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Original Article

Immunohistochemical analysis of Bax and AIF in colorectal tumors

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Abstract: Background/Aims: This study had three aims. The first was to determine whether Bax (Bcl-2-associated X protein) and AIF (apoptosis-inducing factor) are expressed in tissue sections of colorectal tumors. The second was to ascertain whether there is any difference in Bax and AIF expression between colorectal polyps, adenomas, and carcinomas. The third aim was to determine whether there is any difference between Bax and AIF expression in colorectal tumors. Materials and methods: Bax and AIF expression were determined in 20 hyperplastic polyps (HPs), 20 adenomatous polyps (APs), 20 samples of colorectal carcinomas, and 20 samples of normal mucosa by immunohistochemical methods. Results: The staining level of Bax and AIF in adenomas and carcinomas was significantly higher than in normal tissues ($P<0.01$). There was also a significant difference between HPs and APs ($P<0.01$). The level of Bax and AIF in carcinomas was higher than in adenomas, and the difference was of statistical significance ($P<0.01$). Conclusion: This study may be of interest in future research to confirm whether the changed expression of Bax and/or AIF between benign and malignant tumors can provide valuable information for the evaluation of colon or other tumors.

Keywords: Colorectal neoplasms, apoptosis, apoptosis-inducing factor, Bcl-2-associated X protein, immunohistochemistry

Introduction

Apoptosis, also defined as programmed cell death, is a mechanism that plays a role in immune regulation and normal tissue homeostasis, cellular differentiation, and development. It is characterized by morphological, biological, and molecular genetic modifications [1-3] and by several morphologic nuclear changes, such as chromatin condensation and extensive fragmentation of chromosomal DNA [4, 5].

Apoptosis-inducing factor (AIF) was first identified as a protein involved in caspase-independent apoptosis [2, 6]. When cells are induced to undergo apoptosis, AIF is cleaved by calpain and then translocated from mitochondria to the cytosol and the nucleus [7-9].

AIF causes caspase-independent chromatin condensation and DNA fragmentation, demonstrating that it plays an important role in apoptosis [5, 7, 9]. In addition, AIF is a flavoprotein that can oxidize NADH and NADPH *in vitro* and may participate in the detoxification of reactive oxygen species [7]. A recent study showed that the oxidoreductase activity of AIF conferred resistance to oxidative stress and maintenance of transformation status in colon cancer cells. Because AIF plays important roles both in apoptosis and the survival of cells that are essential for cancer development, it can be hypothesized that AIF is altered by somatic mutation or aberrant expression in human cancers [10].

The Bcl-2 family also plays an important role in regulating the mitochondrial pathway for apoptosis. Bax is a proapoptotic and functions as a promotor of apoptosis. The Bcl-2-associated X protein (Bax) gene creates a 21-kD protein product that exists in both the cytoplasm and the mitochondria in all tissues. As a response to an apoptotic signal, most of the soluble cyto-

Table 1. Results of Bax Immunostaining of colorectal lesions

	HP n=20	Adenomatous Polyp (AP) n=20			Adenocarcinoma (AC) n=20			NCM n=20
		TA n=10	TVA n=6	VA n=4	WD-AC n=8	MD-AC n=7	PD-AC n=5	
Score 0	15	0	0	0	0	0	0	20
Score 1	5	4	0	0	0	0	0	0
Score 2	0	4	3	0	5	2	1	0
Score 3	0	2	2	4	0	4	4	0
Score 4	0	0	1	0	3	1	0	0

HP: Hyperplastic polyp, TA: Tubular adenoma, TVA: Tubulovillous adenoma, VA: Villous adenoma, WD-AC: Well differatiated adenocarcinoma, MD-AC: Moderately differantiated adenocarcinoma, PD-AC: Poorly differatiated adenocarcinoma, NCM: Normal colon mucosa.

Table 2. Results of AIF Immunostaining of colorectal lesions

	HP n=20	Adenomatous Polyp (AP) n=20			Adenocarcinoma (AC) n=20			NCM n=20
		TA n=10	TVA n=6	VA n=4	WD-AC n=8	MD-AC n=7	PD-AC n=5	
Score 0	14	1	0	0	0	0	0	20
Score 1	5	5	0	0	0	0	0	0
Score 2	1	4	2	4	3	0	0	0
Score 3	0	0	0	0	5	4	0	0
Score 4	0	0	4	0	0	3	5	0

HP: Hyperplastic polyp, TA: Tubular adenoma, TVA: Tubulovillous adenoma, VA: Villous adenoma, WD-AC: Well differatiated adenocarcinoma, MD-AC: Moderately differantiated adenocarcinoma, PD-AC: Poorly differatiated adenocarcinoma, NCM: Normal colon mucosa.

plasmic Bax moves to the mitochondria to form membrane-bound dimers [1, 11, 12]. It is currently believed that both the survival and the death of cells play important roles in the pathogenesis of tumors [2, 13]. Loss of apoptosis could allow for the survival of transformed cells that are prone to undergo further genetic damage, thereby playing an important role in the pathogenesis of tumors [2, 7]. Somatic mutations and/or aberrant expression of apoptosis-related genes have been reported in human cancers [14, 15].

