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Anti-apoptotic proteins, apoptotic and proliferative parameters and their prognostic significance in cervical carcinoma

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Abstract

The inhibitor of apoptosis proteins (IAP) suppress apoptosis induced by a variety of stimuli. The aims of this study were to: (a) compare the expression of X-linked IAP (Xiap) and Human IAP-2 (Hiap-2) in cervical carcinoma cells and normal cervix, (b) determine the correlation between IAP expression and tumour apoptosis or proliferation, and (c) assess their prognostic significance in cervical carcinomas. Paraffin-embedded tissue sections were retrieved from 77 patients with cervical squamous carcinomas prior to treatments and 47 normal subjects. Tumour apoptosis was determined by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuracil triphosphate (dUTP) nick-end labelling (TUNEL) and apoptotic index (AI), and the proliferative rate was measured by Ki-67 and mitotic (MI) indices. Immunoreactive Xiap and Hiap-2 were found in both cervical cancer cells and normal tissues. IAP expressions in cancers did not correlate with apoptotic and proliferative parameters, disease stage and patient survival. The lower AI and Ki-67 index were associated with a better survival. In conclusion, the basal expression levels of IAPs have no prognostic significance, but AI and Ki-67 expression are potential prognostic indicators in cervical carcinoma.

Keywords: Inhibitor of apoptotic protein (IAP); Apoptotic (AI) and mitotic indices (MI); Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuracil triphosphate (dUTP) nick-end labelling (TUNEL); Ki-67 index; Immunohistochemical staining; Cervical carcinoma and prognosis

1. Introduction

It is now accepted that tumour growth is the result of a balance between cell proliferation and physiologically occurring cell death, often referred to as apoptosis [1–4]. Apoptosis is a physiological process critical for organ development, tissue homeostasis and elimination of defective or potentially dangerous cells in a complex organism [5]. It can be initiated by a wide variety of stimuli and regulated by the balance of pro-apoptotic and anti-apoptotic activities within the cell. Deregulation of the apoptotic pathway has been implicated in the pathogenesis of many human diseases, including cancer and autoimmune diseases [6].

The inhibitor of apoptosis proteins (IAP) is a family of anti-apoptotic intracellular proteins that are highly conserved across several species. The baculovirus IAPs, Cp-IAP and Op-IAP, were the first members of this family to be discovered based on their ability to block apoptosis induced by a wide number of apoptotic triggers [7,8]. Five human IAPs (neuronal apoptosis inhibitory protein (NAIP), Human IAP-1 (Hiap-1), human IAP-2 (Hiap-2), X-linked IAP (Xiap) and survivin) have since been identified [9–13]. Although the mechanism by which the IAPs suppress cell death remains unclear, it has been reported that IAPs play a central role in the regulation of a number of physiological and pathological processes by inhibiting a family of 'death' proteases called the caspases. Xiap, Hiap-1 and Hiap-2 suppress apoptosis by binding and inhibiting the activation of caspase-3 and caspase-7 [14,15]. Hiap-1 and Hiap-2 inhibit tumour necrosis factor (TNF) receptor signalling

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by binding to the TNF receptor associated factor [9]. Survivin, normally expressed in fetal and not in adult differentiated tissues, is highly expressed in most cancers including those of the lung, colon, breast, prostate, pancreas and stomach, and has been suggested to be an important aetiological factor in oncogenesis and of prognostic significance in some cancers [13,16].

Although apoptosis has been suggested to play an important role in the development and progression of cervical carcinomas [2,3,17,18], the role of the IAP family in cervical carcinomas has not yet been investigated. In this study, we have assessed the expression of Xiap and Hiap-2, as well as apoptotic and proliferative parameters in cervical carcinoma and normal cervix, to determine whether basal IAP expression correlates with tumour apoptosis and cell proliferation, and their prognostic significance in cervical carcinoma.

