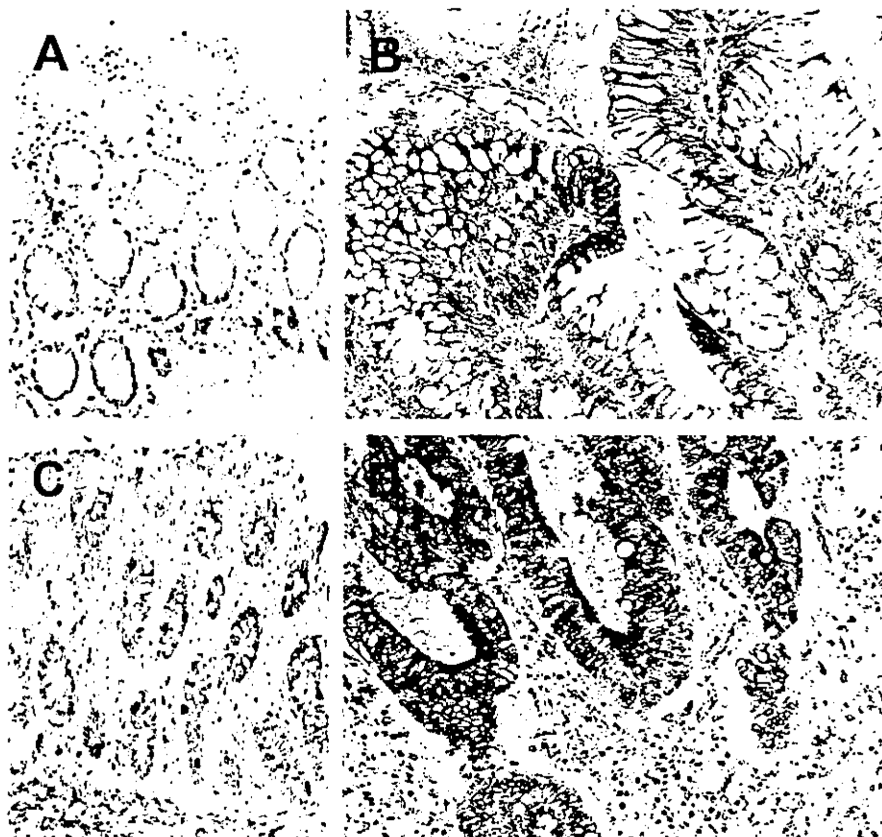


Fig. 2A–D Immunohistochemical localization of survivin in the colon from normal mucosa (A) (×100), well-differentiated adenocarcinoma of a young patient (B) (×200), and normal mucosa (C) (×100), well-differentiated adenocarcinoma of a elderly patient (D) (×200). The expression of survivin was observed in a few scattered cells of the normal colonic epithelia, mainly at the bottom of the glands (A, C). The subcellular distribution was dominantly cytoplasmic. In contrast, survivin-positive cells were diffusely distributed in cancerous tissues, and staining was cytoplasmic at the cellular level (B, D)

Immunohistochemical localization of survivin in colon cancers

To investigate the distribution of survivin, immunohistochemical staining was performed using normal colonic mucosa and cancerous tissues. As shown in Fig. 2A (young) and C (elderly), survivin was mainly detected in the bottom of the glands of normal mucosa, and subcellular localization was mainly cytoplasmic. The staining intensity of positive cells in normal mucosa varied between different samples. In contrast, the majority of samples from colon cancer showed a diffuse localization of survivin, although the staining intensity varied from case to case. At the cellular level, survivin signals in colon cancer cells were predominantly localized in the cytoplasm. The distribution of survivin was similar in well-differentiated and moderately differentiated adenocarcinomas as well as in cancers from young and elderly patients (Fig. 2B, D). Tissue sections that reacted with preimmune rabbit antibody with irrelevant specificity showed no significant staining for any of samples (not shown).

Discussion

For the expression of survivin in colon cancer, a previous study revealed its overexpression in cancer tissue while normal colonic epithelia exhibited weak signals [7]. However, little is known about the potential roles of IAPs in the homeostasis of normal epithelia as well as the pathogenesis of colon cancers among different age groups. In the colon cancer samples in the present study, cancerous tissues had a tendency to exhibit higher levels of survivin but lower levels of cIAP1, cIAP2, and NAIP than the normal mucosa. Immunohistochemical staining revealed a high degree of survivin expression in many cancer cells in the majority of cases, although in normal mucosa, the number of positive cells was small. Thus, differences in the positive cell ratio influenced the intensity and overall expression of survivin mRNA. Kawasaki et al. [7] reported that the expression of survivin correlated with apoptosis, proliferation, and angiogenesis during human colorectal tumorigenesis. We also confirmed the progression-related overexpression of survivin by our findings that moderately differentiated adenocarcinomas expressed higher levels of survivin than well-differentiated adenocarcinomas. Well and moderately differentiated adenocarcinomas, cancerous tissues from elderly patients, demonstrated a significantly higher degree of survivin expression than those from young patients. It would be possible that chances for genetic mutations might increase associated with progression of cancer as well as aging and might result in dysregulation in controlling survivin expression. However, further studies are necessary to determine whether age-related overexpression and differentiation-related

overexpression of survivin in colon cancers occurs through the same mechanisms.

In addition to its antiapoptotic function, survivin also plays a role in the regulation of cell cycle progression during mitosis [8]. The highly proliferative activity and low frequency of apoptosis in colon cancer cells is associated with the significant expression of survivin [7]. We recently reported that the proliferative activity and apoptotic frequency of cancer cells from colorectal cancers exhibited a positive correlation with age [17]. However, in the present study, survivin expression in colon cancers was significantly higher in elderly patients than in young patients. Thus, increased apoptosis in cancers of elderly patients is not attributable to a lack of antiapoptotic regulatory mechanisms by survivin. The manner of age-related changes in the expression of survivin might be associated with the proliferative activities of colon cancers rather than apoptosis. We can also postulate the other possibility that increased apoptosis in cancers of the elderly might be controlled by the caspase-independent mechanisms and, thus, survivin would be overexpressed as the feedback mechanisms of the cells.

Wild-type p53, and not mutant p53, represses survivin expression at the mRNA and protein level [18]. Modification of chromatin within the survivin promoter would explain the silencing of survivin gene transcription by p53 [19]. On the other hand, the overexpression of exogenous survivin protein rescues cells from p53-induced apoptosis in a dose-dependent manner, suggesting that the loss of survivin in part mediates the p53-dependent apoptotic pathway [19]. As there is a high frequency of p53 mutations in many solid cancers, p53 mutation may play a role in controlling the overexpression of survivin in colon cancer. Regarding the age-dependent changes in expression of tumor tissues, a microarray experiment revealed an activation of p53 and some of the genes controlled by p53 at more advanced age [20]. Thus, higher expression of survivin might be induced in response to the increased p53-dependent apoptosis in cancers of the elderly group. However, mutations/loss of p53 gene may occur more frequently with aging in certain settings [21] and, therefore, the p53-associated mechanisms controlling aging and carcinogenesis are complicated.

Using an in vitro cell culture system of colon carcinoma cell lines, Wang et al. [22] reported that the expression of cIAP2 was induced by PKC/NF-κB-dependent pathways. However, the present study revealed that cIAP2 expression was not elevated in colon cancers in vivo. Instead, cIAP1, cIAP2, and NAIP exhibited lower expression in cancerous tissues than in normal mucosa. Further studies are necessary to clarify the function of these molecules in the colonic mucosa as well as in carcinogenesis of the colon.

