Overexpression of cellular FLICE-inhibitory protein (FLIP) in gastric adenocarcinoma

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ABSTRACT

The aim of the present study was to investigate the prevalence of c-FLIP [cellular FLICE-inhibitory protein, where FLICE is Fas-associated death domain (FADD)-like interleukin-1β-converting enzyme] expression in gastric adenocarcinoma and its possible implications for the progression of the cancer. Expression of c-FLIP in 48 gastric adenocarcinomas and their normal counterparts was analysed by reverse transcriptase PCR, Western blotting and immunohistochemistry. In situ cell apoptosis was detected by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) assay. As a result, c-FLIP transcripts were constitutively expressed in gastric adenocarcinomas and their levels were significantly higher than those in matched normal tissues (P<0.01). Immunohistochemically, the c-FLIP protein was also found to be expressed in all gastric adenocarcinomas (48/48), and 68.8% (33/48) showed an intense immunostaining; in contrast, only 75% (36/48) of normal gastric mucosa showed positive staining and none of them immunostained intensely. The abundance of c-FLIP protein was significantly higher in carcinoma than in normal gastric mucosa (6.93±0.58 versus 3.19±0.26, P<0.01) and showed a reverse correlation with apoptotic index in adenocarcinoma, but not in normal mucosa. In addition, abundance of c-FLIP was significantly associated with lymph node metastasis at both mRNA level (P<0.05) and protein level (P<0.01). Western-blot analysis showed that the expression levels of the long form of c-FLIP and the short form of c-FLIP in adenocarcinomas were 2.6-fold and 2.8-fold (P<0.01) higher than those in normal tissues respectively. However, no significant difference was found between the expression levels of the two isoforms in both adenocarcinomas or normal tissues. In conclusion, overexpression of c-FLIP is tumour specific, which may be one of the in vivo mechanisms by which tumour cells escape from apoptotic death during the malignant transformation, and plays an important role in lymph node metastasis of gastric adenocarcinoma, which ultimately contributes to the tumour progression.

INTRODUCTION

Gastric cancer is one of the most frequent neoplasms and a leading cause of death worldwide [1]. The overall survival in patients with gastric cancer has improved as a result of the high detection rate of early cancer and wider implementation of radical surgery. However, a significant number of patients with gastric carcinoma still die due to primary treatment failure or presentation at a late stage of the disease [2]. New molecular markers and therapeutic

Key words: adenocarcinoma, apoptosis, FLICE-inhibitory protein (FLIP), gastric adenocarcinoma, metastasis, tumour progression.

Abbreviations: AI, apoptotic index; FADD, Fas-associated death domain; FLICE, FADD-like interleukin-1β-converting enzyme; c-FLIP, cellular FLICE-inhibitory protein; c-FLIPL/S, long and short forms of cellular FLICE-inhibitory protein; DED, death effect domain; DISC, death-inducing signalling complex; RT-PCR, reverse transcriptase PCR; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling; UICC, International Union Against Cancer; VEGF, vascular endothelial growth factor.

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principle are needed to allow early detection and to treat these patients appropriately, but this relies on further elucidation of the pathogenesis of gastric carcinoma.

Apoptosis is a highly regulated mechanism that plays a pivotal role in the physiology of organisms and possibly participates in the defence against pathogens and cancer. It is essential for the maintenance of normal structural and functional tissue homoeostasis. Dysregulated apoptosis, resulting in excessive or insufficient cell death, is fundamental to the pathogenesis of diverse diseases including cancers, autoimmune diseases, and degenerative disorders [3,4]. In the last decade, basic cancer research has been focused on the deregulation of apoptosis as a central event in the process of carcinogenesis and a number of studies showed a central role of apoptosis for the tumorigenesis of gastric carcinoma, various regulators of apoptosis, including p53, survivin, caspase-3 and Fas, have been implicated in the development of gastric cancer [5-8]. Fas is a cell surface receptor which triggers death signals when ligated by natural ligand FasL or cross-linking antibody. Fas protein is constitutively expressed in immune, epithelial and some mesenchymal cells [9,10]. Fas–FasL interaction leads to cleavage of procaspase-8 to caspase-8 [also known as FLICE (Fas-associated death domain)-like interleukin-1β-converting enzyme]. Activated caspase-8, the most upstream caspase in the Fas apoptotic pathway, promotes caspase-3 activation and DNA fragmentation. According to previous reports, Fas and FasL are highly expressed in gastric carcinomas, either at the protein level or mRNA level [11-13], but only a subpopulation of tumour cells undergoes apoptosis. Furthermore, co-expression of Fas and FasL, which occurs over large areas of the tumours, did not result in an enhanced rate of tumour cell apoptosis [13]. So, it is reasonable to harbour the view that some inhibitors may be present in the pathway of Fas signal transduction.

