Influence of survivin and caspase-3 on cell apoptosis and prognosis in gastric carcinoma

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AIM: To evaluate the role of survivin and caspase-3 in apoptosis of gastric carcinoma, as well as in prognosis of patients with gastric carcinoma.

METHODS: Expressions of survivin and caspase-3 were investigated immunohistochemically in 80 gastric carcinoma patients without a history of chemo-radiation therapy. Tumor cell apoptosis was examined by TUNEL method.

RESULTS: Immunohistochemical analysis showed that survivin expression was positive in 61 of 80 patients (76%) with gastric carcinoma. In contrast, no expression of survivin was detected. Expression level of survivin was associated with histological grades and pathological stages. Expression of caspase-3 was significantly associated with histological stages, but not with the pathological stages. Although survivin expression in carcinoma was not inversely related to caspase-3, patients with survivin (-) and caspase-3(+) had the maximum apoptosis index.

CONCLUSION: Expression level of survivin was associated with histological grades and pathological stages of the tumor, indicating that survivin may be a poor prognosis factor for gastric carcinoma. Unlike caspase-3, survivin (an apoptosis inhibitor) can markedly inhibit the apoptosis of tumor cells.

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INTRODUCTION
Abnormalities in cell death control are implicated as a cause or contributing factor in a range of diseases, including cancer, autoimmunity, and degenerative disorders[11]. This control involves several proteins that promote or inhibit apoptosis and an evolutionarily conserved multistep cascade[2]. A number of proteins, such as Bcl-2, Fas and Bax affect upstream of the cascade[3,4]. Survivin, a recently discovered inhibitor of apoptosis, may prolong cell survival by targeting the terminal effector caspase-3[5,6]. Located at the end of cascade, caspase-3 acts as both initiators and executors in the apoptotic process. So survivin and caspase-3 have been the focus of debate regarding apoptosis.

In the last decade, molecular abnormalities of tumor cells have emerged as important prognostic indicators of gastric carcinoma. As a candidate molecule to influence the apoptosis balance, survivin has unique properties such as undetectable in normal adults tissues and overexpression in a variety of human cancers in vivo[7]. Although studies indicated that survivin was a prognostic tumor marker[8-13], little is known about its potential role in gastric carcinoma. In this study we sought to investigate the expression of survivin and caspase-3 in gastric carcinoma and to dissect their potential prognostic value, and discuss the relationship between survivin, caspase-3 and tumor cell apoptosis.

MATERIALS AND METHODS

Patients and samples
A total of 80 patients with gastric adenocarcinoma did not receive any treatment prior to surgery. Of them 56 were males and 24 were females, with a mean age of 60 years. Surgically resected specimens were fixed in 10% neutral formalin, embedded in paraffin, and stained by haematoxylin-eosin. Histological grades and pathological stages were conformed to the criteria of UICC (Figure 1). The subjects consisted of 17 cases in stage I, 34 cases in stage II, and 29 cases in stage III. Tumor tissues and normal tissues from every patient were detected.

Immunohistochemical staining for survivin and caspase-3
A pilot study using the anti-survivin antibody and anti-caspase-3 antibody was conducted on various neoplasms, including gastric carcinoma, lung cancer, breast cancer and non-Hodgkin’s lymphoma to determine an appropriate dilution. The immunostaining was performed, and negative control slides processed without primary antibody were incubated for each staining. Paraffin-embedded slides were deparaffinized and put in 400 mL EDTA solution (0.001 mol/L, pH 6.0). Then the solution was heated in a pressure cooker and boiled for 2 min while maintaining the pressure. After cooling the slides were incubated with the primary antibody (mouse anti-human survivin or caspase-3 monoclonal antibody purchased from NEO MAEKERS) overnight at 0 °C and rinsed by PBS (0.01 mol/L, pH 7.4) three times. Then the slides were incubated with an anti-mouse conjugate containing horseradish peroxidase at 37 °C for 30 min and rinsed by PBS three times. Finally 3,3-diaminobenzidine was used for color development and hematoxylin was used for counterstaining. The mean percentage of positive tumor cells was determined in at least five areas at 400-fold magnification and assigned to one of the following five categories[14]: -, <5%; +, 5-25%; ++, 26-50%; ++++, 51-75%; +++++, >75%.
Histochemical detection of apoptosis

All cases received detection of apoptosis except those with both survivin and caspase-3 negativities. Apoptotic cells and apoptotic bodies were detected by in situ labeling using a TUNEL kit purchased from Borrinman Company. In brief, deparaffinized and rehydrated sections were digested with proteinase K for 20 min at room temperature and washed. After quenching in 30 mL/L hydrogen peroxide for 10 min and washing with PBS, terminal deoxynucleotidyl transferase enzyme was pipetted onto the sections, which were then incubated at 37 °C for 1 h. After stopping the reaction by putting sections in PBS and washing, anti-digoxin-peroxidase was added to the slides. Finally slides were washed with PBS, stained with 3,3-diaminobenzidine, and counterstained with hematoxylin. Substitution for terminal deoxynucleotidyl transferase with distilled water was used as negative control. Positive cells were determined according to the method described previously [15]. In brief, positive cells had dark or dark brown nuclei and some morphological characteristics, including chromatin condensation, nuclear disintegration, and formation of crescent caps of condensed chromatin at the nuclear periphery. Counting method was the same as described previously.