Signaling pathways involved in survival responses may attenuate the apoptosis response to the cytotoxic tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) in colon carcinoma cell lines. Some lines are sensitive, while others are resistant to TRAIL-induced apoptosis [23, 24]. The mechanisms of this resistance include blocking caspase processing by XIAP. However, in the present study, changes in XIAP expression were not notable among normal/cancerous tissues, well/moderately differentiated adenocarcinomas, and cancers of young/elderly patients. Thus, XIAP function in in vivo colon cancer might not be essential for the survival of cancer cells.

In conclusion, we demonstrated the differentiation-related and age-related overexpression of survivin in colon cancer samples. Further studies are warranted to clarify the regulatory mechanisms of IAP expression in colon cancer in association with the apoptotic/proliferative signaling pathways.

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References

1. LaCasse EC, Baird S, Korneluk RG et al (1998) The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene 17:3247–3259
2. Tamm I, Wang Y, Sausville E et al (1998) IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res 58:5315–5320
3. Shin S, Sung B-J, Cho Y-S et al (2001) An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7. Biochemistry 40:1117–1123
4. Adida C, Crotty PL, McGrath J, Berrebi D et al (1998) Developmentally regulated expression of the novel cancer anti-apoptotic gene survivin in human and mouse differentiation. Am J Pathol 152:43–49
5. Ambrosini G, Adida C, Altieri DC (1997) A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 3:917–921
6. Velculescu VE, Madden S, Zhang L et al (1999) Analysis of human transcriptomes. Nature Genet 23:387–388
7. Kawasaki H, Toyoda M, Shinohara H et al (2001) Expression of survivin correlates with apoptosis, proliferation, and angiogenesis during human colorectal tumorigenesis. Cancer 91:2026–2032
8. Gianani R, Jarboe E, Orlicky D et al (2001) Expression of survivin in normal, hyperplastic, and neoplastic colonic mucosa. Hum Pathol 32:119–125
9. Okada E, Murai Y, Matsui K et al (2001) Survivin expression in tumor cell nuclei is predictive of a favorable prognosis in gastric cancer patients. Cancer Lett 163:109–116
10. Rodriguez JA, Span SW, Ferreira CGM et al (2002) CRM1-mediated nuclear export determines the cytoplasmic localization of the antiapoptotic protein survivin. Exp Cell Res 275:44–53
11. Nakagawa Y, Yamaguchi S, Hasegawa M et al (2004) Differential expression of survivin in bone marrow cells from patients with acute lymphocytic leukemia and chronic lymphocytic leukemia. Leuk Res (in press)
12. Duckett CS, Nava VE, Gedrich RW et al (1996) A conserved family of cellular genes related to the baculovirus IAP gene and encoding apoptosis inhibitors. EMBO J 15:2685–2694
13. Liston P, Roy N, Tamai K et al (1996) Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature 379:349–353

14. Rothe M, Pan MG, Henzei WJ et al (1995) The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell 83:1243–1152
15. Roy N, Dereraux QL, Takahashi R et al (1997) The cIAP-1 and cIAP-2 proteins are distinct inhibitors of specific caspases. EMBO J 16:6914–6925
16. Liston P, Fong WG, Korneluk RG (2003) The inhibitor of apoptosis: there is more to life than Bcl2. Oncogene 22:8568–8580
17. Tanaka K, Nagaoka S, Takemura T et al (2003) Incidence of apoptosis increases with age in colorectal cancer. Exp Gerontol 37:1469–1479
18. Hoffman WH, Biade S, Zilfou JT et al (2002) Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. J Biol Chem 277:3247–3257
19. Mirza A, McGuirk M, Hockenberry TN et al (2002) Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. Oncogene 21:2613–2622
20. Kirschner M, Pujol G, Radu A (2002) Oliginucleotide microarray data mining: search for age-dependent gene expression. Biochem Biophys Res Commun 298:772–778
21. Sharpless NE, DePinho RA (2004) Telomeres, stem cells, senescence, and cancer. J Clin Invest 113:160–168
22. Wang Q, Wang X, Evers BM (2003) Induction of cIAP-2 in human colon cancer cells through PKC delta/NF-kappa B. J Biol Chem 278:51091–51099
23. Deng Y, Lin Y, Wu X (2002) TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO. Genes Dev 16:33–45
24. Tillman DM, Izeradjene K, Szucs KS et al (2003) Rottlerin sensitizes colon carcinoma cells to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis via uncoupling of the mitochondria independent of protein kinase C. Cancer Res 63:5118–5125

Chemosensitivity profile of cancer cell lines and identification of genes determining chemosensitivity by an integrated bioinformatical approach using cDNA arrays

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Abstract

We have established a panel of 45 human cancer cell lines (JFCR-45) to explore genes that determine the chemosensitivity of these cell lines to anticancer drugs. JFCR-45 comprises cancer cell lines derived from tumors of three different organs: breast, liver, and stomach. The inclusion of cell lines derived from gastric and hepatic cancers is a major point of novelty of this study. We determined the concentration of 53 anticancer drugs that could induce 50% growth inhibition (GI_{50}) in each cell line. Cluster analysis using the GI_{50}s indicated that JFCR-45 could allow classification of the drugs based on their modes of action, which coincides with previous findings in NCI-60 and JFCR-39. We next investigated gene expression in JFCR-45 and developed an integrated database of chemosensitivity and gene expression in this panel of cell lines. We applied a correlation analysis between gene expression profiles and chemosensitivity profiles, which revealed many candidate genes related to the sensitivity of cancer cells to anticancer drugs. To identify genes that directly determine chemosensitivity, we further tested the ability of these candidate genes to alter sensitivity to anticancer drugs after individually overexpressing each gene in human fibrosarcoma HT1080. We observed that transfection of HT1080 cells with the *HSPA1A* and *JUN* genes actually enhanced the sensitivity to mitomycin C, suggesting the direct participation of these genes in mitomycin C sensitivity. These results suggest that an integrated bioinformatical approach using chemosensitivity and gene expression profiling is useful for the identification of genes determining chemosensitivity of cancer cells. [Mol Cancer Ther 2005;4(3):399–412]

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Introduction

Predicting the chemosensitivity of individual patients is important to improve the efficacy of cancer chemotherapy. An approach to this end is to understand the genes that determine the chemosensitivity of cancer cells. Many genes have been described that determine the sensitivity to multiple drugs, including drug transporters (1-3) and metabolizing enzymes (4-6). Genes determining the sensitivity to specific drugs have also been reported. For example, increased activities of γ-glutamyl hydrolase (7) and dihydrofolate reductase (8) are resistant factors for methotrexate; increased activities of thymidylate synthase (9), metallothionein (10), and cytidine deaminase (11) are resistant factors for 5-fluorouracil (5-FU), cisplatin, 1-β-D-arabinofuranosylcytosine, respectively; and increased activity of NQO1 (12) is a sensitive factor for mitomycin C (MMC). However, the chemosensitivity of cancer cells is not determined by a handful of genes. These genes are not sufficient to explain the variation of the chemosensitivity of cancer cells.

Recently, attempts were made to predict the chemosensitivity of cancers using genome-wide expression profile analyses, such as cDNA microarray and single nucleotide polymorphisms (13–18). For example, Scherf et al. (18) and Zembutsu et al. (15) reported the analysis of genes associated with sensitivity to anticancer drugs in a panel of human cancer cell lines and in human cancer xenografts, respectively. Tanaka et al. (17) presented prediction models of anticancer efficacy of eight drugs using real-time PCR expression analysis of 12 genes in cancer cell lines and clinical samples. We also analyzed chemosensitivity-related genes in 39 human cancer cell lines (JFCR-39; ref. 19) and validated the association of some of these genes to chemosensitivity using additional cancer cell lines (20). These genes can be used as markers to predict chemosensitivity. Moreover, some of these genes may directly determine the chemosensitivity of cancer cells.