Sensitivity toward Fas-mediated apoptosis can be modulated by various anti-apoptotic proteins. A new class of virus-encoded apoptosis inhibitory molecules, designated viral FLICE (caspase-8) inhibitory protein (v-FLIP), has been described previously [14–16]. These molecules comprise two DEDs (death effect domains) [14]. Cellular homologues of v-FLIP have been identified by different groups and have been termed c-FLIP (cellular FLIP) [17], CASH [18], Casper [19], CLARP (caspase-like apoptosis regulatory protein) [20], FLAME-1 (FADD-like anti-apoptotic molecule-1) [21], I-FLICE [15], MRIT (FADD-associated CED-3 homologue-related inducer of toxicity) [22] and Usurpin [23]. At the mRNA level, c-FLIP exist as multiple splice variants, but at the protein level only two endogenous forms, c-FLIP_{long} (c-FLIP_L) and c-FLIP_{short} (c-FLIP_S) have been detected to date [17,19,24]. c-FLIP_{long} is a 55 kDa protein and structurally similar to procaspase-8; it contains two DEDs and a caspase-like domain. This domain lacks residues that are important for its catalytic activity, most notably a cysteine residue within the active site. The short form of c-FLIP is a 28 kDa protein and structurally resembles v-FLIP, containing two DEDs. Both c-FLIP species were found to be recruited to the DISC (death-inducing signalling complex) [24]. A number of studies support the notion that both forms of FLIP can prevent Fas/CD95-mediated apoptosis by interacting with either FADD and/or procaspase-8 [14,17,25].

In the present study, we performed an expression analysis of c-FLIP at mRNA and protein levels to determine its prevalence in gastric adenocarcinomas and normal gastric mucosas by immunohistochemistry, semi-quantitative RT-PCR (reverse transcriptase-PCR) and Western blotting, and the association of c-FLIP abundance with clinicopathological parameters and apoptotic index (AI) of patients was also investigated.

**MATERIALS AND METHODS**

**Tissue specimens**

Forty eight fresh gastric adenocarcinoma tissues, together with matched normal gastric mucosas from patients with disparate pathological stages (Table 1), were collected and freshly frozen in liquid N2 after surgical resections performed at the Renmin Hospital, Wuhan University (Wuhan, China) from 1998 to 2000. Of these, 28 were male and 20 were female. The mean age was 58.5 years (S.D. 23.7; range 29–80 years). None of the patients had received chemo-, radio- or immuno-therapy before resection. Parts of each specimen were routinely processed, fixed in 10 % buffered formalin, and embedded in paraffin for histopathological analysis (haematoxylin and eosin stain) and for immunohistochemical staining and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) assay. All tissues were scored by one pathologist who was blinded to culture results and disease status. To allocate a TNM (tumour node metastasis) stage, a median of 13 (range 6–33) lymph nodes was counted. This research was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association, and has been approved by the Ethics Committee of the Wuhan University.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded tissue blocks were serially sectioned at 4 µm. Then the sections were mounted on to the histostick-coated slides and kept in an oven at 72°C for 2 h. Sections were deparaffinized in xylene and rehydrated before analysis. Endogenous peroxidase was quenched with 3.0 % H2O2 in methanol for 10 min. Antigen retrieval was performed by heating...
Table 1 Clinicohistopathological characteristics of patients with gastric adenocarcinoma and their association with c-FLIP<sub>L/S</sub> protein and mRNA expression

c-FLIP<sub>L/S</sub> protein and mRNA levels are expressed as the means ± S.D.