Statistical analysis

Differences of positivity rates between different groups were assessed by t-test. Kruskal-Wallis rank sum test was used to assess the differences between ranked data. Linear correlation hypothesis test was used to evaluate the extent of correlation between two groups. All of the statistical analyses were performed with SAS statistical package.

RESULTS

Immunohistochemical staining revealed that anti-survivin mAb 8E2 specifically reacted with gastric carcinoma cells, with positive staining in cytoplasm and near the Golgi apparatus, whereas no expression of survivin was observed in adjacent normal tissues. A total of 61 cases of gastric carcinoma in this series were defined as positive staining (76%, Figure 2), with the mean percentage of 29.83%.

Of the 80 cases of gastric carcinomas, 75 cases (94%) of the adjacent normal tissues were positive for caspase-3, while 68 cases (85%) of the tumors were caspase-3 positive (Figure 3). Student’s t-test showed that caspase-3 expressed higher in normal tissue than in carcinoma. Survivin and caspase-3 were not positive at the same position in cancer cells. Expression of survivin in carcinomas showed a negative but not linear correlation with that of caspase-3 ($r=-0.18, P>0.05$).

Through Kruskal-Wallis rank sum test, we found that the expression of both survivin and caspase-3 had significant differences between tissues with different histological grades (Tables 1, 3). The expression of survivin was significantly associated with pathological stages, but caspase-3 was not (Tables 2, 4).
Table 1  Correlation between histological grades and survivin expression

| Positive degree | Poorly differentiated | Moderately differentiated | Well differentiated | Sum | Mean ranks |
|-----------------|-----------------------|---------------------------|--------------------|-----|------------|
| +               | 1                     | 13                        | 4                  | 18  | 9.5        |
| ++              | 8                     | 11                        | 1                  | 20  | 28.5       |
| +++             | 10                    | 5                         | 1                  | 16  | 46.5       |
| ++++            | 4                     | 3                         | 0                  | 7   | 58         |
| n               | 23                    | 32                        | 6                  | 61  |             |

Hc=12.8, P<0.005 between poorly, moderately and well differentiated gastric carcinomas.

Table 2  Correlation between pathological stages and expression of survivin

| Positive degree | Stage I | Stage II | Stage III | Sum | Mean ranks |
|-----------------|---------|----------|-----------|-----|------------|
| +               | 8       | 6        | 4         | 18  | 9.5        |
| ++              | 3       | 15       | 2         | 20  | 28.5       |
| +++             | 1       | 3        | 12        | 16  | 46.5       |
| ++++            | 13      | 26       | 22        | 61  |             |

Hc=15.1, P<0.005 between stages I, II and III.

Figure 3  Expression of caspase-3 in gastric carcinoma. A: well differentiated gastric carcinoma; B: Moderately differentiated gastric carcinoma; C: Poorly differentiated gastric carcinoma; D: Substitution for antibody with PBS as negative control (Original magnification: ×200).

Table 3  Correlation between histological grades and expression of caspase-3

| Positive degree | Poorly differentiated | Moderately differentiated | Well differentiated | Sum | Mean ranks |
|-----------------|-----------------------|---------------------------|--------------------|-----|------------|
| +               | 6                     | 2                         | 0                  | 8   | 3.5        |
| ++              | 10                    | 14                        | 1                  | 25  | 17.5       |
| +++             | 8                     | 19                        | 2                  | 29  | 42.5       |
| ++++            | 1                     | 1                         | 4                  | 6   | 63         |
| n               | 25                    | 36                        | 7                  | 68  |             |

Hc=11.7, P<0.005 between poorly, moderately and well differentiated gastric carcinomas.

Figure 4  Apoptosis in gastric carcinoma. A: Positive; B: Substitution for terminal deoxynucleotidyl transferase with distilled water as negative control (Original magnification: ×200).