In the present study, we established a new panel of 45 human cancer cell lines (JFCR-45) derived from tumors from three different organs: breast, liver, and stomach. Using JFCR-45, we attempted to analyze the heterogeneity of chemosensitivity in breast, liver, and stomach cancers. We assessed their sensitivity to 53 anticancer drugs and

developed a database of chemosensitivity. Then, we analyzed gene expression in 42 human cancer cell lines using cDNA arrays and stored them in the gene expression database. Using these two databases, we extracted genes whose expression was correlated to chemosensitivity. We further screened them to identify genes that could change the sensitivity to anticancer drugs using an *in vitro* gene transfection assay.

Materials and Methods

Cell Lines and Cell Cultures

We established a panel of JFCR-45 that included a portion of JFCR-39 and the 12 stomach cancer cell lines described previously (19, 20). They consist of the following cell lines: breast cancer cells HBC-4, BSY1, HBC-5, MCF-7, MDA-MB-231, KPL-3C (21), KPL-4, KPL-1, T-47D (22), HBC-9, ZR-75-1 (23), and HBC-8; liver cancer cells HepG2, Hep3B, Li-7, PLC/PRF/5, HuH7, HLE, HLF (24), HuH6 (25), RBE, SSP-25 (26), HuL-1 (27), and JHH-1 (28); and stomach cancer cells St-4, MKN1, MKN7, MKN28, MKN45, MKN74, GCIY, GT3TKB, HGC27, AZ521 (29), 4-1ST, NUGC-3, NUGC-3/5-FU, HSC-42, AGS, KWS-1, TGS-11, OKIBA, ISt-1, ALF, and AOTO. The AZ521 cell line was obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The 4-1ST, OKIBA, and AOTO cell lines were provided by Dr. Tokuji Kawaguchi (Department of Pathology, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan). All cell lines were cultured in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) with 5% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C under 5% CO_2.

Determination of the Sensitivity to Anticancer Drugs

Growth inhibition experiments were done to assess the chemosensitivity to anticancer drugs. Growth inhibition was measured by determining the changes in the amounts of total cellular protein after 48 hours of drug treatment using a sulforhodamine B assay. The GI_{50} values, which represent 50% growth inhibition concentration, were evaluated as described before (30, 31). Several experiments were done to determine the median GI_{50} value for each drug. Absolute values were then log transformed for further analysis.

Anticancer Drugs and Compounds

Actinomycin D, 5-FU, tamoxifen, cytarabine, radicicol, melphalan, 6-mercaptopurine, 6-thioguanine, and colchicine were purchased from Sigma (St. Louis, MO). The anticancer agents in clinical use were obtained from the company specified in parentheses, and those under development were kindly provided by the company specified as described below: aclarubicin and neocarzinostatin (Yamanouchi Pharmaceutical, Tokyo, Japan); oxaliplatin (Asahi Kasei, Tokyo, Japan), HCFU (Nihon Schering, Osaka, Japan); doxifluridine (Chugai Pharmaceutical, Tokyo, Japan); toremifene, bleomycin, and estramustine (Nippon Kayaku, Tokyo, Japan); daunorubicin and pirarubicin (Meiji, Tokyo, Japan); doxorubicin, epirubicin, MMC, vinorelbine, and L-asparaginase (Kyowa Hakko Kogyo, Tokyo, Japan); peplomycin, etoposide, NK109, and NK611 (Nippon Kayaku); vinblastine, vincrinstine, IFN-γ, and 4-hydroperoxycyclophosphamide (Shionogi, Tokyo, Japan); carboplatin and cisplatin (Bristol-Myers Squibb, New York, NY); mitoxantrone and methotrexate (Wyeth Lederie Japan, Tokyo, Japan); cladribine (Janssen Pharmaceutical, Titusville, NJ); amsacrine (Pfizer Pharmaceutical, formerly Warner Lambert, Plymouth, MI); camptothecin, irinotecan, and SN-38 (Yakult, Tokyo, Japan); paclitaxel (Bristol-Myers Squibb); docetaxel and topotecan (Aventis Pharma, Strasbourg, France); IFN-α (Sumitomo Pharmaceutical, Osaka, Japan); IFN-β (Daiichi Pharmaceutics, Tokyo, Japan); gemcitabine (Eli Lilly Japan, Kobe, Japan); E7010 and E7070 (Eisai, Tokyo, Japan); dolastatine 10 (Teikoku Hormone MFG, Tokyo, Japan); and TAS103 (Taiho Pharmaceutical Co., Tokyo, Japan).

Gene Expression Profiles by cDNA Array

Expression profiles of 3,537 genes in 42 human cancer cell lines were examined using Atlas Human 3.6 Array (BD Biosciences Clontech, Inc., Franklin Lakes, NJ) in duplicates. Experiments were done according to the manufacturer's instructions. Briefly, cell lines were harvested in log phase. Total RNA was extracted with TRIzol reagent (Invitrogen, Inc., Carlsbad, CA) and purified with Atlas Pure Total RNA Labeling System. Purified total RNAs were converted to ^{32}P-labeled cDNA probe by SuperScript II (Invitrogen). cDNA probe was hybridized to the Atlas Array overnight at 68°C and washed. Hybridized array was detected with PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). Scanned data were transformed to the numerical value with Atlas Image 2.0 software (BD Biosciences Clontech) and normalized by dividing by the value of 90% percentile of all genes in each experiment. Then, the intensities of the genes were defined by the average of intensities of duplicate results. The genes whose expression levels differed more than twice between the duplicates were eliminated from subsequent analysis. When the intensities of gene expression in both arrays were below the threshold value, they were given the value of threshold and were used for analysis. We determined the values of threshold of the normalized data as 30% of the value of 90% percentile. Then, $\log_2$ was calculated for each expression value.

Hierarchical Clustering

Hierarchical clustering using average linkage method was done by "Gene Spring" software (Silicon Genetics, Inc., Redwood, CA). Pearson correlation coefficients were used to determine the degree of similarity.

Correlation Analysis between Gene Expression and Chemosensitivity Profiles

The genes whose expressions were observed in >50% of all cell lines examined were selected for the correlation analysis. The degree of similarity between chemosensitivity and gene expression were calculated using the following Pearson correlation coefficient formula:

$$r = \frac{\sum_i (x_i - x_m)(y_i - y_m)}{\sqrt{\sum_i (x_i - x_m)^2 \sum_i (y_i - y_m)^2}}$$

where x_i is the log expression data of the gene x in cell i, y_i is the log sensitivity $|\log_{10}GI_{50}|$ of cell i to drug y, x_m is the mean of the log expression data of the gene x, and y_m is the mean sensitivity $|\log_{10}GI_{50}|$ of drug y. A significant correlation was defined as $P < 0.05$.

