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Advances in Brief

Identification of *cIAP1* As a Candidate Target Gene within an Amplicon at 11q22 in Esophageal Squamous Cell Carcinomas[1]

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Abstract

Amplification of chromosomal DNA is thought to be one of the mechanisms that activate cancer-related genes in tumors. In a recent study, we identified high copy-number amplification at 11q21-q23 in cell lines derived from esophageal squamous cell carcinomas (ESCs) using comparative genomic hybridization. Because 11q21-q23 amplification has been reported in tumors of various other types as well, gene(s) associated with tumor progression may lie within this chromosomal region. To identify the most likely target(s) for amplification at 11q21-q23, we determined the extent of the amplicon by fluorescence *in situ* hybridization and then analyzed ESC cell lines for expression levels of 11 known genes and one uncharacterized transcript present within the 1.8-Mb commonly amplified region. Only *cIAP1*, a member of the IAP (antiapoptotic) gene family, was consistently overexpressed in cell lines that showed amplification. Additionally, the cIAP1 protein was overexpressed in the primary tumors from which those cell lines had been established. The ESC cell lines with *cIAP1* amplification were resistant to apoptosis induced by chemotherapeutic reagents. An increase in *cIAP1* copy number was also detected in 4 of 42 (9.5%) primary ESC tumors that were not related to the cell lines examined. Because inhibition of apoptosis seems to be an important feature of carcinogenesis, *cIAP1* is likely to be a target for 11q21–23 amplification and may be involved in the progression of ESC, as well as other malignancies.

Introduction

Gene amplification, a phenomenon characteristic of numerous human cancers, appears to be a key mechanism whereby a cancer cell activates molecules that confer a selective advantage (1). Many oncogenes or other cancer-related genes have been identified in amplified chromosomal regions. Therefore, characterization of high-copy-number amplifications and the genes affected by them represents an excellent route toward identification of novel genes involved in growth control and carcinogenesis.

Oncogenes, such as *EGFR* (7p12), *MYC* (8q24), and *CCND1* (11q13), have already been identified as amplification targets associated with development, progression, or metastasis of ESCs[3] (2–4). Additional chromosomal amplicons have been identified in ESCs by means of CGH analysis (5–7), although the target genes for those amplicons remain largely unknown. If we are to achieve novel insights into the pathogenesis of ESC and design improved protocols for its clinical management, candidate genes within these amplicons must be explored and characterized.

Our CGH analyses have revealed amplification at 11q21-q23 in ESC cell lines (7) and in primary ESC tumors (5). This amplicon is of particular interest because, although infrequent, amplification within this region has been implicated in various other malignancies, including medulloblastoma (8), renal-cell carcinoma (9, 10), glioblastoma (11), and gastric cancer (12). Therefore, this region may harbor gene(s) that, when activated by the amplification mechanism, are involved in carcinogenesis regardless of the type of tissue involved, although no specific gene has been proposed as a target.

In the study reported here, we performed a detailed molecular characterization of the 11q21-q23 amplicon using ESC cell lines, with the goal of identifying gene(s) involved in tumorigenesis. By determining levels of amplification and expression of 12 transcribed elements located in this amplicon, we successfully identified one candidate, *cIAP1*, a gene that encodes an antiapoptotic molecule. Cell lines that overexpressed this gene were resistant to the apoptosis induced by chemotherapeutic reagents. Because any mechanism that aberrantly prolongs the life span of a cell may contribute to carcinogenesis, our findings indicate that *cIAP1* is a potential target for 11q21–23 amplification on the basis of both position and function.

Materials and Methods

ESC Cell Lines and Tumors. All 31 human ESC cell lines of the KYSE series had been established from surgically resected tumors (13) and maintained in RPMI 1640 supplemented with 10% FCS. Data from CGH analyses involving 29 of those lines have been reported elsewhere (7).

ESC tumor samples from 42 patients were provided by the Kyoto University Hospital, with written consent from each patient in the formal style and after approval by the local ethics committee.