2. Patients and methods

2.1. Patients

This retrospective study was carried out on 77 patients with squamous cell carcinoma of the uterine cervix from the Department of Obstetrics and Gynaecology, Queen Mary Hospital, the University of Hong Kong between 1987 and 1991. Among them, 28 patients (36%) were early stage (I–IIa) and 49 (64%) were advanced stage (IIb and above) according to the criteria of the International Federation of Gynaecology and Obstetrics (FIGO) classification [19]. The ages of patients ranged from 37 to 89 years, with a mean age of 62.7 years. All patients were treated with radiotherapy and 12 of them (16%) had additional treatment of chemotherapy and/or surgery. Normal cervical tissues from 47 patients undergoing hysterectomy for uterine fibroid were also included as normal controls. Their ages ranged from 40 to 64 years, with a mean age of 48.9 years.

Formalin-fixed and paraffin-embedded cervical tissues of the patients prior to any treatment were retrieved from the Department of Pathology after review of histological diagnoses on haematoxylin and eosin (H&E) stained sections. Sections (5 μm) were cut and mounted on APES (Aminopropyltriethoxysilane, Sigma, USA)-coated slides.

2.2. Immunohistochemistry (IHC)

2.2.1. IAP expression

A standard three-step streptavidin–biotin complex immunohistochemical assay was performed on paraffin sections for the detection of IAPs. Sections were deparaffinised and rehydrated in graded concentrations of ethanol and then in distilled water. Antigen retrieval was performed by microwave pretreatment in 0.01 M citrate buffer for 20 min. After cooling, sections were treated with 3% H_2O_2 to quench the endogenous peroxidase activity. Non-specific binding of the antibody was blocked by incubation in the blocking buffer (LSAB kit, DAKO, Glostrup, Denmark) for 30 min. Specific polyclonal antibodies of Xiap and Hiap-2 (kindly provided by Dr Eric LaCasse, ApoptoGen Inc., Ottawa, Ontario, Canada) were diluted 1:50 and applied for a 2 h incubation at 37°C. After extensive washes with phosphate-buffered solution (PBS), the sections were incubated with biotinylated linked antibody and subsequently with peroxidase-labelled streptavidin (LSAB kit) for 30 min for each. The peroxidase was then developed in DAB (Diaminobenzidine, Amresco Inc. OH, USA) with H_2O_2. The sections were counterstained with haematoxylin, dehydrated in graded ethanol, cleared in xylene and mounted. Two cervical cancer sections with known expressions of IAPs were used as positive controls, as well as negative controls where the primary antibody was omitted.

2.2.2. Ki-67 labelling

Tumour growth faction was ascertained by IHC with the monoclonal antibody Ki-67 (Zymed, San Francisco, CA, USA). The IHC procedure was essentially the same as described above for the IAPs. Briefly, antigen retrieval was performed by microwave pretreatment with 0.01 M citrate buffer for 7 min. After non-specific blocking with 10% rabbit serum, Ki-67 antibody was applied in a 1:4 dilution and incubated at 4°C overnight. A subsequent reaction with biotinylated rabbit antimouse (DAKO) and StreptABComplexes/HRP (DAKO) was carried out in dilutions of 1:100 for both. One known cervical cancer sample was included for positive or negative (primary antibody replaced with 10% rabbit serum) controls.

2.3. TUNEL Assay

Apoptotic cells were detected by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuracil triphosphate (dUTP) nick-end labelling (TUNEL), using an *in situ* cell death detection kit (Boehringer Mannheim, Mannheim, Germany). The assay was performed as per the manufacturer's instructions, with minor modifications. Briefly, after routine deparaffinisation and treatment with 3% H_2O_2, sections were digested with proteinase K (20 mg/ml; 15 min) and incubated with the reaction mixture (1:100; 30 min) at 37°C. Incorporated fluorescein was detected with an antifluorescein antibody conjugated with horse-radish peroxidase (POD) after a 30 min incubation at 37°C and subsequent colour development with DAB. One known cervical cancer sample was used as positive or negative (absence of reaction mixture) controls.

2.4. Assessment of apoptotic cells and mitotic figures

Seventy-four samples with sufficient numbers of tumour cells for assessment were stained with H&E. Apoptotic cells and mitotic figures were counted from at least 10 randomly chosen high-power fields (HPFs). Cells were considered apoptotic according to the following criteria [20]: dense condensed nodular hyperchromatic nuclei, fragmentation of chromatin, deep eosinophilia of the cytoplasm, cell shrinkage, and absence of lymphocyte infiltration and inflammation.