Screening of the Genes That Determine Chemosensitivity

Candidate genes related to the chemosensitivity were cloned into the vector pcDNA3.1/*myc*-His A (Invitrogen). Transfection of HT1080 cells with the plasmid DNA was carried out using LipofectAMINE Plus reagent (Invitrogen). The transfection efficiency was monitored by green fluorescent protein fluorescence. The fluorescence of green fluorescent protein was observed in >90% of the green fluorescent protein–transfected HT1080 (data not shown). Twenty-four hours after the transfection, proper concentrations of MMC were added and the cells were treated for 24 hours. Efficacies of anticancer drugs were determined by measuring the growth inhibition. Cell growth was measured by following [^{3}H]thymidine incorporation. [^{3}H]thymidine (0.067 MBq) was added to each well and incubated at 37°C for 45 minutes. Cells were washed with prewarmed PBS(−) and fixed with 10% TCA on ice for 2 hours. After fixing, cells were washed with 10% TCA and lysed with 0.1% SDS-0.2 N NaOH solution. After incubation at 37°C, the lysed mixture was neutralized with 0.25 mol/L acetic acid solution. [^{3}H]thymidine incorporated into the cells was determined using scintillation counter. All experiments, except for interleukin (IL)-18, were done four times.

Results

Sensitivity of JFCR-45 to 53 Anticancer Drugs

Sensitivity to 53 drugs was assessed as described in Materials and Methods. The known modes of actions and the value of $|\log_{10}GI_{50}|$ of 53 anticancer drugs in each of the 45 cell lines are summarized in Table 1. The $|\log_{10}GI_{50}|$ indicated here is the median value of multiple experiments. The chemosensitivity of the cell lines differed even among those derived from the same organ. These data were stored in a chemosensitivity database. Figure 1 shows the classification of the anticancer drugs by hierarchical clustering analysis based on chemosensitivity, $|\log_{10}GI_{50}|$, of JFCR-45. As shown, the 53 drugs were classified into several clusters, each consisting of drugs with similar modes of action [e.g., one cluster included topoisomerase (topo) I inhibitors, such as camptothecin, topotecan, and SN-38]. The second cluster comprised tubulin binders, including taxanes and *Vinca* alkaloids. 5-FU and its derivatives were also clustered into a single group. These results indicated that our system using JFCR-45 was able to classify the drugs based on their modes of action, which is in agreement with previous findings using NCI-60 and JFCR-39 (18, 19, 32).

Classification of 42 Human Cancer Cell Lines According to Gene Expression Profiles

Using a cDNA array, we examined the expression of 3,537 genes in 42 cell lines of JFCR-45. Based on these expression profiles, hierarchical clustering was done. In a few experiments, cell lines derived from the same organ were clustered into a group (Fig. 2). Breast cancer cell lines, except KPL-4, formed one cluster. Liver and stomach

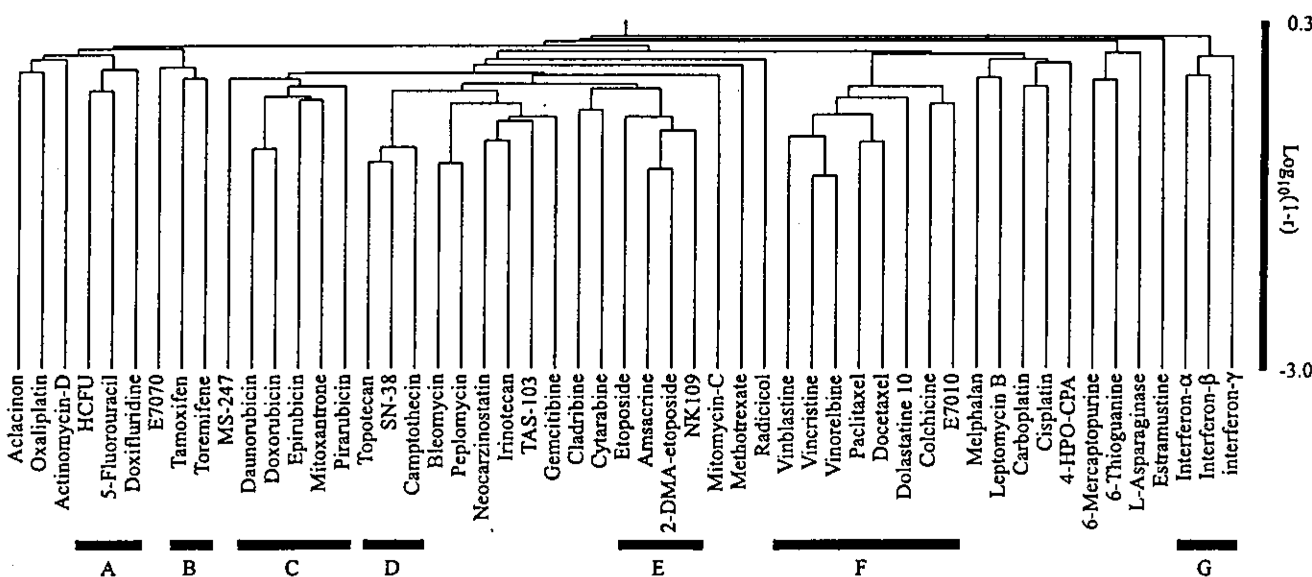

Figure 1. Hierarchical clustering of 53 anticancer drugs based on their activity on 45 human cancer cell lines. Hierarchical clustering method was "average linkage method" using Pearson correlation as distance. Fifty-three drugs were classified into several clusters, each consisting of drugs with similar modes of action or targets: (A) 5-FU derivatives, (B) estrogen receptor, (C) DNA synthesis/topo II inhibitors, (D) topo I inhibitors, (E) topo II inhibitors, (F) tubulin binders, and (G) IFN.

Table 1. The mode of actions and the median value of $|\log_{10}GI_{50}|$ of 53 anticancer drugs in each of the 45 cell lines