FISH. Metaphase chromosome slides were prepared, and FISH experiments were carried out in the manner described previously (14, 15). The locations of BACs (RPCI-11 library) within the region of interest were compiled from information archived by the UCSC[4] and the National Center for Biotechnology Information.[5] Relative positions of the selected BACs on a map of the 11q22 region are indicated in Fig. 1. Probes were labeled by nick-translation with biotin-16-dUTP or digoxigenin-11-dUTP (Roche Diagnostics, Tokyo, Japan). Chromosomal *in situ* suppression hybridization and fluorescent detection of hybridization signals were carried out as described elsewhere (14, 15). The copy number and molecular organization of the region of interest were assessed according to the hybridization patterns observed on both met-

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[3] The abbreviations used are: ESC, esophageal squamous cell carcinoma; CGH, comparative genomic hybridization; FISH, fluorescence *in situ* hybridization; BAC, bacterial artificial chromosome; DAPI, 4′,6′-diamidino-2-phenylindole; HSR, homogeneously staining region; FIC, fluorescent immunocytochemistry; IHC, immunohistochemistry; UCSC, University of California at Santa Cruz; cDDP, *cis*-platinum; CPT, camptothecin; SRO, smallest amplified region of overlap; IAP, inhibitor of apoptosis; MALT, mucosa-associated lymphoid tissue; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

[4] Internet address: http://genome.ucsc.edu/.

[5] Internet address: http://www.ncbi.nlm.nih.gov/.

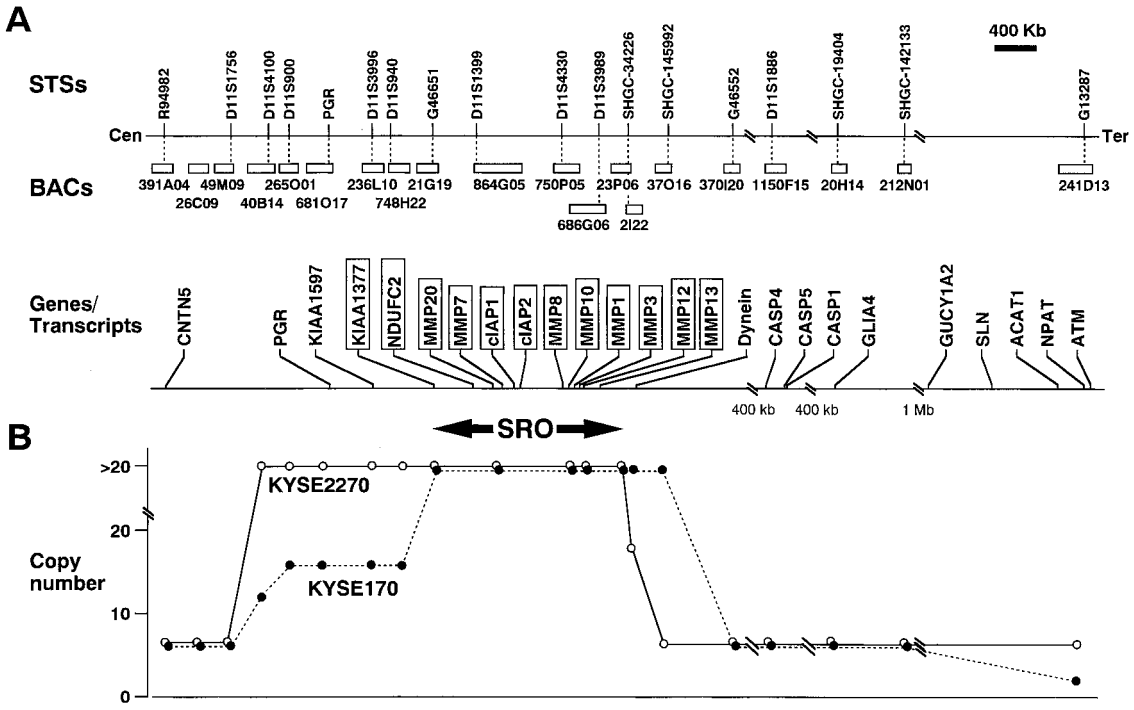

Fig. 1. *A*, map of the 11q22 region between two markers, R94982 and *ATM*. The *top scale* shows the ordering of selected sequence-tagged site (*STS*) markers mapped on this region. The 20 BACs used as probes in FISH analysis are indicated as *horizontal open bars*. The position of each STS, and the position and relative length of each BAC, were compiled from information archived by the UCSC and the National Center for Biotechnology Information. Genes and uncharacterized transcripts mapped in this region, including the 12 transcripts we analyzed (*boxed*) and *ATM* as reference gene, are listed on the *bottom map* (also see Table 1). *B*, summarized results of DNA sequence copy-number analysis by FISH in two different cell lines. The *vertical axis* shows the number of FISH signals achieved with the BAC probes indicated above. The number of signals was truncated at 20 because it was difficult to enumerate them above this level. Lines connect the measurements made for each cell line. The smallest overlapping region (*SRO*) with maximal amplification in both cell lines is indicated.

aphase and interphase chromosomes. Precise localization of each BAC was confirmed using normal metaphase chromosomes.