2.5. Evaluation

Sections were examined at ×400 magnification. The immunostaining of IAPs was estimated and graded as percentages of positive cancer cells as follows: 0, negative; 1, ⩽5%; 2, 6–25%; 3, 26–50%; 4, 51–75% and 5, >75%. The intensity of the positive staining was scored as: 1, weak; 2, moderate and 3, intense. The immunoreactivity score for each case was the sum of the values of the two parameters.

Cells with clear brown nuclear labelling were defined as Ki-67- or TUNEL-positive. The Ki-67 index and TUNEL labelling were expressed as percentages of positive cancer cells and graded into six groups as above. Apoptotic (AI) and mitotic (MI) indices were defined as the total numbers of apoptotic cells and mitotic figures seen in cancer cells per ten HPFs on H&E stained sections, respectively.

2.6. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS). Kaplan–Meier test was used for the survival estimation. Chi-Square test was applied for nominal data and Mann–Whitney U test was used to compare between groups. A P value of less than 0.05 ($P<0.05$) was considered as statistically significant.

3. Results

3.1. Expression of IAPs

Seventy-seven cervical carcinomas and 47 normal cervices were screened for IAPs. The staining patterns of both Xiap and Hiap-2 were diffused and heterogeneous. The immunoreactivities were mainly localised to the cell cytoplasm, although nuclear staining was sometimes observed (Fig. 1a–d). Both IAPs were highly expressed in cancer cells and in the majority of the normal epithelial cells. The expression of Hiap-2 protein in normal cervical tissue was primarily present in the intermediate epithelial cells, less in the basal and superficial layers. However, Xiap expression was spread more evenly among the three layers (Fig. 1c and d).

Table 1
The expression of Hiap-2 and Xiap proteins in cervical carcinomas and normal cervices

	Hiap-2		Xiap	
IHC	Cancer (77) *n* (%)	Normal (47) *n* (%)	Cancer (77) *n* (%)	Normal (47) *n* (%)
Positive				
>25	50[a] (65)	11[a] (23)	70[b] (91)	42[b] (89)
⩽25	2 (3)	20 (43)	2 (3)	5 (11)
Negative	25 (32)	16 (34)	5 (6)	0

IHC, immunohistochemistry.
[a] $P<0.001$.
[b] $P>0.05$.

Table 1 summarises the distribution of Hiap-2 and Xiap in carcinomas and normal cervical samples. Hiap-2 expression was similar in both cancer and normal groups (68 versus 66%, respectively). However there was significantly more cancer samples with over 25% reactivity than normal cervical samples (65 versus 23%; $P<0.001$). High expression of Xiap was observed in most of the cancers (94%) and in all normal (100%) samples. No difference was found between these two groups.

3.2. Tumour apoptosis

3.2.1. TUNEL labelling

TUNEL signal was defined by distinct nuclear staining (Fig. 1e). The sections were considered TUNEL positive if >5% of the cancer cells showed immunoreactivity. Among the 16 normal cervices assessed, five (31%) showed <5% immunoreactivity and the rest were negative. Positive TUNEL labelling was observed in 52 out of 74 cancers (70%; Table 2). Thirty-eight cancer samples (51%) exhibited equal to and more than 5% of cells being TUNEL positive, a response significantly higher than that in the normal samples

Table 2
TUNEL labelling and Ki-67 index in cervical carcinomas and normal cervices

	TUNEL		Ki-67	
IHC	Cancer (74) *n* (%)	Normal (16) *n* (%)	Cancer (74) *n* (%)	Normal (16) *n* (%)
Positive				
>5	38[a] (51)	0[a]	40[b] (54)	0[b]
<5	14 (19)	5 (31)	31 (42)	16 (100)
Negative	22 (30)	11 (69)	3 (4)	0

IHC, immunohistochemistry; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuracil triphosphate (dUTP) nick-end labelling.
[a] $P<0.001$.
[b] $P<0.001$.

($P<0.001$). Fourteen (19%) and 22 (30%) cancers showed only background ($<5\%$) or negative apoptosis.