Drug name	Target/ mode of action	Breast											
		HBC-4	BSY1	HBC-5	MCF-7	MDA-MB-231	KPL-3C	KPL-4	KPL-1	T-47D	HBC-9	ZR-75-1	HBC-8
Aclarubicin	DNA/RNA synthesis	7.04	8.69	7.92	7.86	7.83	7.11	7.63	7.95	7.39	7.08	8.03	7.93
Oxaliplatin	DNA cross-linker	5.79	5.75	5.40	5.69	4.75	5.04	5.20	4.78	5.17	4.10	5.08	6.17
Actinomycin D	RNA synthesis	9.20	9.10	8.85	9.45	8.71	8.90	9.05	9.04	8.89	8.24	8.98	9.60
HCFU	Pyrimidine	4.36	5.17	4.44	5.13	4.57	4.65	5.55	4.41	4.97	4.22	4.68	4.84
5-FU	Pyrimidine	4.43	4.87	4.40	5.12	4.18	4.00	5.23	4.00	4.13	4.00	4.70	5.11
Doxifluridine	Pyrimidine	4.00	4.42	4.00	4.00	4.00	4.00	4.09	4.00	4.00	4.00	4.14	4.19
E7070	Cell cycle inhibitor	4.50	6.20	4.22	4.50	4.35	4.94	5.01	4.74	4.69	4.00	4.38	4.98
Tamoxifen	Estrogen receptor	4.95	5.42	5.01	5.04	4.90	5.14	5.49	4.93	5.31	4.90	4.95	5.53
Toremifene	Estrogen receptor	4.81	5.12	4.87	4.96	4.85	4.93	5.13	4.88	5.17	4.89	4.88	4.86
MS-247	DNA synthesis	6.08	6.79	5.32	6.78	5.98	6.09	6.16	5.86	6.63	6.42	6.88	6.71
Daunorubicin	DNA synthesis/topo II	6.96	7.34	6.82	7.68	6.83	6.77	7.25	6.84	7.41	6.92	7.39	7.97
Doxorubicin	DNA synthesis/topo II	7.13	7.26	6.85	7.58	6.66	6.74	7.38	6.76	7.36	6.94	7.12	7.85
Epirubicin	DNA synthesis/topo II	6.08	6.90	6.59	7.08	6.42	6.50	7.03	6.83	7.26	6.73	7.90	7.19
Mitoxantrone	DNA synthesis	6.28	7.12	6.00	8.06	6.50	6.40	6.83	6.38	7.11	6.96	8.02	7.44
Pirarubicin	DNA synthesis/topo II	8.97	9.00	8.34	9.00	8.47	8.62	9.00	8.39	9.00	8.22	9.00	9.00
Topotecan	Topo I	5.84	6.57	5.10	8.00	5.55	6.37	6.71	5.90	7.51	6.18	7.20	7.61
SN-38	Topo I	7.98	7.52	5.56	8.56	6.12	6.75	7.40	6.60	8.25	6.13	7.92	7.75
Camptothecin	Topo I	5.92	6.57	6.04	7.63	5.86	6.67	6.60	6.70	7.12	5.80	7.21	6.92
Bleomycin	DNA synthesis	4.81	4.89	4.00	4.48	4.00	4.00	5.59	4.00	5.46	4.46	4.22	4.37
Peplomycin	DNA synthesis	4.90	5.84	4.00	5.22	4.27	4.61	6.29	4.08	5.37	4.52	4.72	5.25
Neocarzinostatin	DNA synthesis	7.35	8.00	6.03	8.17	6.55	6.42	7.61	6.18	7.26	7.06	7.26	8.10
Irinotecan	Topo I	4.86	5.09	4.00	5.46	4.28	4.30	4.91	4.11	5.21	4.15	4.47	5.24
TAS103	Topo	6.81	7.22	6.37	7.66	6.57	6.45	7.20	6.17	7.25	6.16	7.13	7.60
Gemcitabine	Pyrimidine	6.74	5.62	4.00	8.00	5.20	4.00	7.25	4.00	7.18	5.15	4.71	5.75
Cladribine	Pyrimidine	4.00	4.00	4.00	5.41	4.05	4.60	4.73	4.00	4.83	4.23	4.00	4.68
Cytarabine	Pyrimidine	4.00	4.00	4.00	6.40	4.00	4.00	5.02	4.00	4.00	4.00	4.00	4.54
Etoposide	Topo II	4.88	5.48	4.39	6.15	4.66	4.00	5.42	4.68	5.93	4.48	5.11	4.72
Amsacrine	Topo II	5.20	5.78	5.29	6.56	5.25	4.89	5.69	4.93	5.97	5.14	6.56	5.70
2-Dimethylaminoetoposide	Topo II	4.67	4.82	4.02	6.02	4.48	4.00	5.03	4.00	5.05	4.89	5.74	4.71
NK109	Topo II	5.69	5.88	5.27	6.37	6.04	5.49	6.31	5.56	6.30	5.57	6.08	5.81
MMC	DNA alkylator	5.90	6.68	5.68	6.99	5.14	5.46	6.40	5.50	5.42	5.49	5.74	6.69
Methotrexate	DHFR	7.11	5.19	4.00	7.53	4.00	4.00	7.53	5.25	4.00	4.00	4.00	4.00
Radicicol	HSP90/Tyr kinase	5.55	5.80	5.17	7.28	6.55	5.19	6.13	5.28	7.43	5.39	6.18	6.62
Vinblastine	Tubulin	9.22	9.76	9.22	9.68	8.67	9.17	9.77	9.13	9.15	6.00	7.58	7.99
Vincristine	Tubulin	8.77	9.72	9.29	9.42	8.67	9.12	9.57	9.31	9.22	6.00	8.41	6.20
Vinorelbine	Tubulin	8.45	9.23	8.51	8.85	8.23	8.33	9.35	8.93	8.41	6.00	8.16	6.00
Paclitaxel	Tubulin	7.30	8.43	7.94	7.72	7.37	7.38	8.20	7.53	7.90	6.00	7.05	6.59
Docetaxel	Tubulin	8.41	8.98	8.23	8.52	7.88	8.18	8.82	8.19	8.56	6.00	7.15	8.28
Dolastatine 10	Tubulin	9.15	10.83	11.19	10.26	9.07	10.02	10.74	9.44	9.95	8.00	9.46	8.67
Colchicine	Tubulin	6.06	8.68	6.33	6.48	7.24	7.58	8.48	7.89	6.64	5.00	7.84	6.59
E7010	Tubulin	4.37	6.56	4.00	6.14	5.07	5.38	6.69	5.71	6.29	5.50	6.04	4.72
Melphalan	DNA cross-linker	4.20	4.92	4.42	5.09	4.33	4.67	4.04	4.66	4.38	4.08	4.45	4.57
Leptomycin B	Cell cycle inhibitor	9.35	9.64	9.33	9.44	8.91	9.59	9.47	9.63	9.26	8.96	9.78	9.74
Carboplatin	DNA cross-linker	4.00	4.34	4.12	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Cisplatin	DNA cross-linker	4.90	5.69	5.65	5.09	4.56	4.72	5.52	4.63	4.56	5.35	4.71	5.39
4-Hydroperoxycyclo-phosphamide	DNA alkylator	4.78	4.85	5.41	5.58	4.68	4.78	4.54	4.74	4.86	5.18	4.76	4.78
6-Mercaptopurine	Purine	5.41	4.73	4.15	5.88	5.17	5.11	4.50	5.02	6.00	4.27	4.05	4.50
6-Thioguanine	Purine	4.59	5.85	5.40	5.86	5.80	5.92	5.55	5.91	5.81	4.53	5.21	5.66
L-Asparaginase	Protein synthesis	6.55	6.63	4.00	6.43	6.01	6.03	7.20	6.18	6.10	5.49	6.07	6.36
Estramustine	Estradiol	4.09	4.51	4.00	4.00	4.66	4.85	4.56	4.31	4.17	4.74	4.00	4.73
IFN-α	Biological response	4.00	7.71	4.00	4.00	4.23	4.00	4.00	4.00	4.00	4.00	4.00	5.02
IFN-β	Biological response	4.00	8.00	4.00	4.00	6.40	4.23	7.08	4.00	4.00	4.00	4.00	4.56
IFN-γ	Biological response	7.69	7.93	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00

(Continued on the following page)

Table 1. The mode of actions and the median value of $|\log_{10}GI_{50}|$ of 53 anticancer drugs in each of the 45 cell lines (Cont'd)