<table>
<thead>
<tr>
<th>Category</th>
<th>Number</th>
<th>c-FLIP&lt;sub&gt;L/S&lt;/sub&gt; protein level</th>
<th>P-value</th>
<th>c-FLIP&lt;sub&gt;L/S&lt;/sub&gt; mRNA level</th>
<th>P-value</th>
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<tr>
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<td>7.01 ± 0.39</td>
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<td>0.105</td>
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</tr>
<tr>
<td>II</td>
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<td>7.11 ± 0.64</td>
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<td>III</td>
<td>17</td>
<td>6.76 ± 0.55</td>
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<td>IV</td>
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<td>7.14 ± 0.46</td>
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<td>0.64 ± 0.12</td>
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</table>

* Each factor of node, metastasis, and UICC (International Union Against Cancer) stage was determined according to the UICC TNM classification (fifth edition).

in a microwave for 15 min and they were then blocked for 15 min with normal rabbit serum. This was followed by incubation overnight at 4°C with affinity purified rabbit polyclonal anti-human c-FLIP<sub>L/S</sub> specific IgG (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at a dilution of 1:120. Incubation with PBS, instead of the primary antibody, served as a negative control and human colonic adenocarcinoma tissue that had previously shown strong expression for c-FLIP served as the positive control samples [26]. Sections were washed three times with PBS for 2 min each and incubated with biotin-labelled anti-rabbit IgG for 1 h at room temperature (25°C). After three washes with PBS for 2 min each, sections were stained by a streptavidin–peroxidase detection system. Antibody binding was visualized using the diaminobenzidine as chromogen and counterstained with haematoxylin.

Scoring method for c-FLIP<sub>L/S</sub> expression was modified from that described for Bcl-2 expression by Sinicrope et al. [27]. Positive tumour cells were quantified by two independent observers, and a mean percentage of positive tumour cells was determined in at least five areas randomly at a magnification of ×400 and assigned to one of five categories: 0, <5%; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75%. The intensity of c-FLIP<sub>L/S</sub> immunostaining was scored as: weak, 1; moderate, 2; and intense, 3. For tumours showing heterogeneous staining, the predominant pattern was taken into account for scoring. The percentage of positive tumour cells and staining intensity were multiplied to produce a weighted
score for each case. Cases with weighted scores < 1 were defined as negative; otherwise they were defined as positive.

**TUNEL assay**
Gastric epithelial cell apoptosis was determined in situ from paraffin-embedded tissue sections by TUNEL assay, using the Apop Tag in situ detection kit (Intergen Company, Oxford, U.K.) according to the manufacturer’s instructions. All cancerous and matched normal tissue samples were processed under the same conditions with positive and negative controls. Positive control sections were pretreated with DNase I and negative controls were processed without terminal deoxynucleotidyl transferase. Morphological characteristics of apoptosis were chromatin condensation, nuclear disintegration and formation of crescentic caps of condensed chromatin at the nuclear periphery. The AI was expressed as the ratio of positively stained epithelial cells and bodies to all epithelial cells. If possible, 5 areas were randomly selected for counting under × 400 magnification; otherwise, the whole section underwent assessment.

**Semi-quantitative RT-PCR**
After homogenization, total RNA was extracted from 48 fresh primary gastric cancers and paired normal gastric mucosa, using TRIzol reagent (Invitrogen, San Diego, CA, U.S.A.). First-strand cDNA was synthesized from 1 µg of total RNA using oligo-dT primer and Moloney-murine-leukaemia virus reverse transcriptase (Promega, Southampton, UK) according to the manufacturer’s instructions. One tenth of the cDNA synthesized was then amplified by using the following primers: c-FLIPL (sense), 5′-TGT TGC TAT AGA TGT GG-3′ (antisense); β-actin, 5′-AAG GAT CCT TGA GAC TCT-3′ (antisense); β-actin, 5′-TGA CGG GGT CAC CCA CAC TGT GGC-3′ (sense), 5′-CTG CAT CCT GTC GGC AAT GCC AG-3′ (antisense). The conditions of the PCR amplification were: 2 min at 95 °C for one cycle, 45 s at 95 °C, 45 s at 55 °C and 45 s at 72 °C for 35 cycles for β-actin; 2 min at 95 °C for one cycle, 60 s at 95 °C, 60 s at 55 °C and 120 s at 72 °C for 35 cycles for c-FLIPL/s. The size of the PCR amplification products was 512 bp for c-FLIPL/s and 475 bp for β-actin.