In the present study, we analyzed the expression of AIF and Bax protein in colorectal carcinoma tissues immunohistochemically and compared these proteins to each other.

Materials and methods

Formalin-fixed, paraffin-embedded archival tissue blocks from 20 hyperplastic polyps (HPs), 20 adenomatous polyps (APs) (10 tubular adenomas [TAs], 6 tubulovillous adenomas [TVAs], and 4 villous adenomas [VAs]), 20 colorectal carcinomas (8 well differentiated, 7 moderately differentiated, and 5 poorly differentiated) were studied. Twenty samples of normal mucosal tissue were obtained at a distance from tumor or polyp tissue.

Immunohistochemical staining for Bax and AIF was performed on all tissues. Sections 4 m thick were cut and placed on charged slides. For AIF, the slides were deparaffinized and rehydrated, then endogenous peroxidases were blocked by incubation with 3% H_2O_2. Antigen retrieval was accomplished by incubating the slides with 10 mM of citrate buffer at pH 6.0 and microwaving for 20 min. The slides were stained with a primary monoclonal antibody to AIF (1:50; Epitomics).

For Bax, deparaffinization and rehydration were performed using xylene and alcohol. After washing with distilled water, the sections were pretreated in 10 mmol/L of citrate buffer in a microwave oven for 20 minutes. The sections were then treated with 1% H_2O_2 for 20 minutes and a protein block solution (Dako, Carpinteria, CA) for 10 minutes. To determine Bax protein expression, rat monoclonal antibody in 1:50 was used (no.13401A, clone G206-1276, immunoglobulin [Ig] M, 0.5 mg/mL, PharMingen, San Diego, CA) for 90 minutes. Subsequently, the sections were incubated with 1:200 biotinylated anti-rat antibody (Dako A/S, Copenhagen, Denmark) and 1:300 peroxidase-conjugated streptavidin (Dako A/S). Finally, 3,3' diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO) was used for the peroxidase reaction, and hematoxylin for counterstaining.

The intensity of staining was evaluated as follows: score of 0: negative; score of 1: <25% positive cells; score of 2: 26%-50% positive cells; score of 3: 51%-75% positive cells; and score of 4: >75% positive cells.