3.2.2. Apoptotic index

AI was evaluated in 74 cancers with sufficient materials to count 10 consecutive HPFs. The numbers of apoptotic cells ranged from 0 to 18 per 10 HPFs (median = 5). Forty-seven samples (64%) showed apoptotic cells above the median (Table 3).

3.3. Tumour cell proliferation

3.3.1. Ki-67 index

As shown in Fig. 1f, Ki-67 immunoreactivity was clearly evident in the nuclei of proliferating cells. In 16 normal cervical tissues, the Ki-67 labelling (immunoreactivity $<5\%$) was confined to the epithelial cells in the basal and parabasal regions. The intermediate and superficial cell layers were nearly negative. In 74 cancers, the intensity of Ki-67 labelling was markedly higher and reached statistical significance ($P=0.008$) when compared with those in normal tissues (Table 2). Forty cancers (54%) were equal to and more than 5% positive and 31 (42%) displayed a low Ki-67 index ($<5\%$). Negative staining was observed in three cancer samples (4%).

3.3.2. Mitotic index

MI was assessed in 74 cancer samples, which were also scored for AI. The numbers of mitotic figures

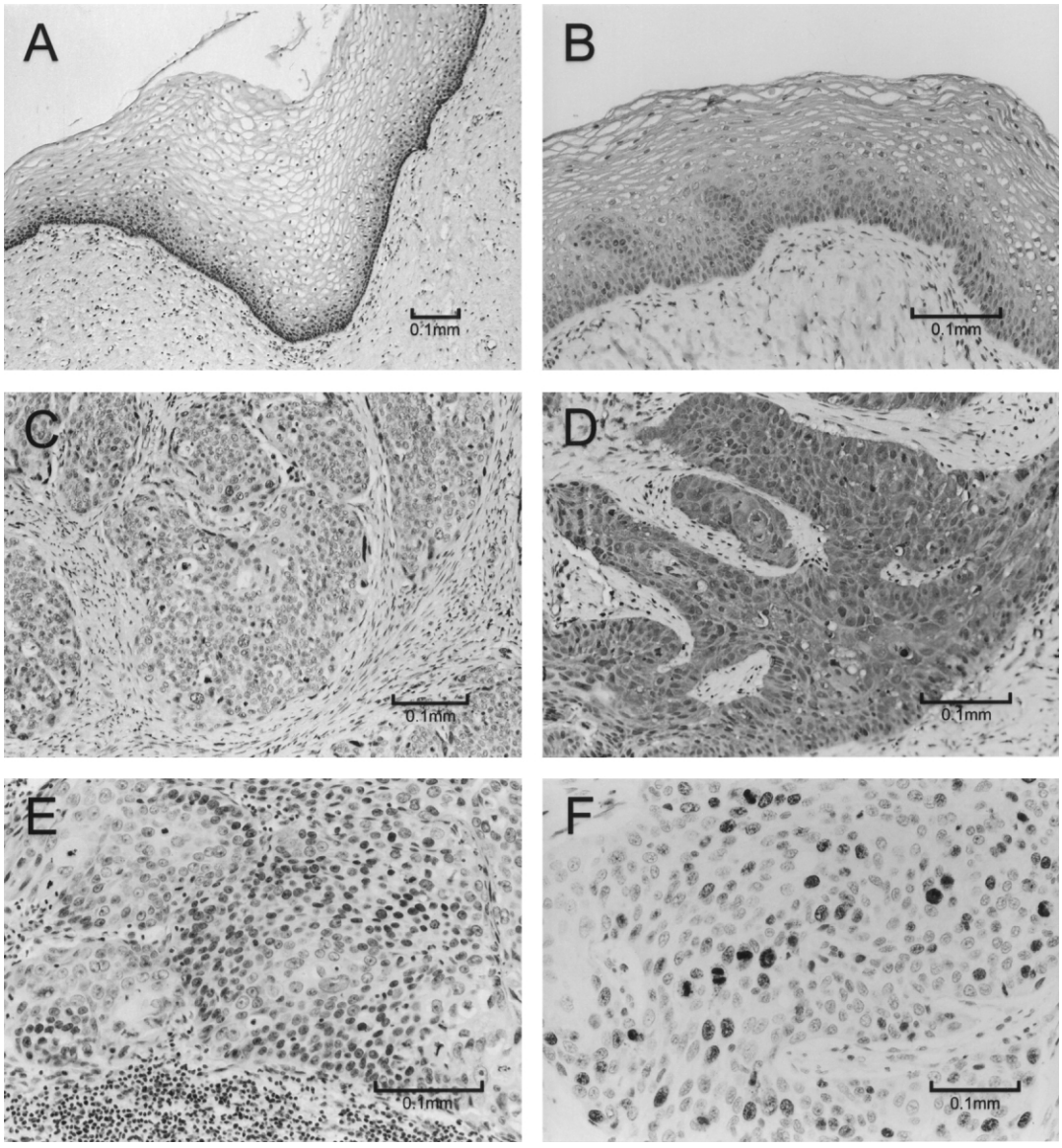

Fig. 1. The expressions of Hiap-2 (a) and Xiap (b) proteins in normal cervical epithelial cells. Immunoreactivity of Hiap-2 was primarily found in the intermediate cells, less in basal and superficial cells, whereas Xiap was presented in three layers; (c) and (d) the expression of Hiap-2 and Xiap in cervical carcinomas; (e) and (f), positive TUNEL and Ki-67 labelling in the cervical carcinomas.

Table 3
Results of AI and MI in cervical carcinomas

Parameters	Cancer (*n*)	Range (*n*/10 HPFs)	Median	<Median *n* (%)	⩾Median *n* (%)
AI	74	0–18	5	27 (36)	47 (64)
MI	74	1–103	14	33 (45)	41 (55)

AI, apoptotic index; MI, mitotic index; HPF, high power field.