The PCR products were analysed on 2 % agarose gels and visualized by ethidium bromide staining. Quantitation of expression levels was achieved after adjustment for the expression levels of the housekeeping gene β-actin by densitometry (Bio-Rad, Hercules, CA, U.S.A.). A 100 base pair DNA ladder (Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.) was used as a molecular mass marker on each gel. The relative level of expression was then represented as the ratio of c-FLIPL/s/β-actin in normal tissues and adenocarcinomas.

**Western blotting**
Sixteen randomly selected frozen gastric adenocarcinomas with matched normal mucosas were homogenized and lysed in buffer containing 40 mM Tris/HCl (pH 8.0), 120 mM NaCl, 0.5 % Nonidet P40, 1 mM PMSF and 10 µg/ml leuipetin. The lysates were incubated on ice for 60 min and centrifuged at 9901 g for 20 min. After denaturation 50 µg of protein was separated by SDS/PAGE (12 % gel) for each sample and then electro-blotted on to nitrocellulose membranes. The nitrocellulose membranes were blocked with 5 % non-fat milk in TBST (10 mM Tris, pH 7.4, 100 mM NaCl and 0.5 % Tween 20) and then incubated with rabbit polyclonal anti-c-FLIPL/s antibody (Santa Cruz Biotechnology) for 1 h with peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) for 1 h. The protein signals were visualized using an enhanced chemiluminescence reaction (ECL® Western blotting detection, Amersham Life Science, Amersham, Bucks, U.K.) and exposed to medical X-ray film for 1 min. β-Actin was used to control equal loading in the same method as above. The developed X-rays were scanned using Epson GT-9500 (Seiko Corp., Nagano, Japan) and quantified by a densitometer (Image PC alpha 9, National Institutes of Health, Bethesda, MD, U.S.A.).

**Statistical analysis**
The statistical software package SPSS 10.0 was used and data were presented as the means ± S.D. The prevalence of c-FLIPL/s mRNA and protein expression in cancers and normal tissues and AI in different groups was compared with Student’s t test. Association between c-FLIPL/s expression in tumours and clinicopathological features was examined by Student’s t test. Correlation parameters were submitted to Pearson and non-parametric Spearman correlations. A P value less than 0.05 was considered to indicate statistical significance.

**RESULTS**

**Expression of c-FLIPL/s protein in gastric adenocarcinoma and its association with AI**
By immunohistochemistry, anti-c-FLIPL/s polyclonal antibody reacted with gastric mucosal epithelial cells of both normal and neoplastic tissues, and the c-FLIPL/s protein was localized to the cytoplasm (Figures 1A and 1B). In addition, c-FLIPL/s protein was also found to express in tumour cells of colonic adenocarcinoma, which served as a positive control (Figure 1C). c-FLIPL/s-positive and -negative areas were found to occur within the same tissue, and the intensity of positive staining was also found to be variable within a case tested. After multiplying the weighted c-FLIPL/s scores, the mean expression scores in normal mucosa were in the
Figure 1 Immunohistochemical staining for c-FLIP<sub>L/S</sub> using a rabbit anti-c-FLIP<sub>L/S</sub> antibody and its association with in situ cell apoptosis in stomach