Table 4  Correlation between pathological stages and expression of caspase-3

| Positive degree | Stage I | Stage II | Stage III | Sum | Mean ranks |
|-----------------|---------|----------|-----------|-----|------------|
| +               | 1       | 5        | 2         | 8   | 4.5        |
| ++              | 7       | 7        | 11        | 25  | 21         |
| +++             | 5       | 14       | 10        | 29  | 48         |
| ++++            | 3       | 2        | 1         | 6   | 65.5       |
| n               | 16      | 28       | 24        | 68  |             |

Hc=0.54, P>0.75 between stages I, II and III.
Apoptotic cells and apoptotic bodies were observed in gastric carcinoma by using in situ labeling (Figure 4). The mean apoptotic index (AI) of the 80 cases was 0.84%. The mean AI in survivin-positive tumors was 0.59%, which was significantly lower than the mean AI of 1.26% observed in survivin-negative tumors ($P < 0.005$). The mean AI in caspase-3-positive tumors (0.97%) was significantly higher than that in caspase-3-negative tumors (0.56%, $P < 0.05$). Tumors with survivin(-) and caspase-3 (+) had the highest AI of 1.58%.

**DISCUSSION**

Recently, several inhibitors of apoptosis (IAP) related to the baculovirus IAP gene have been identified in humans, mice, and *Drosophila*. Recombinant expressions of IAP proteins counteract various forms of apoptosis *in vivo* and *in vitro*. These molecules are thought to block an evolutionarily conserved step in apoptosis. At least in the case of X-linked IAP, this may involve direct inhibition of the terminal effectors caspase-3 and caspase-7 through a BIR-dependent recognition. Among the recently described IAP family, survivin is characterized by a unique structure with a single BIR and no-zinc-binding domain known as the RING finger and by the selective distribution in common human cancers. In this study, specific staining for survivin was detected in 61 cases (76%), with a variable proportion of positive tumor cells (10-85%). In contrast, the adjacent normal tissues or the infiltrating lymphocytes did not express survivin, consistent with similar studies. As the histological differentiation decreased and pathological stage increased, positivity rate and expression level of survivin were elevated. So survivin expression has prognostic value in human gastric carcinoma. Alessandra reported that survivin was expressed in G2-M phase of the cell cycle in a cell cycle-regulated manner and associated with mitotic spindle microtubules. In this study, survivin-positive patients had lower AI as compared with survivin-negative patients, suggesting that the overexpression of survivin in cancer might obliterate the checkpoint of the cell cycle and allow aberrant progression through the G2-M phase checkpoint in gastric carcinoma.

In normal gastric tissues, caspase-3 was mainly expressed in gastric surface mucous cells, being in accord with the gastric cell turnover. Caspase-3 expression was increased in well differentiated tumors and apoptotic cells were increased in apoptotic cells. It is well known that a number of genetic alterations are required for malignant transformation. Therefore we can speculate that abnormal differentiation leads to decreased expression of caspase-3 in tumor cells.

As a key effector molecule of apoptosis, caspase-3 can inactivate number proteins, which are associated with the structure and cycle of normal cells. Survivin showed an inversed function compared with caspase-3, which can be illustrated by the results that cases with survivin (-) and caspase-3 (+) had higher AI than cases with survivin (+) and caspase-3 (-). Then what we wanted to know was, if survivin inhibited apoptosis in tumor cells? This issue was that survivin inhibited caspase pathway of apoptosis, caspase-9 deactivation first of the relevant points to this issue was that survivin induced linear correlation. So we think survivin did not inhibit the caspase-3 in tumor cells.

Abnormal differentiation leads to decreased expression of survivin in colorectal cancer, rates in colorectal cancer. Cancer Res 1998; 58: 5315-5320. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 1997; 3: 917-921. Adida C, Haioun C, G malignancies. Cancer Res 1998; 58: 5071-5074. Islam K. Role of survivin, whose gene is mapped to 17q25, in human neuroblastoma and identification of a novel dominant-negative isoform, survivin-beta'.B. Med Pediatr Oncol 2000; 35: 550-553. Tanaka K, Iwamoto S, Gondal N, Nakagawara A. Expression of caspase-3 promoter apoptosis of tumor cells. It is well known that a number of genetic alterations are required for malignant transformation. Therefore we can speculate that abnormal differentiation leads to decreased expression of caspase-3 in tumor cells.

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As long as survivin affects terminal effector of apoptosis and exists in tumor cells, it would be an ideal target of apoptosis-based therapy. One of the roles of chemotherapy is inducing apoptosis, and caspase-3 has been proved to participate in this process, so acting on this target might also enhance sensitivity to chemotherapy or reduce the effect of drug-resistance. A recent *in vitro* study demonstrated that anti-survivin RNA down-regulated the expression of endogenous survivin in transformed cells and induced apoptotic cell death. Targeted antagonists of survivin may offer a new therapeutic method for gastric carcinoma. A homeland study revealed that antisense oligonucleotide targeting survivin induced decrease of survivin expression, increase of cell apoptosis, inhibition of cell proliferation in hepG2 cells. It also has been reported that survivin-based plasmids could induce apoptosis in gastric cancer cells and sensitize gastric cancer cells to chemotherapeutic agents. Gene therapy targeting survivin gene expression may offer a new approach to cancer therapy.
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