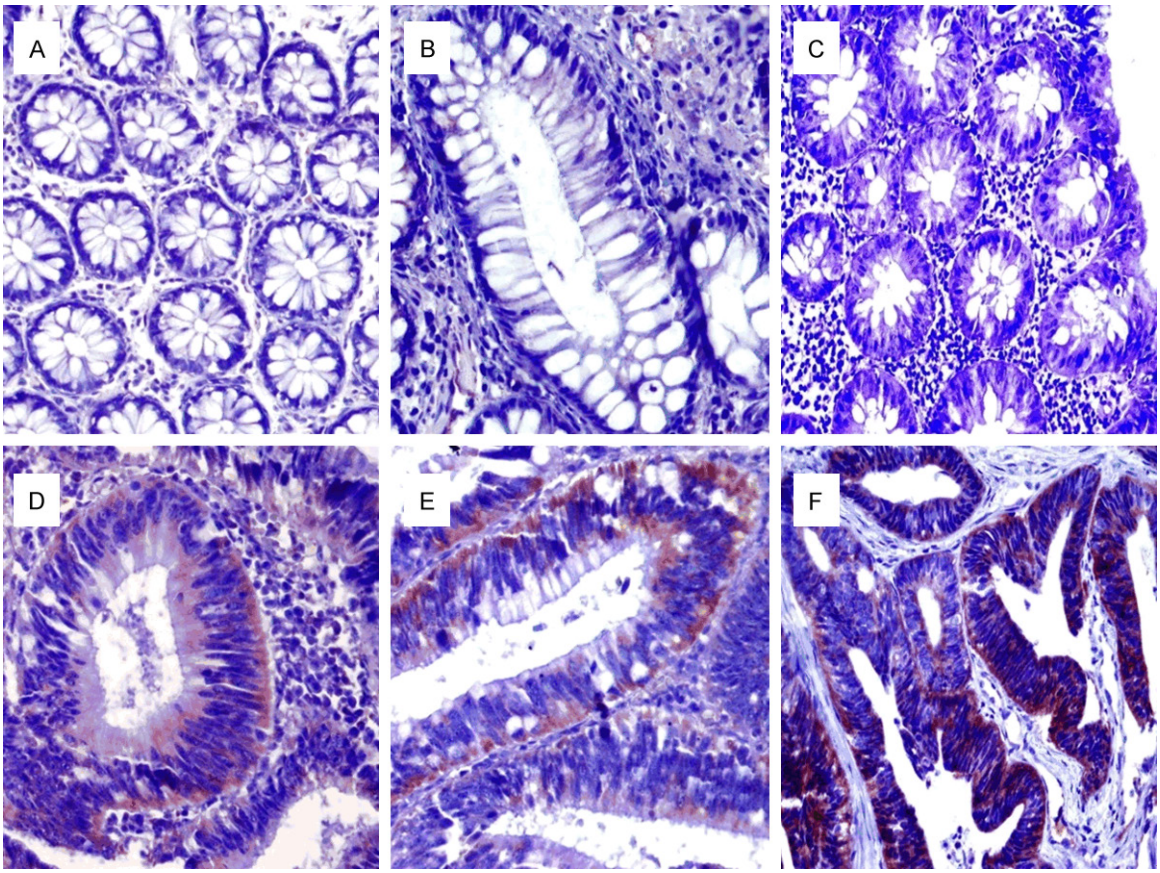

Figure 1. A-F. Normal mucosa (A), Hyperplastic Polyp (B), Tubular Adenoma (C), Tubulovillous Adenoma (D), Villous Adenoma (E), Well differantiated Adenocarcinoma (F), Bax staining × 400.

This study was performed on archived blocks and for this reason did not require ethical approval.

Statistical analysis

For statistical analysis, we used SPSS version 12.0 for Windows (SPSS Inc., Chicago, IL). The results were analyzed using the Kruskal-Wallis test and the Mann-Whitney U test. A value of $P<0.01$ was considered statistically significant.

Results

The intensities of Bax and AIF protein expression in colorectal tumors are presented in **Tables 1** and **2**. In general, the lowest amount of AIF and Bax staining was seen in HPs, with only 5 of 20 HP samples (25%) being weakly positive (score of 1) for Bax staining. Fifteen of 20 samples (75%) did not have any Bax staining (score of 0). AIF was also weakly stained, with 5 of 20 samples (25%) being weakly positive (score of 1). Only one sample (5%) had a score of 2, and 14 of 20 examples (70%) did not have any AIF staining (score of 0). AIF staining was identified in all TVAs, VAs, and carcinomas. The staining intensity varied between the different types of colorectal tumors. Normal colon mucosa did not show significant activity.

For the TAs, 1 of 10 cases (10%) was negative for AIF, 4 of 10 (40%) had stain scores of 2, and 5 of 10 (50%) had weak (score of 1) staining. Bax was also positive all TA cases: 2 of 10 (20%) stained at a score of 3, 4 of 10 (40%) stained at a score of 2, and 4 of 10 (40%) stained at a score of 1.

Of the 6 TVA cases, 4 (66.66%) stained strongly (score of 4), and the remainder (33.33%) stained moderately (score of 2) for AIF. Only one case (16.66%) showed strong staining (score of 4) for Bax. Two of 6 cases (33.33%) stained for score a 3, and 3 of 6 cases (50%) had a score of 2 for Bax. All VAs (100%) stained for AIF and