(A) c-FLIP<sub>L/S</sub> expression in normal gastric mucosa with moderate cytoplasmic immunostaining (brown staining, ×400 magnification). (B) Immunohistochemical staining of c-FLIP<sub>L/S</sub> in tissue sections obtained from gastric adenocarcinoma. c-FLIP<sub>L/S</sub> was mainly expressed in the cytoplasm of cancer cells (brown staining, ×400 magnification). (C) Expression of c-FLIP<sub>L/S</sub> in colonic adenocarcinoma which served as a positive control. c-FLIP<sub>L/S</sub> was mainly expressed in the cytoplasm of cancer cells (brown staining, ×400 magnification). (D) Photomicrograph showing apoptotic bodies within well-differentiated gastric adenocarcinoma cells (×400 magnification). (E) No correlation between the AI and c-FLIP<sub>L/S</sub> level was found in normal gastric mucosa. (F) Gastric adenocarcinomas with higher level of c-FLIP<sub>L/S</sub> showed significantly lower AI when compared with those with lower level of c-FLIP<sub>L/S</sub>.

range 0 to 8 (3.19 ± 0.26); 75% (36/48) of all cases showed either a weak or a moderate intensity and 25% (12/48) showed negative reactivity. c-FLIP<sub>L/S</sub> protein was constitutively expressed in all adenocarcinomas (48/48) and the mean scores were in the range 1 to 12 (6.93 ± 0.58); 68.8% (33/48) of adenocarcinomas were intensely immunostained and only 6.3% (3/48) were weakly immunostained (Table 2). Moreover, its level was
Table 2  c-FLIP<sub>L/S</sub> expression in gastric adenocarcinoma and matched normal gastric mucosa

<table>
<thead>
<tr>
<th>Protein and mRNA levels are expressed as the means ± S.D.</th>
</tr>
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<tbody>
<tr>
<td>Number</td>
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<tr>
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<tr>
<td>Normal mucosa</td>
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<tr>
<td>Adenocarcinoma</td>
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much higher than that of matched normal gastric mucosa, which was statistically significant (P < 0.001).

To investigate whether the presence of c-FLIP<sub>L/S</sub> is related to apoptosis, TUNEL staining was performed in consecutive tissue sections of 48 gastric adenocarcinomas and matched normal mucosas (Figure 1D). Cancerous samples and normal samples were divided into high groups and low groups respectively according to the mean c-FLIP<sub>L/S</sub> immunohistochemical expression level (high, ≥ mean value; low, < mean value). As a result, a higher AI was found in normal gastric mucosa than that of adenocarcinoma (6.12 ± 1.37 % versus 2.09 ± 1.02 %, P < 0.001). We failed to find a significant difference in AI between high-level and low-level c-FLIP<sub>L/S</sub> groups of normal gastric tissue samples; however, the AI of 26 tumours with high c-FLIP<sub>L/S</sub> level was significantly lower than that of 22 tumours with low c-FLIP<sub>L/S</sub> level (1.36 ± 0.11 % versus 2.96 ± 0.15 %, P < 0.01) (Figures 1E and 1F).

Expression of c-FLIP<sub>L/S</sub> mRNA in stomach and its association with protein expression

We examined mean expression levels of c-FLIP<sub>L/S</sub> mRNA in 48 gastric adenocarcinomas and their matched normal gastric mucosa using a semi-quantitative RT-PCR assay. The RT-PCR was controlled by equalization of input RNA for each sample and comparable amplification efficiencies were validated by the uniformity of control β-actin RT-PCR product yields. The RT-PCR results showed that normal mucosa (not influenced or connected with the tumour region) and adenocarcinoma constitutively expressed c-FLIP<sub>L/S</sub> mRNA (Figure 2A), but the mean expression levels varied between these tissues. While the mean expression levels of c-FLIP<sub>L/S</sub> mRNA (c-FLIP<sub>L/S</sub>/β-actin ratio) in 48 normal gastric mucosa were in the range 0.10 to 0.54 (0.24 ± 0.13), the levels in 48 carcinomas ranged from 0.35 to 0.81 (0.59 ± 0.16) (Table 2). Mean c-FLIP<sub>L/S</sub> mRNA expression in adenocarcinoma was significantly higher than in normal gastric mucosa (P < 0.001).