ranged from 1 to 103 per 10 HPF (median = 14). Samples were divided into those with a MI equal to and above or below the median. Forty-one (55%) had a MI equal to and above the median and 33 (45%) were below it (Table 3).

3.4. Correlation of clinical and laboratory parameters

The expression of Xiap and Hiap-2 in cervical carcinomas correlated neither with tumour apoptosis (including TUNEL labelling and AI) nor tumour proliferation (including Ki-67 index and MI). The IAPs did not show prognostic significance in terms of disease stages (Xiap, $P = 0.71$; Hiap-2, $P = 0.34$) and patient survival (Xiap, $P = 0.28$; Hiap-2, $P = 0.16$). Tumour apoptosis was determined by TUNEL and AI. Increased AI, but not TUNEL labelling, was positively correlated with MI ($P = 0.001$; Fig. 2). The patients with a tumour AI equal to and above the median had a worse outcome than those with an AI below the median ($P = 0.04$; Fig. 3a), although there was no significant correlation between AI and disease stage ($P = 0.28$). Unlike AI, increased TUNEL labelling (>25%) was significantly associated with advanced stage of disease ($P = 0.04$), but not with patient survival ($P = 0.21$) (data not shown). Tumour proliferation was assessed by Ki-67 index and MI. A low Ki-67 index was associated with better survival ($P = 0.005$; Fig. 3b). MI showed a similar trend with survival, but did not reach statistical significance ($P = 0.24$, data not shown). The survival rate for early stage (I–IIa) disease was 77.8% which was significantly higher than the advanced stage (IIb and above) disease of 48% ($P = 0.007$).

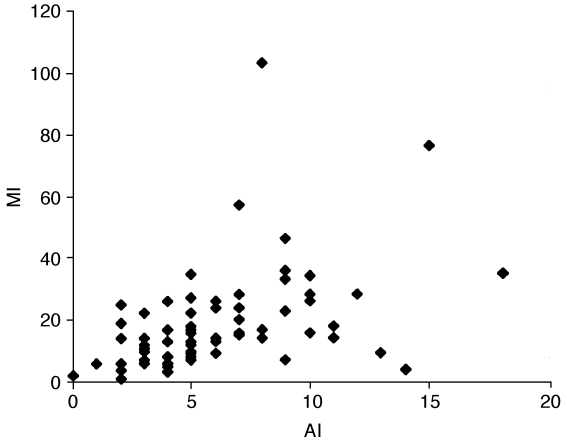

Fig. 2. Scatter plots of apoptotic index (AI) versus mitotic index (MI) obtained from cervical carcinomas ($n = 74$). There was a positive correlation between them ($P = 0.001$).