Southern-, Dot-, and Northern-Blot Hybridizations. We used Southern and Northern blotting to investigate the status of amplification and overexpression of 11 known genes and one uncharacterized transcript that lies within the 11q22 amplicon we had observed in ESC cell lines (Fig. 1*A*; Table 1). *ATM* (cDNA probe: clone 1367928) telomeric to the 11q22 amplicon was used as a reference in each analysis (Fig. 1*A*). For Southern blots, 10 μg of genomic DNA from each cell line or from normal lymphocytes were digested with *Eco*RI and separated on 0.8% agarose, then transferred onto a nylon membrane (BIODYNE B; Nihon Pall, Tokyo, Japan). For analyzing primary ESC tissues, we prepared Southern blots with 5 μg instead of 10 μg of genomic DNA or used dot blots, because the amount of available DNA was limited. For dot blots, 2 μg of DNA from each tumor, cell line, or normal lymphocyte was denatured with 0.4 N NaOH, then transferred to a nylon membrane (BIODYNE B; Nihon Pall). For Northern blots, 10 μg of total RNA extracted from each cell line was electrophoresed in a 1.0% agarose/0.67 M formaldehyde gel and transferred to a positively charged nylon membrane (Hybond N+; Amersham Pharmacia Biotech, Tokyo, Japan). Membranes were hybridized under appropriate conditions with [α^{32}P]-dCTP-labeled cDNA probes prepared from expressed-sequence tag clones (Table 1) purchased from Incyte Genomics, Inc. (St. Louis, MO). The blots were washed in a solution of 0.1XSSC/0.1% SDS before exposure to X-ray film for 24–84 h at −80°C.

FIC and IHC. We detected expression of cIAP1 protein in ESC cell lines by indirect FIC, as described elsewhere (16, 17). In brief, cultured cells were fixed with acetone/methanol (1:1 volume for volume), blocked with antibody-dilution buffer (1% BSA in PBS), and incubated with 1 μg/ml antihuman cIAP1 polyclonal antibody (H-83; Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min at room temperature. Normal rabbit serum was used as a negative control for the first antibody. Binding was detected by incubation with FITC-conjugated goat antirabbit IgG (ICN Pharmaceuticals, Aurora, OH) diluted 1:200 with antibody-dilution buffer. The cells were counterstained with DAPI and observed under a fluorescence microscope (Nikon, Tokyo, Japan).

Expression of cIAP1 protein in formalin-fixed, paraffin-embedded tissue sections from primary ESC tumors was detected by indirect IHC, as described elsewhere (18). Dewaxed and rehydrated sections were incubated in 3%

Table 1 *Genes and transcripts analyzed in the present study*

Gene/transcript		GenBank accession no.	Probe for hybridization	
Symbol	Name		EST no.	Clone ID
KIAA1377		AB037798	AA401311	743150
NDUFC2	NADH-ubiquinone oxido-reductase subunit B14.5B	AL050278	R05628	125219
MMP20	Enamelysin	XM_006268	AW593141	2945121
MMP7	Matrilysin	X07819	AI095584	1697164
cIAP1/BIRC2	Cellular inhibitor of apoptosis 1	XM_006266	BE886741	3908352
cIAP2/BIRC3	Cellular inhibitor of apoptosis 2	XM_006267	AI978668	2491858
MMP8	Neutrophil collagenase	NM_002424	AW236358	2700136
MMP10	Stromelysin	XM_006269	AI829878	2407015
MMP1	Interstitial collagenase	XM_006270	AA182830	624545
MMP3	Stromelysin1, progelatinase	XM_006271	AA586839	1088958
MMP12	Macrophage elastase	XM_006272	AI368844	1989528
MMP13	Collagenase 3	XM_006273	AA926873	1486567