Drug name	Target/ mode of action	Liver											
		HepG2	Hep3B	Li-7	PLC/ PRF/5	HuH7	HLE	HLF	HuH6	RBE	SSP-25	HuL-1	JHH-1
Aclarubicin	DNA/RNA synthesis	8.13	7.77	7.39	7.68	8.29	7.49	7.86	7.70	7.87	7.39	7.97	8.23
Oxaliplatin	DNA cross-linker	7.07	5.39	5.78	5.61	6.44	4.90	4.75	5.60	5.19	4.58	6.04	6.01
Actinomycin D	RNA synthesis	9.03	8.61	8.24	8.04	8.99	8.13	8.45	8.75	8.25	8.47	8.78	9.00
HCFU	Pyrimidine	5.28	4.80	4.79	4.56	4.99	4.67	4.70	4.50	4.92	4.69	4.87	4.63
5-FU	Pyrimidine	5.27	4.20	4.26	4.21	5.08	4.00	4.19	4.00	4.60	4.00	5.29	4.72
Doxifluridine	Pyrimidine	4.49	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.04	4.00
E7070	Cell cycle inhibitor	5.47	4.99	4.77	4.44	5.36	4.61	4.43	4.74	5.09	4.29	4.29	4.87
Tamoxifen	Estrogen receptor	5.45	5.30	5.23	4.79	5.09	5.02	4.97	5.38	4.90	5.11	4.87	4.97
Toremifene	Estrogen receptor	5.06	4.97	4.92	4.82	4.99	5.09	4.91	4.95	4.92	5.00	4.80	5.10
MS-247	DNA synthesis	6.33	5.84	6.35	5.23	6.02	6.58	6.42	5.82	5.66	6.37	5.67	6.82
Daunorubicin	DNA synthesis/topo II	7.48	7.10	6.83	6.39	7.29	7.55	7.49	6.98	7.18	6.73	7.08	7.51
Doxorubicin	DNA synthesis/topo II	7.29	6.77	6.88	5.83	7.04	7.39	7.25	6.87	6.89	6.68	6.89	7.31
Epirubicin	DNA synthesis/topo II	7.33	6.86	6.87	6.29	7.31	7.21	7.25	6.91	6.84	6.73	6.74	7.03
Mitoxantrone	DNA synthesis	7.95	6.51	7.88	6.51	6.76	7.60	7.67	6.71	7.37	7.59	6.11	7.15
Pirarubicin	DNA synthesis/topo II	9.00	8.58	9.00	8.26	9.00	9.00	9.00	8.59	8.98	9.00	8.95	9.00
Topotecan	Topo I	7.93	5.81	7.70	5.64	6.07	7.73	7.73	5.72	6.83	6.74	5.30	6.99
SN-38	Topo I	8.43	6.37	8.21	6.03	6.75	8.28	8.31	5.91	7.05	7.47	5.69	7.74
Camptothecin	Topo I	7.44	6.19	7.48	5.86	6.35	7.42	7.53	6.10	6.69	6.79	6.16	6.92
Bleomycin	DNA synthesis	6.02	4.38	5.66	4.00	4.85	6.04	6.59	4.15	4.73	4.97	5.10	4.94
Peplomycin	DNA synthesis	6.73	4.72	6.40	4.45	5.46	5.86	6.56	4.01	5.12	5.83	5.35	5.34
Neocarzinostatin	DNA synthesis	8.22	6.72	7.81	6.34	6.92	7.60	7.80	6.57	7.27	7.53	6.67	7.09
Irinotecan	Topo I	5.18	4.36	5.61	4.00	4.33	5.25	5.13	4.11	4.37	4.64	4.05	4.78
TAS103	Topo	7.56	6.57	7.68	6.64	6.95	7.81	7.87	6.55	7.32	6.89	6.95	6.94
Gemcitabine	Pyrimidine	8.00	4.63	8.00	4.00	6.16	7.83	8.00	4.19	6.56	7.24	5.60	5.85
Cladribine	Pyrimidine	6.30	4.00	4.86	4.00	4.00	5.85	5.45	4.00	4.86	5.30	4.00	4.00
Cytarabine	Pyrimidine	6.22	4.00	4.00	4.00	4.00	5.22	5.41	4.00	4.00	4.00	4.00	4.00
Etoposide	Topo II	5.62	4.86	5.56	4.60	4.92	5.80	5.70	5.05	4.85	5.35	5.35	5.09
Amsacrine	Topo II	6.41	5.56	6.66	5.47	5.77	6.58	6.61	5.43	5.90	5.98	5.71	5.46
2-Dimethylaminoetoposide	Topo II	5.56	4.66	5.70	4.54	4.73	5.75	5.84	4.57	5.20	5.54	4.75	4.66
NK109	Topo II	6.56	5.96	6.72	5.85	6.05	6.83	6.77	5.84	6.24	6.39	5.92	6.09
MMC	DNA alkylator	6.56	5.04	7.09	5.63	5.73	6.15	6.31	5.38	5.32	6.20	5.50	5.99
Methotrexate	DHFR	7.47	4.00	6.11	4.00	6.12	6.64	6.83	4.00	6.71	4.06	4.00	5.13
Radicicol	HSP90/Tyr kinase	7.87	7.08	6.43	6.16	6.46	6.63	6.83	6.03	5.52	5.61	5.94	5.68
Vinblastine	Tubulin	8.18	6.50	9.30	7.73	9.35	9.73	9.20	7.22	6.00	9.51	9.11	9.66
Vincristine	Tubulin	7.93	6.00	7.70	6.00	8.52	8.76	8.40	6.00	6.00	8.27	8.38	9.11
Vinorelbine	Tubulin	7.98	6.00	8.15	6.00	8.43	8.75	8.28	7.05	6.00	8.51	8.65	9.21
Paclitaxel	Tubulin	7.35	6.84	7.41	6.48	7.44	7.50	7.27	6.00	6.73	7.80	8.22	7.94
Docetaxel	Tubulin	8.08	7.11	7.83	6.80	8.23	8.09	8.08	6.00	6.14	8.50	8.54	8.50
Dolastatine 10	Tubulin	10.42	8.94	10.71	9.50	10.12	10.19	9.94	8.60	8.00	10.30	9.68	10.61
Colchicine	Tubulin	7.16	5.40	7.25	6.43	7.62	7.77	7.39	5.54	5.00	7.50	7.45	8.17
E7010	Tubulin	6.28	4.62	6.38	6.23	6.35	6.47	6.35	4.79	4.00	6.50	6.44	6.50
Melphalan	DNA cross-linker	4.76	4.47	4.62	4.00	4.44	4.59	4.81	4.03	4.39	4.40	4.84	4.86
Leptomycin B	Cell cycle inhibitor	9.67	9.32	9.44	9.19	9.10	9.31	9.37	9.00	9.29	9.51	9.54	9.66
Carboplatin	DNA cross-linker	4.18	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.53
Cisplatin	DNA cross-linker	5.53	5.32	5.51	4.75	5.63	5.36	5.45	5.26	4.73	4.94	5.41	5.86
4-Hydroperoxycyclo-phosphamide	DNA alkylator	4.92	4.74	4.88	4.65	4.84	4.87	5.04	4.82	4.69	4.90	4.76	5.30
6-Mercaptopurine	Purine	5.01	4.10	5.12	4.42	4.00	4.17	4.49	4.90	5.29	4.58	4.82	5.10
6-Thioguanine	Purine	5.08	4.57	5.23	5.37	4.70	4.22	5.14	6.04	5.76	5.18	5.92	6.14
L-Asparaginase	Protein synthesis	6.40	4.78	8.00	6.49	4.00	6.91	6.63	4.00	6.35	8.00	6.61	4.42
Estramustine	Estradiol	4.00	4.00	4.27	4.24	4.05	4.37	4.03	4.10	4.14	4.18	4.09	4.14
IFN-α	Biological response	4.00	4.00	4.20	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
IFN-β	Biological response	4.00	4.00	7.15	6.17	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
IFN-γ	Biological response	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	7.93

(Continued on the following page)

Table 1. The mode of actions and the median value of $|\log_{10}GI_{50}|$ of 53 anticancer drugs in each of the 45 cell lines (Cont'd)