4. Discussion

Suppression of apoptosis is thought to contribute to tumorigenesis by aberrantly prolonging cellular life span, permitting growth factor-dependent cell survival, promoting resistance to immune-based cytotoxicity, and allowing disobeyance of cell cycle checkpoints that would normally induce apoptosis [21]. Several genes critical in the regulation of apoptosis have been identified, including the anti-apoptotic gene family IAPs. Recent studies in various cancer cells have suggested that the expression of survivin may play an important role in oncogenesis [13,16]. However, the expression of

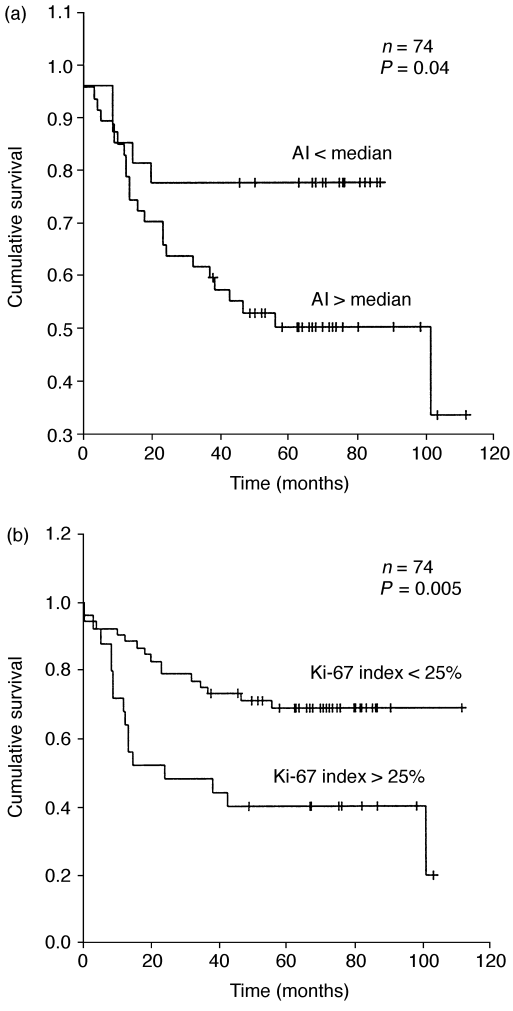

Fig. 3. Cumulative patient survival as a function of apoptotic index (a) stratified as above or below the median AI, or Ki-67 index (b) stratified as immuoreactivity more than or less than 25%. Survival was significantly worse if AI > median ($P = 0.04$) or ($P = 0.005$).

other IAPs has not been thoroughly investigated in tumours. Liston and colleagues [11] reported high Hiap-1 and Hiap-2 expression in many normal tissues including kidney, small intestine, liver and lung, and low expression in the central nervous system. Similarly, *Xiap* mRNA was also observed in most of adult and fetal tissues, except peripheral blood leucocyte [11]. In ovarian cancer cell lines, expression of Xiap and Hiap-2 was high in the proliferating cells and low in the apoptotic ones, suggesting that these intracellular proteins may play a significant role in suppressing apoptosis and may be an important determinant in the chemosensitivity of ovarian cancer cells [22].

In order to study the function of IAPs in cervical carcinomas, we assessed the expression of Xiap and Hiap-2 in normal cervical tissues and cervical squamous carcinomas, prior to surgery or radiotherapy. We found that both IAPs were highly expressed in cancers and most of normal cervices, although Hiap-2 expression (immunoreactivity > 25%) in cancer tissues was significantly higher. Expression of these IAPs was neither correlated with tumour apoptosis nor proliferation, disease stage and patient survival. Our finding suggested that these two IAPs might function differently in the suppression of apoptosis in cervical cancer. Whether they play a role in cervical carcinogenesis and tumour progression is not known. In addition, IAPs have been shown to be capable of blocking apoptosis induced by a wide spectrum of traumas, including radiation and chemotherapeutic drugs. Whether the expression of Xiap and Hiap-2 in cervical cancer would function in protecting tumours from apoptosis induced by radiotherapy and play some roles in the radioresistance of cancers remain to be investigated.