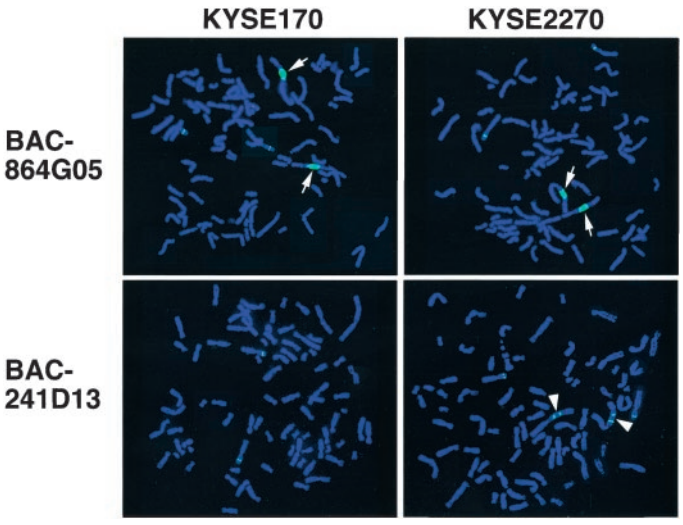

Fig. 2. Representative images of FISH analysis on metaphase chromosomes from KYSE170 and 2270. The images show HSR with BAC864G05, which contains *cIAP1* on each marker chromosome in both cell lines (*arrows*); BAC241D13, containing the *ATM* gene that is located outside the SRO, shows either no (*KYSE170*) or two signals (*KYSE2270, arrowheads*) on each marker chromosome.

hydrogen peroxide to block endogenous peroxidase, then reacted overnight at 4°C with 0.67 μg/ml antihuman cIAP1 polyclonal antibody (H-83) or normal rabbit serum. After rinsing, the sections were incubated with rabbit EnVision+ peroxidase (Dako, Carpinteria, CA), and staining was developed with 0.05% hydrogen peroxide and 3,3′-diaminobenzidine. The sections were counterstained with hematoxylin.

Estimation of Viability and Apoptotic Cell Death. Sensitivity of ESC cells to cDDP or CPT was assessed using a colorimetric assay on microtiter plates (cell-counting kit-8; Dojindo Laboratories, Kumamoto, Japan), which measures the ability of viable cells to cleave a tetrazolium salt (WST-8) to a water-soluble formazan. In brief, cells were seeded at 10^4 cells/well in 96-well plates. After plating (24 h), cells were exposed to various concentrations of cDDP or CPT for 48 h. WST-8 was added 2 h before the end of culture, and absorbance was measured at 450 nm using a microplate reader (Benchmark; Bio-Rad Laboratories, Hercules, CA). Experiments were repeated three times, and each series was performed in triplicate.

For nuclear staining, cells were plated onto glass coverslips and incubated with or without 10 μg/ml cDDP or CPT for 24 h. Cells were washed, fixed, and stained with DAPI. Nuclear morphology was examined with a fluorescence microscope (Nikon).

For observation of DNA fragmentation, cells were plated on 60-mm dishes and incubated with or without 10 μg/ml cDDP or CPT for 24 h. DNA was extracted from washed cells with the Quick Apoptosis DNA Ladder Detection Kit (Medical & Biological Laboratories, Nagoya, Japan), separated through a 2% agarose gel, and visualized after staining with ethidium bromide.

Results

Definition of the 11q21-q23 Amplicon by FISH. In our previous CGH analysis, we had detected a high-level gain of copy number on 11q21-q23 in 2 of the 29 ESC cell lines examined (KYSE170 and 2270; Ref. 7). This region was clearly distinguished from 11q13, a region frequently amplified in ESCs. To define a map of the novel amplicon, we performed FISH analyses in those two lines using 20 BACs located on 11q21-q23 (Fig. 1*A*). We assessed the copy number as well as molecular organization of the amplicon by analyzing the hybridization patterns on metaphase and interphase chromosomes.

In both cell lines, five BACs (21G19, 864G05, 750P05, 686G06, and 23P06) produced the highest number of signals as HSRs on two marker chromosomes (Figs. 1*B* and 2). By contrast, no HSR pattern and less than two FISH signals on each marker chromosome were detected with eight other BACs (391A04, 26C09, 49M09, 103C15, 130N12, 20H14, 212N01, and 241D13), suggesting that these eight lay outside the amplicon. The remaining seven BACs examined (40B14, 265O01, 681O17, 236L10, 748H22, 2I22, and 37O16) demonstrated different patterns and/or numbers of signals between KYSE170 and KYSE2270, but all of them showed fewer signals than BACs 21G19, 864G05, 750P05, 686G06, or 23P06 on each marker chromosome in at least one cell line. Taken together, as summarized in Fig. 1, we defined the SRO between BACs 21G19 and 23P06. The estimated extent of this region was 1.8 Mb on 11q22, on the basis of the genome database archived by the UCSC.