Drug name	Target/ mode of action	Stomach											
		St-4	MKN1	MKN7	MKN28	MKN45	MKN74	GCIY	GT3 TKB	HGC27	AZ521	4-1ST	NUGC -3
Aclarubicin	DNA/RNA synthesis	7.88	8.09	7.73	7.25	8.59	7.43	8.00	7.86	7.13	8.49	7.96	9.04
Oxaliplatin	DNA cross-linker	4.75	5.04	4.42	4.58	6.84	4.93	5.71	5.31	5.10	6.16	5.17	6.18
Actinomycin D	RNA synthesis	7.99	8.74	8.77	9.02	9.39	9.20	8.24	9.12	8.76	9.55	8.80	8.85
HCFU	Pyrimidine	4.17	4.70	4.82	4.77	5.56	4.86	4.77	5.09	4.74	5.21	4.84	4.74
5-FU	Pyrimidine	4.35	4.40	4.26	4.27	5.46	4.22	4.60	5.09	4.34	5.12	4.04	4.67
Doxifluridine	Pyrimidine	4.00	4.00	4.01	4.00	4.20	4.00	4.00	4.00	4.00	4.00	4.00	4.02
E7070	Cell cycle inhibitor	4.43	6.03	4.90	5.48	4.55	5.20	5.04	4.82	5.69	6.02	4.88	5.75
Tamoxifen	Estrogen receptor	4.95	4.89	5.44	5.23	5.13	5.67	4.92	5.19	5.25	5.11	4.87	5.06
Toremifene	Estrogen receptor	4.81	4.92	4.90	4.82	4.93	5.23	4.85	4.92	5.07	5.09	4.87	4.96
MS-247	DNA synthesis	5.66	5.72	6.27	5.59	7.32	6.62	5.71	6.88	6.76	7.58	7.09	6.62
Daunorubicin	DNA synthesis/topo II	6.60	7.30	6.98	7.03	7.66	6.88	6.79	7.55	7.17	7.98	7.18	7.74
Doxorubicin	DNA synthesis/topo II	6.39	7.45	6.79	6.71	7.32	6.70	6.39	7.14	6.86	7.87	6.68	7.66
Epirubicin	DNA synthesis/topo II	7.21	7.53	6.85	6.60	7.35	6.60	6.53	7.10	6.71	8.00	7.02	7.68
Mitoxantrone	DNA synthesis	6.82	7.52	6.57	6.52	7.79	6.68	6.87	7.82	6.83	8.79	7.38	7.59
Pirarubicin	DNA synthesis/topo II	8.31	8.97	8.55	8.57	9.00	8.53	8.81	9.00	8.56	9.00	8.86	9.00
Topotecan	Topo I	7.21	6.27	5.54	5.81	8.00	5.62	6.61	7.83	5.64	7.74	8.00	7.68
SN-38	Topo I	6.83	6.63	6.16	6.16	8.71	6.17	6.89	8.49	6.04	8.49	8.78	8.28
Camptothecin	Topo I	7.13	6.39	5.82	5.50	7.99	5.62	6.81	7.53	5.49	7.61	7.75	7.73
Bleomycin	DNA synthesis	4.00	4.61	4.03	4.00	4.54	4.22	4.00	6.21	4.22	7.18	6.03	4.75
Peplomycin	DNA synthesis	4.00	4.80	4.56	4.09	5.18	4.82	4.39	5.96	4.68	7.32	6.16	4.92
Neocarzinostatin	DNA synthesis	6.17	6.92	6.58	6.47	8.38	7.19	6.95	7.74	6.92	8.58	7.59	8.00
Irinotecan	Topo I	4.00	4.41	4.29	4.02	5.41	4.26	4.44	5.24	4.00	5.58	5.39	5.41
TAS103	Topo	5.75	7.54	6.50	6.56	7.50	6.43	6.96	7.97	6.81	8.51	7.40	7.76
Gemcitabine	Pyrimidine	4.09	6.17	4.45	4.00	8.00	5.38	6.18	7.57	4.00	8.00	6.68	7.70
Cladribine	Pyrimidine	4.11	4.51	4.00	4.00	6.88	4.00	4.00	5.56	4.00	6.52	4.43	5.42
Cytarabine	Pyrimidine	4.00	4.00	4.00	4.00	6.41	4.00	4.00	6.38	4.00	6.56	5.68	5.76
Etoposide	Topo II	4.67	5.79	4.59	4.51	5.43	4.22	4.96	5.55	5.22	6.23	5.80	5.90
Amsacrine	Topo II	5.30	6.24	5.01	4.96	6.43	5.34	5.75	6.55	5.50	6.98	6.44	6.68
2-Dimethylaminoetoposide	Topo II	4.70	5.63	4.57	4.37	5.67	4.29	4.97	5.75	5.05	5.99	5.72	6.14
NK109	Topo II	6.02	6.66	5.88	5.76	6.51	5.62	6.58	6.92	6.29	6.90	6.66	6.78
MMC	DNA alkylator	4.93	5.00	5.33	5.10	7.09	5.56	5.75	6.17	5.74	6.45	5.99	7.28
Methotrexate	DHFR	7.27	7.04	4.00	4.00	7.15	4.00	7.06	7.04	7.49	7.37	7.33	7.32
Radicicol	HSP90/Tyr kinase	6.96	6.59	5.88	5.66	6.44	6.15	6.40	6.89	6.00	6.63	7.42	6.08
Vinblastine	Tubulin	6.17	9.62	7.60	9.64	9.04	9.25	8.58	9.88	9.37	9.76	9.85	9.53
Vincristine	Tubulin	6.37	9.36	8.60	8.58	8.42	9.13	8.12	9.30	8.91	9.36	9.61	8.94
Vinorelbine	Tubulin	6.00	8.60	8.51	8.59	8.42	8.53	7.96	9.22	8.37	8.89	8.83	8.87
Paclitaxel	Tubulin	6.87	7.68	7.50	7.48	7.89	7.16	6.77	8.15	7.70	8.09	7.86	8.15
Docetaxel	Tubulin	7.05	8.06	8.10	8.32	8.47	7.71	6.93	8.85	8.19	9.08	8.50	8.51
Dolastatine 10	Tubulin	9.41	9.56	10.27	10.18	9.75	10.29	10.51	10.60	9.23	10.42	10.53	10.35
Colchicine	Tubulin	7.76	7.99	7.28	7.90	7.75	7.51	7.34	7.78	7.65	7.70	8.69	7.53
E7010	Tubulin	6.06	6.21	6.26	6.35	6.02	6.15	6.39	6.69	6.08	6.69	6.67	6.40
Melphalan	DNA cross-linker	4.47	4.70	4.19	4.00	4.79	4.36	4.55	4.59	4.72	5.18	5.26	5.32
Leptomycin B	Cell cycle inhibitor	9.45	9.44	9.36	9.25	9.45	9.50	9.15	9.48	9.57	9.81	9.69	9.54
Carboplatin	DNA cross-linker	4.00	4.25	4.00	4.00	4.00	4.00	4.00	4.14	4.00	4.00	4.24	4.97
Cisplatin	DNA cross-linker	4.78	5.61	5.07	4.66	5.47	4.48	5.35	5.46	4.75	5.12	5.60	6.52
4-Hydroperoxycyclo-phosphamide	DNA alkylator	4.37	4.77	4.81	4.92	5.13	4.76	4.85	4.81	4.80	5.30	5.25	5.33
6-Mercaptopurine	Purine	4.21	5.58	4.67	5.21	5.39	5.86	4.45	5.21	5.47	5.54	5.97	5.03
6-Thioguanine	Purine	6.18	6.13	5.49	5.46	5.66	5.74	5.83	5.57	5.83	6.21	6.53	5.36
L-Asparaginase	Protein synthesis	6.32	6.41	6.64	6.54	6.65	6.91	5.30	6.70	5.78	6.72	6.34	6.51
Estramustine	Estradiol	4.21	4.26	4.00	4.00	4.20	4.72	4.29	4.45	4.34	4.20	5.11	4.48
IFN-α	Biological response	4.00	4.00	4.00	4.00	4.00	4.51	4.00	4.00	4.00	4.00	4.00	4.00
IFN-β	Biological response	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
IFN-γ	Biological response	4.00	4.07	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00