Association of c-FLIP<sub>L/S</sub> expression with clinicopathological characteristics

As shown in Table 1, expression of c-FLIP<sub>L/S</sub> protein and mRNA was independent of age, gender and site of origin, as well as other histopathological characteristics, such as degree of differentiation, Lauren classification, tumour size, distance metastasis and tumour stage. However, the difference of c-FLIP<sub>L/S</sub> protein abundance between tumours with different status of lymph node metastasis reached statistical significance. The abundance of c-FLIP<sub>L/S</sub> protein was significantly associated with...
Western-blot analysis was performed on adenocarcinoma and matched normal mucosa of human stomach using polyclonal anti-c-FLIP\textsubscript{L/S} antibodies

Both forms of c-FLIP protein levels are higher in adenocarcinoma samples when compared with the normal mucosas. No significant difference was found between the expression of c-FLIP\textsubscript{L} and c-FLIP\textsubscript{S} either in cancerous samples or in normal tissues. \(\beta\)-actin blot shows equal sample loading in all the lanes. N, normal mucosa; C, adenocarcinoma.

lymph node metastasis (\(P<0.01\)). It was also found that c-FLIP\textsubscript{L/S} gene had a higher transcriotional level in tumours with positive lymph node metastasis (\(P<0.05\)).

**Western-blot analysis of the expression pattern of the two isoforms of c-FLIP protein**

To semi-quantitatively compare the long and short forms of c-FLIP protein in normal and tumour tissue samples of stomach, and also to confirm the specificity of the c-FLIP\textsubscript{L/S} antibody used in the immunohistochemistry experiments, Western-blot analysis was performed on 16 randomly selected gastric adenocarcinomas with matched normal mucosas. Two protein bands corresponding to c-FLIP\textsubscript{L} and c-FLIP\textsubscript{S} were just detectable in all gastric cancer tissue samples; in contrast, 13 matched normal tissues showed positive, but relatively weaker, expression of c-FLIPL and c-FLIPS when compared with tumour tissues (Figure 3). The relative mean c-FLIPL and c-FLIPS densities when compared with that of \(\beta\)-actin in adenocarcinomas were 2.6-fold (\(P<0.01\)) and 2.8-fold (\(P<0.01\)) higher than those in normal tissues respectively. However, densitometric analysis showed no significant difference between c-FLIPL and c-FLIPS protein levels either in adenocarcinomas or in normal tissues.

**DISCUSSION**

Inhibition of apoptosis confers a survival advantage on cells harbouring genetic alterations, and may promote acquisition of further mutations to cause neoplastic progression and also contribute to the development of resistance to chemotherapy [28,29]. A novel anti-apoptosis gene, designated c-FLIP, has been demonstrated to be over-expressed in several human cancers, suggesting an important role of c-FLIP in cancer development [26,30,31]. However, the exact expression status of c-FLIP and its possible implications have not been determined in gastric adenocarcinoma.

In the present study, we first demonstrated that the expression of c-FLIP\textsubscript{L/S} was frequently elevated in gastric adenocarcinomas at both mRNA and protein levels, and comparisons with matched normal tissues revealed that over-expression of c-FLIP\textsubscript{L/S} was a tumour-specific phenomenon. Furthermore, the prevalence of c-FLIP\textsubscript{L/S} mRNA expression in gastric adenocarcinomas correlates well with expression of the c-FLIP\textsubscript{L/S} protein, but not in normal gastric mucosa. This was also found in colonic adenocarcinoma [26]. Most recently, Lee et al. [32] reported that c-FLIP expression was detected by an immunohistochemistry approach in 90 % of stomach carcinomas and considerable numbers of the carcinomas (90 %) showed co-expression of Fas and c-FLIP, so it is reasonable to hypothesize that most gastric cancer in vivo may need c-FLIP expression for inhibition of apoptosis. However, the exclusion of normal gastric tissue in their study resulted in failing to determine whether the over-expression of c-FLIP was a tumour-specific phenomenon. In addition, their positive rate of c-FLIP protein expressed in gastric cancer was lower than that of the present study (90 % versus 100 %). It was found to be at least partially due to the different antibody used in immunostaining. The antibody used in the previous study was designed only to recognize the long form of c-FLIP; however, the antibody used in our study was expected to recognize both forms of c-FLIP (c-FLIPL\textsubscript{L} and c-FLIPL\textsubscript{S}). The results of Western-blot analysis in our present study, that both forms of c-FLIP were detected in gastric adenocarcinoma without significant difference, has further validated it.