Analysis of Positional Candidate Genes in ESC Cell Lines. To explore genes that might be targets of the 11q22 amplification, we analyzed the status of amplification and expression of transcripts located on this amplicon, especially within the SRO. On the basis of amplicon mapping by FISH, 12 transcripts consisting of 11 known genes and 1 uncharacterized transcript were selected from the genome database archived by the UCSC (Fig. 1*A*; Table 1). Among them, only *cIAP1* was consistently overexpressed in the two cell lines that showed amplification (Fig. 3), strongly suggesting that this gene is a potential target within the 11q22 amplicon. On the other hand, *MMP10* and *MMP13* were highly expressed in KYSE2270 but not in KYSE170 (Fig. 3*B*). None of the remaining genes within the SRO, including *cIAP2* and the uncharacterized transcript, were overexpressed in both cell lines (data not shown). Therefore, none of the examined transcripts, except for *cIAP1*, are likely to be targets for 11q22 amplification.

Overexpression of cIAP1 Protein in ESC Cell Lines and Parent Tumors. Overexpression of cIAP1 was observed not only at the mRNA level but at the protein level as well. FIC experiments clearly showed expression of cIAP1 in the cytoplasm of KYSE170 and 2270 cells, the two ESC lines in which concomitant amplification with overexpression of cIAP1 had been detected (Fig. 3*C*). Cell lines without amplification of cIAP1 showed a considerably lesser degree of cytoplasmic staining for this molecule, in accord with the results of Northern blotting (Fig. 3*C*).

Next, we performed IHC analysis of cIAP1 using tissue sections of the primary ESC tumors from which the KYSE series of ESC cell lines had been established. As shown in Fig. 3*D*, moderate to strong staining of cIAP1 was clearly observed in >10% of cancer cells but not in normal epithelial or stromal cells in parent tumors of the KYSE 170 and 2270 lines. In both primary tumors, most immunoreactions occurred in the cytoplasm of cancer cells, at the frontier of stromal invasion. The cIAP1-immunopositive carcinoma cells were usually pleomorphic; they infiltrated into the stroma as single cells or in the form of small nests or strands. In the primary tumor of KYSE2270, strong cIAP1 staining was predominant at the periphery of the small nests (Fig. 3*D*, *right panel*). By contrast, we detected no or weak staining of cIAP1 in primary ESC tumors of KYSE cell lines without 11q22 amplification or in breast tumors used as controls (data not shown). These results strongly suggest that amplification of the *cIAP1* gene, resulting in overproduction of this protein, had already occurred in the primary tumors, from which KYSE 170 and 2270 were derived.

Resistance of ESC Cell Lines with Amplified *cIAP1* to Drug-induced Apoptosis. Because cIAP1 is a member of the IAP family of proteins, and because some IAPs are thought to be involved in carcinogenesis through their antiapoptotic activity (19), we investigated whether the two ESC cell lines with amplification of *cIAP1* were resistant to apoptotic stimuli. As shown in Fig. 4*A*, both KYSE 170 and 2270 exhibited resistance to cDDP and CPT as compared with control cell lines (KYSE200 and 960) in which *cIAP1* was not overexpressed. The resistance of KYSE170 and 2270 cells to these anticancer drugs may reflect resistance to chemotherapeutic agent-induced apoptosis, because 10 μg/ml cDDP or CPT induced remark-