Apoptosis occurs spontaneously in normal adult tissues to maintain homeostasis. It is also seen in untreated malignant tumours and the frequency of occurrence varies between experimental tumours [2,23]. It has been proposed that spontaneous apoptosis is a mechanism of cell loss in untreated tumours. Since chemo- and radiotherapy enhance the apoptotic process, the possibility exists that their pre-treatment levels may be predictive of the tumour response to the treatment [24,25]. On the contrary, studies have also suggested that high apoptotic cell counts might be linked to high mitotic index in tumour, which having a high proliferative rate, were in fact less responsive to radiation [2,26,27]. Thus, whether apoptosis is associated with tumour progression and poor clinical outcome is unclear. Findings on the prognostic role of apoptosis parameters on clinical outcome in patients with cervical carcinomas treated by radiotherapy are contradictory [2,17]. In a retrospective study by Wheeler and colleagues [17], patients with an AI above the median were found to have a better survival than those with a lower AI level. An opposite conclusion was however reached by Levine and coworkers [2] who demonstrated that increased apoptosis was associated with poor prognosis and AI acted as an independent prognostic factor. A correlation of AI with Ki-67 labelling was observed, but the latter was not predictive of outcome. Our present results are consistent with those of Levine and colleagues [2], in that patients with an AI below the median of 5 were found to have a better survival than those with a higher AI value ($P = 0.04$), although our TUNEL results showed no prognostic significance. AI is therefore an indicator for prognosis in cervical cancer.

The differences between the results obtained from TUNEL and AI may be due to differences in the two methods used. The TUNEL technique is based on the specific binding of TdT to 3′-OH of DNA fragments in apoptotic nuclei, permitting the *in situ* visualisation of apoptotic cells [28]. It can identify cells not only with well established apoptosis, but also those at an early stage of the cell death process when they often appear morphologically healthy and normal. Necrotic cells, however, are sometimes weakly stained by TUNEL method [29]. In contast, morphological assessment on H&E sections mainly depends on detectable changes in cell morphology. The detection could be sometimes missed by the presence of cells at early stages of apoptosis which do not exhibit typical apoptotic features, apoptotic bodies, infiltrated lymphocytes and neutrophils and their fragments, as well as keratinised squamous cancer cells [20]. Using both TUNEL and AI would therefore give a clearer and more comprehensive assessment of apoptosis.

Our results have demonstrated a positive correlation between AI and MI in cervical cancers ($P = 0.001$). Apoptosis is recognised as a distinctive form of genetically programmed cell death and represents the counterpart of mitosis [30]. While a balance between mitotic activity and apoptosis exists in the maintenance tissue homeostasis under normal physiological conditions, it is possible that the observed increased apoptotic activity in cervical tumours may represent a compensatory response to the increased mitotic activity in the neighbouring malignant cells in an attempt to limit tissue growth. Although no correlation was found in our present study between Ki-67 index and AI or TUNEL labelling, this could be due to different cell populations assessed in the determination of the mitotic figure and Ki-67 labelling. The former is equivalent to the fraction of cells at any given moment that are in mitosis, whereas the latter indicates a fraction of cells expressing the nuclear antigen in all phases of the cell cycle except G0. Nevertheless, together they represent cell proliferation and mitotic activity in tumour. Ki-67 index revealed a prognostic significance in cervical cancer by showing a reversed correlation with patient survival.

In conclusion, the present study demonstrated both cervical carcinomas and normal cervical epithelial cells

express Xiap and Hiap-2. Basal expression of these IAPs did not correlate with any apoptotic and proliferative parameters detected and had no prognostic significance. AI and Ki-67 index were potential prognostic indicators in cervical cancer. Further studies are needed to identify whether the expression of Xiap and Hiap-2 may change in post-treatment cancers. They may improve the current understanding of the function of IAPs, particularly in relation to their role in the regulation of apoptosis in response to the radiation treatment.

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