In previous studies in vitro, both forms have been demonstrated to act as inhibitors of apoptosis [14,17,25]. We found that a high level of c-FLIP\textsubscript{L/S} was closely associated with a low apoptosis rate in adenocarcinoma, but in normal mucosa, the presence of c-FLIP\textsubscript{L/S} appears insufficient to block apoptosis, possibly because of its low levels and/or the possibility that molecules other than c-FLIP\textsubscript{L/S} play major roles in the anti-apoptosis mechanisms. We speculate that both forms of c-FLIP protein can inhibit apoptosis of gastric adenocarcinoma, since they showed nearly equal expression levels in the present study. Further studies in vitro are needed to clarify this.

As c-FLIP\textsubscript{L/S} was specifically highly expressed in gastric adenocarcinoma, we set out to determine if the expression of c-FLIP\textsubscript{L/S} was correlated with clinicopathological parameters. In the present study, no correlation was found between c-FLIP\textsubscript{L/S} expression and the clinicopathological parameters, such as patient’s age, sex,
site of tumour, histological grade, Lauren classification, tumour size, distance of metastasis or UICC stage. But c-FLIPL S mRNA and protein expression were significantly stronger in lymph-node-metastasis-positive tumours than in negative ones. Notably, tumours with lymph node metastasis all showed intense immunostaining of c-FLIPL. The possible explanation for this phenomenon may be as follows. (a) Metastasis of cancer cells from the primary tumour, which ultimately results in recurrence of disease, involves detachment of cells from the matrix. This event triggers apoptosis in most adherent cells; however, cells that express anti-apoptotic genes may survive and continue to divide [33]. These cells migrate and re-attach in secondary sites. Such micrometastasis remains dormant when tumour cell proliferation is balanced by an equivalent rate of apoptosis, but exhibits rapid growth when apoptosis is inhibited [34]. It is possible that apoptotic inhibition due to increased expression of c-FLIPL S may provide more diverse survival advantages for detached gastric cancer cells from the primary tumour and further promote their growth in secondary sites, such as lymph node. (b) Recently, a number of studies [35–42], including our previous results, strongly suggested that VEGF (vascular endothelial growth factor) in gastric cancer might have a higher potential for the invasation of cancer cells into the lymphatic vessels, leading to lymph node involvement. Interestingly, Suhara et al. [43] found that VEGF can promote c-FLIP expression via the phosphoinositide 3-kinase signalling pathway, resulting in protection of endothelial cells against Fas-mediated apoptosis. It has also been shown that the matrix-derived angiogenesis inhibitor canstatin specifically induces apoptosis in endothelial cells, and this toxicity has been correlated with a down-regulation of c-FLIP [44]. So it is reasonable to deduce that increased expression of c-FLIP may inhibit the pro-apoptosis effect of canstatin on endothelial cells and co-operate with VEGF to promote angiogenesis and lymphogenesis. Of course, further studies in gastric cancer cell lines and tissues are needed to validate this.

In summary, our results suggest that the over-expression of c-FLIPL S is tumour specific, which may be one of the in vivo mechanisms by which tumour cells escape from apoptotic death during malignant transformation. Furthermore, c-FLIPL S may play an important role in lymph node metastasis of gastric adenocarcinoma, which ultimately contributes to the tumour progression.

ACKNOWLEDGMENTS

This work was supported by the Scientific Research Foundation (SRF) for the Returned Overseas Chinese Scholars (ROCS), State Education Ministry, China [grant number (2003)14].

REFERENCES

FLICE-inhibitory protein in gastric cancer


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Received 15 July 2003/28 October 2003; accepted 24 November 2003
Published as Immediate Publication 24 November 2003, DOI 10.1042/CS20030238