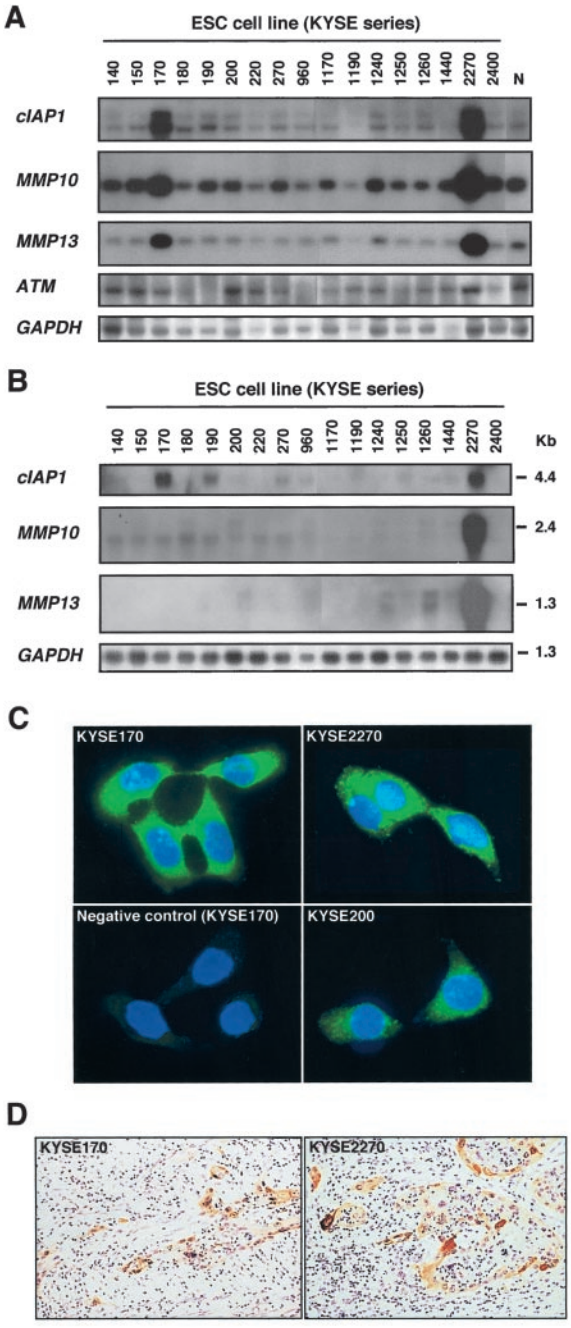

Fig. 3. *A*, representative results of Southern-blot analyses using *cIAP1*, *MMP10*, *MMP13*, and *ATM* probes and a control (*GAPDH*) in ESC cell lines. DNA from peripheral blood lymphocytes of a healthy donor served as a normal control (*N*). *cIAP1*, *MMP10*, and *MMP13* were clearly amplified in both KYSE170 and 2270. Note that an increased signal of *ATM* was also detected in KYSE2270. *B*, representative results of Northern-blot analyses using *cIAP1*, *MMP10*, and *MMP13* probes and control (*GAPDH*) probe in ESC cell lines. Only *cIAP1* was consistently overexpressed in the cell lines that showed amplification (*KYSE170* and *2270*). *C*, immunofluorescent staining of cIAP1 protein in the same two ESC lines. Cells were counterstained with DAPI. cIAP1 protein was overexpressed in the cytoplasm of KYSE170 and 2270 but not in KYSE200 cells. *Negative control*, KYSE170 stained with normal rabbit serum. Magnification, ×600. *D*, immunohistochemical staining of cIAP1 protein in the primary tumors from which KYSE170 and 2270 cell lines had been established. In both tumors, enhanced staining of cIAP1 was observed in the cytoplasm of ESC cells, indicating overexpression of this protein. Magnification, ×200.

able apoptotic changes in control cells but had little effect on KYSE 170 or 2270 cells. As shown in Fig. 4 (*B* and *C*), typical apoptotic changes, such as condensation of nuclear chromatin and laddering of DNA, were observed in KYSE 200 after 24 h of treatment with cDDP. In KYSE170 (data not shown) and 2270 cells, however, few apoptotic cells and little fragmented DNA were observed after the same treatment. The same results were obtained with CPT (data not shown).

Amplification of *cIAP1* in Primary Tumors. Because *cIAP1* seems to be a target for amplification at 11q22, we examined 42 primary ESC tumors that were unrelated to the cell lines to determine whether amplification of *cIAP1* had occurred in any of them. By Southern- or dot-blot analysis, an increased signal of the *cIAP1* gene was detected in 4 of the 42 tumors examined (9.5%; Fig. 5).

Discussion

The 11q22 amplicon we delineated in this study is distinct from the one at 11q13 that has been recognized as commonly amplified in ESCs and other types of tumors (20, 21). Amplification around 11q22 also has been demonstrated by other CGH experiments in a wide variety of human cancers, including ESC, though infrequently (5, 7–12). Primary (9) and metastatic renal-cell carcinomas with sarcomatoid changes (10) sometimes exhibit this alteration. Therefore, the 11q22 region may harbor one or more genes that are activated by amplification and might be associated with progression and/or specific tumor phenotypes. However, neither defined mapping nor screening of putative target genes for the 11q21-q23 amplification has been carried out. This is the first report describing experiments to evaluate potential target genes within that amplicon.

As the first step in exploring the 11q21-q23 region for candidate genes, we constructed a map of the amplicon by FISH to define a relatively small chromosomal region that would make a positional search possible. The criterion we used to define the amplicon is that the best candidate tumor-associated genes, which are selected during amplification process, are located at narrow regions of highest copy number (22). Our determination of the SRO using this criterion and two different cell lines helped to narrow even further the region likely to harbor target gene(s). Even after defined mapping, however, many genes must still exist on an amplicon. The common criterion for designating a gene as a putative target is that amplification leads to its overexpression (23, 24). On the basis of that criterion, we used Northern blots to identify transcripts that were consistently overexpressed in two ESC cell lines where the defined region at 11q22 was amplified (KYSE 170 and 2270) and successfully identified *cIAP1* as a strong candidate target for this amplification. Amplification of *cIAP1* was detected as well in 4 of 42 unrelated primary ESC tumors, indicating that this genetic alteration is not cell line specific.