(Continued on the following page)

Table 1. The mode of actions and the median value of $|\log_{10}GI_{50}|$ of 53 anticancer drugs in each of the 45 cell lines (Cont'd)

Drug name	Target/ mode of action	Stomach								
		NUGC -3/5-FU	HSC-42	AGS	KWS-1	TGS-11	OKIBA	ISt-1	ALF	AOTO
Aclarubicin	DNA/RNA synthesis	7.51	8.21	8.27	7.96	8.31	7.20	7.19	8.54	7.57
Oxaliplatin	DNA cross-linker	5.23	5.98	5.58	6.26	7.02	5.85	5.14	5.46	4.78
Actinomycin D	RNA synthesis	8.56	9.32	8.99	9.22	9.55	9.35	8.77	9.39	8.88
HCFU	Pyrimidine	4.36	4.89	5.00	4.71	4.27	5.10	4.15	4.23	4.44
5-FU	Pyrimidine	4.00	4.40	5.02	4.50	4.06	6.38	4.00	4.42	4.09
Doxifluridine	Pyrimidine	4.00	4.00	4.26	4.00	4.00	4.18	4.00	4.00	4.00
E7070	Cell cycle inhibitor	4.39	4.81	4.46	5.25	4.96	6.05	4.83	6.69	4.97
Tamoxifen	Estrogen receptor	4.86	4.89	5.59	4.93	5.20	5.58	4.93	5.43	5.13
Toremifene	Estrogen receptor	4.85	4.88	5.00	4.93	5.07	5.58	4.88	5.50	5.24
MS-247	DNA synthesis	5.64	7.11	7.01	6.74	6.67	6.20	5.70	5.70	5.63
Daunorubicin	DNA synthesis/topo II	6.85	7.57	7.42	6.99	6.93	7.59	6.37	6.94	6.80
Doxorubicin	DNA synthesis/topo II	6.47	7.33	7.53	6.91	6.90	8.00	6.01	6.34	6.54
Epirubicin	DNA synthesis/topo II	6.13	7.61	8.02	7.12	6.91	7.12	5.99	7.00	6.51
Mitoxantrone	DNA synthesis	6.18	7.70	7.75	7.21	6.74	8.56	5.76	6.14	6.37
Pirarubicin	DNA synthesis/topo II	8.65	9.00	9.00	8.99	8.58	8.81	8.16	8.68	8.57
Topotecan	Topo I	5.82	8.00	7.54	6.07	6.39	6.10	6.70	6.90	6.85
SN-38	Topo I	6.31	8.61	8.70	6.81	6.66	7.07	7.29	7.46	7.28
Camptothecin	Topo I	6.00	7.76	7.23	6.36	6.64	6.81	6.43	6.72	6.96
Bleomycin	DNA synthesis	4.00	5.66	5.19	4.00	4.00	5.55	4.00	4.81	4.58
Peplomycin	DNA synthesis	4.05	6.00	5.82	4.65	4.08	5.92	4.23	5.04	4.78
Neocarzinostatin	DNA synthesis	6.54	7.89	7.78	6.84	6.60	7.05	6.54	6.74	7.24
Irinotecan	Topo I	4.06	5.48	5.50	4.25	4.58	4.64	4.42	4.56	4.71
TAS103	Topo	6.45	7.66	7.98	6.94	6.45	6.89	6.24	6.45	7.74
Gemcitabine	Pyrimidine	4.00	6.77	6.65	4.00	4.06	6.76	4.86	5.82	7.27
Cladribine	Pyrimidine	4.00	4.46	4.56	4.00	4.00	6.41	4.00	4.00	4.24
Cytarabine	Pyrimidine	4.00	5.96	5.60	4.00	4.00	7.32	4.00	5.58	4.00
Etoposide	Topo II	4.72	6.11	6.13	5.13	4.41	8.00	4.73	5.10	5.79
Amsacrine	Topo II	4.91	6.53	6.30	5.71	4.99	6.60	5.06	5.57	6.29
2-Dimethylaminoetoposide	Topo II	4.12	5.94	5.17	4.78	4.36	6.25	4.57	4.80	5.75
NK109	Topo II	5.95	6.70	6.47	6.63	5.68	7.27	5.79	5.91	6.86
MMC	DNA alkylator	5.58	6.27	6.23	5.86	5.75	5.56	5.32	6.03	5.86
Methotrexate	DHFR	4.00	7.38	7.53	7.81	4.00	6.66	4.00	4.00	4.00
Radicicol	HSP90/Tyr kinase	5.71	7.63	7.07	6.78	6.80	6.80	5.76	6.38	6.74
Vinblastine	Tubulin	8.20	9.85	9.69	9.80	9.28	9.71	7.04	8.12	8.33
Vincristine	Tubulin	7.12	9.70	9.24	9.35	9.41	10.00	6.00	7.46	8.20
Vinorelbine	Tubulin	7.13	9.32	8.86	8.87	8.58	9.79	6.00	8.25	8.64
Paclitaxel	Tubulin	6.49	8.07	7.74	7.96	8.03	8.29	6.52	7.79	7.52
Docetaxel	Tubulin	7.21	8.86	8.63	8.47	8.49	8.46	7.33	8.68	8.27
Dolastatine 10	Tubulin	8.89	10.69	10.50	10.44	10.13	11.86	8.69	10.09	10.26
Colchicine	Tubulin	5.98	8.59	8.19	8.34	7.45	8.74	6.05	7.56	7.84
E7010	Tubulin	4.37	6.69	6.47	6.64	6.27	6.88	4.51	5.50	5.36
Melphalan	DNA cross-linker	4.56	5.34	5.27	4.00	5.00	4.62	4.15	4.73	4.67
Leptomycin B	Cell cycle inhibitor	9.12	9.64	9.53	8.66	9.16	9.71	8.82	9.76	9.49
Carboplatin	DNA cross-linker	4.00	4.36	4.16	4.00	4.00	4.62	4.00	4.00	4.26
Cisplatin	DNA cross-linker	4.80	5.64	5.55	4.74	5.71	5.79	5.43	5.57	5.51
4-Hydroperoxycyclo-phosphamide	DNA alkylator	4.78	5.50	5.44	4.70	4.68	5.17	4.61	4.66	4.78
6-Mercaptopurine	Purine	5.19	5.90	5.86	4.95	4.55	4.85	4.00	4.00	4.00
6-Thioguanine	Purine	5.50	6.54	5.61	5.79	5.92	6.10	4.00	4.46	4.36
L-Asparaginase	Protein synthesis	6.63	6.47	6.93	6.51	4.94	6.52	4.00	5.56	4.00
Estramustine	Estradiol	4.08	5.03	4.74	4.42	4.02	4.79	4.59	4.95	4.76
IFN-α	Biological response	4.00	4.00	4.00	4.00	4.51	4.20	4.62	4.62	4.16
IFN-β	Biological response	4.00	4.00	4.00	4.00	6.02	4.93	4.77	6.28	6.54
IFN-γ	Biological response	4.00	4.00	4.00	4.00	4.00	4.00	4.00	5.06	4.00