The *cIAP1* gene product, designated variously as HIAP2, AIP1, MIHB, or BIRC2, was originally identified as a protein recruited to the cytosolic domain of p80 tumor necrosis factor II via its association with tumor necrosis factor-associated factors-1 and -2 (25). cIAP1 protein, like cIAP2 and XIAP in the same subfamily of IAPs, contains three baculovirus IAP repeat domains in the NH_2-terminal region and a RING (real interesting new gene) finger domain close to the COOH terminus. Ectopic expression of *cIAP1* in mammalian cells can inhibit apoptosis induced by serum deprivation or by a variety of stimuli (19, 26). Consistent with those published observations, our ESC cell lines showing amplification of *cIAP1* were resistant to apoptosis induced by chemotherapeutic agents. Our preliminary study demonstrated that those cell lines with amplification of *cIAP1* were also resistant to apoptosis induced by radiation, suggesting that overexpressed cIAP1 inhibits apoptosis, regardless of the type of apoptosis-inducing stimulus.[6] Although the mechanism by which cIAP1 suppresses apoptosis is still debated, several studies have provided insights into the biochemical functions of this intriguing protein (19). The central mech-

[6] I. Imoto *et al.*, unpublished data.

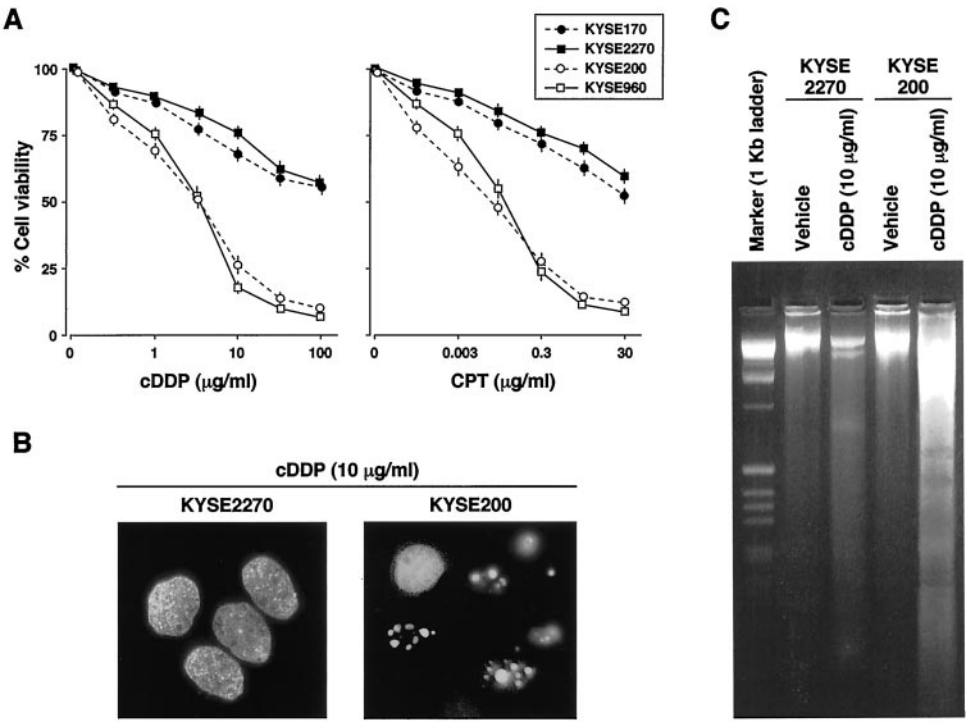

Fig. 4. *A*, drug resistance of ESC cell lines having amplification and overexpression of *cIAP1*. KYSE170, 2270, 200, and 960 were treated with the indicated doses of cDDP (*left*) or CPT (*right*). After 48 h, cell viability was determined by colorimetry. Note that KYSE170 and 2270 exhibited resistance to both anticancer drugs, as compared with control lines KYSE200 and 960. Data represent the mean ±SD of three separate experiments, each performed in triplicate. *B* and *C*, resistance of KYSE2270 cells to drug-induced apoptosis. Typical nuclear morphological changes (*B*) and DNA fragmentation (*C*) occurred in control cell line KYSE200 after 24 h of treatment with 10 μg/ml cDDP. Note the decrease in apoptotic changes in KYSE2270 compared with KYSE200. The same resistance to drug-induced apoptosis was observed in KYSE 170 (data not shown).

anism appears to be direct inhibition of caspase and procaspase; cIAP1 binds directly to caspases 3 and 7 (27, 28) and also inhibits activation of procaspases 8 and 9 (29, 30). cIAP1 appears to inhibit apoptosis through noncaspase mechanisms as well, *e.g.*, by activation of nuclear factor κB (30) and c-Jun-NH_2-terminal kinase (31).

Although evidence for a direct oncogenic role for cIAP1 has yet to emerge, our results indicate that this potent regulator of cell death is likely to play an important role in carcinogenesis. Inhibition of, or increased resistance to, apoptosis is a common property of cancer cells, as it increases their survival time and facilitates their escape from immune surveillance and cytotoxic therapies (19). Therefore, a constitutive activation of antiapoptotic molecules via genetic or epigenetic mechanisms, including gene amplification, may well be involved in carcinogenesis. In follicular and diffuse large B-cell types of non-Hodgkin's lymphoma, *e.g.*, overexpression of BCL2 through amplification or translocation appears to be associated with progression of the disease (32, 33). Moreover, survivin, another member of the IAP family that is not expressed in normally differentiated tissues, is specifically overexpressed in some cancers (34). Tamm *et al.* (35) reported that *cIAP1* was expressed in most of the cancer cell lines they tested (a panel of 60 human cancer cell lines maintained by the National Cancer Institute), and its expression level was correlated with resistance to several anticancer drugs. Those results and our present findings encourage additional investigation of the functional role of cIAP1 in the genesis of various types of cancer, including ESC, as well as its prognostic relevance.

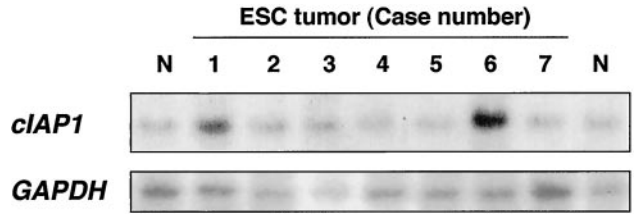

Fig. 5. Representative results of Southern-blot analysis of *cIAP1* and control (*GAPDH*) in primary ESC tumors. Cases 1 and 6 showed strongly amplified signals with *cIAP1*. *N*, normal control from peripheral blood leukocytes of a healthy donor.

Our immunocytochemical study using ESC cell lines clearly showed that the amplification mechanism activated *cIAP1* and led to overproduction of its product. Moreover, our immunohistochemical study of the archived primary tumors from which those lines had been established indicated that activation of cIAP1 via gene amplification had already occurred *in vivo*. We infer that activated cIAP1 might confer malignant phenotypes, including invasiveness, metastasis, and drug resistance. The cIAP1-immunopositive cancer cells were observed at the frontier of stromal invasion, and they proliferated into the stroma as single cells or in the form of small nests or strands. These findings suggest that increased immunoreactivity of cIAP1 may reflect a highly invasive potential of ESC cells. Ono *et al.* (36) observed a similar pattern of immunohistochemical staining for laminin-5 γ2 chain in squamous cell carcinomas of the tongue. Their analysis of 67 such tumors demonstrated that strong cytoplasmic localization of the laminin-5 γ2 chain was significantly associated with poor prognosis for patients with squamous cell carcinomas of the tongue (36). Accordingly, it will be important to determine the clinicopathological significance of cIAP1 expression in squamous cell carcinomas arising in other organs as well as the esophagus.

Of the 12 transcripts we screened, 11 were excluded as candidates because they were not detectably or consistently expressed in the panel of ESC cell lines we examined. However, of the excluded candidates, *cIAP2*, which encodes another member of the IAP family, has been identified as a target gene at the breakpoint of a translocation observed often in MALT-type lymphomas (37–39). In MALT lymphomas, cIAP2 is fused to MALT1, and the chimeric product may be involved in enhanced resistance to apoptosis, although its actual function remains unknown. However, *cIAP2* was not overexpressed in any of cell lines we tested, including the two lines having 11q22 amplification. MMP-10 (stromelysin-2) and MMP-13 (collagenase-3) were each highly expressed in only one (KYSE2270) of those two cell lines, although MMP-10 and MMP-13 are often overexpressed in tumor cells, and that feature is correlated with tumor invasion and

aggressiveness, respectively (40, 41). Expression of MMP-10 and/or MMP-13 may be inhibited in KYSE170 by some mechanism, possibly epigenetic. Thus, additional functional and biochemical studies might be needed to absolutely exclude these two genes as amplification targets.

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