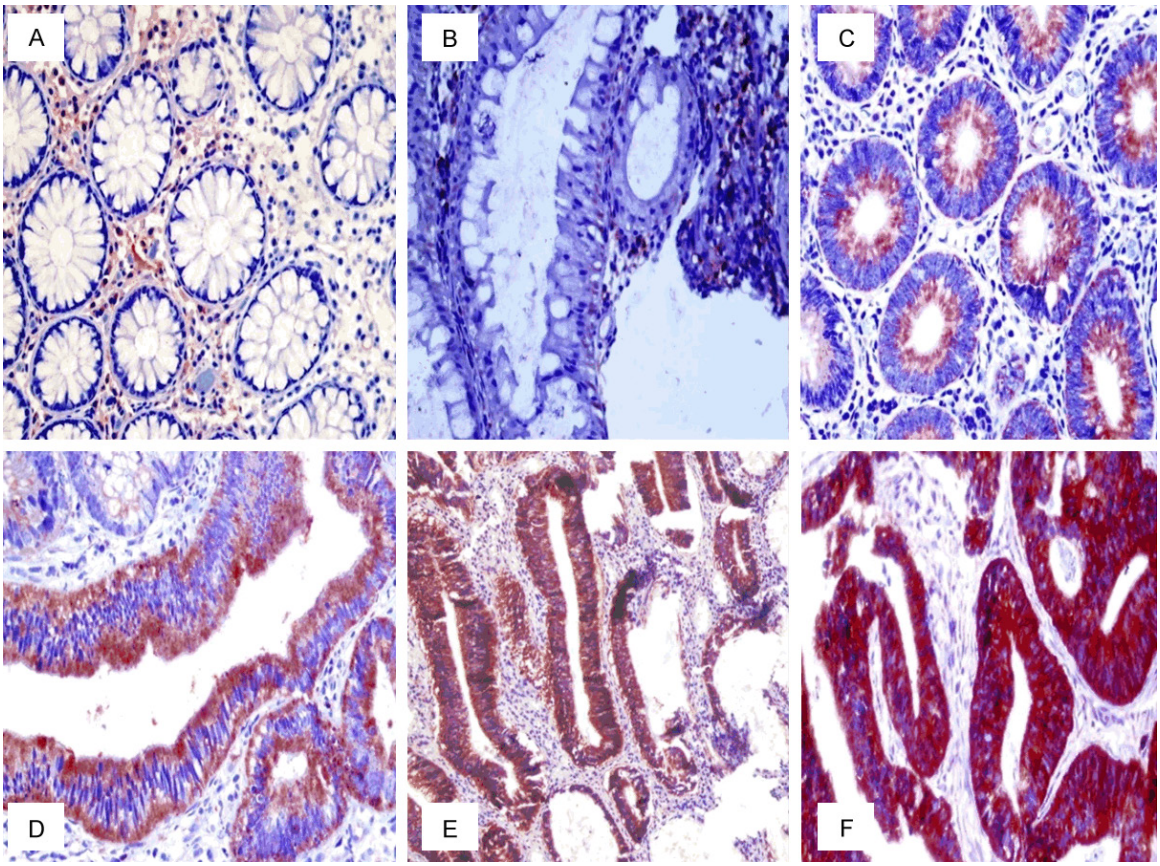

Figure 2. A-F. Normal mucosa (A), Hyperplastic Polyp (B), Tubular Adenoma (C), Tubulovillous Adenoma (D), Villous Adenoma (E), Well differantiated Adenocarcinoma (F), AIF staining × 400.

Bax, with scores of 2 and 3, respectively. AIF and Bax staining was also identified in all colorectal carcinomas.

In the well-differentiated carcinomas, AIF expression was found in 62.5% (5/8) at a score of 3 and in 37.50% (3/8) at a score of 2; Bax was found in 37.50% (3/8) at a score of 4 and in 62.5% (5/8) at a score of 2. There were 7 moderately differentiated carcinomas. Three of these (42.85%) possessed strong staining (score of 4) and 4 (57.14%) stained at a score of 3 with AIF. Bax was found in 14.28% (1/7) at a score of 4, in 57.14% (4/7) at a score of 3, and in 28.57% (2/7) at a score of 2. Of the 5 poorly differentiated carcinomas, all were strongly positive for AIF and Bax, with scores of 4 and 3, respectively. AIF and Bax expression increased when the histological grade was greater.

The staining levels of AIF and Bax in adenomas and carcinomas were significantly higher than in normal tissues ($P<0.01$). There was also a significant difference between HPs and APs ($P<0.01$). The level of AIF and Bax in carcinomas was higher than in adenomas and the difference was of statistical significance ($P<0.01$). For Bax, the differences between HPs and APs were more significant than the differences between APs and carcinomas.

The staining levels for Bax and AIF protein expression in colorectal tumors is presented in **Figures 1** and **2**.

Discussion

This study proves that Bax and AIF can be reliably detected in paraffin-embedded tissue sections in a variety of colorectal lesions, including HPs, APs, and carcinomas.

We had three aims. The first was to determine whether AIF and Bax are expressed in tissue sections of colorectal tumors; the second was to ascertain whether there is any difference in AIF and Bax expression between colorectal polyps, adenomas, and carcinomas; and the third

was to determine whether there is any difference between AIF and Bax expression in colorectal tumors.

To our knowledge, little has been reported about Bax and AIF expression in colorectal tumors, and we could not find any studies on the association between Bax and AIF expression and the distribution of these antibodies in the spectrum of colorectal lesions.

Enhanced Bax expression from normal mucosa to primary colonic carcinoma has been reported [16-18]. Douglas et al. [5] reported that AIF protein expression in cancer cells strongly expressed AIF. Our data also showed that Bax and AIF expression increased from benign tumors to malignant tumors.

During the development and progression of cancer, cancer cells acquire the capability to evade apoptosis, thereby protecting themselves from cell-death stimuli. Cancer cells without this capability may die. AIF expression in colorectal carcinomas might contribute to the promotion of apoptosis, in this way producing selective pressure for apoptosis during progression [7, 13].

There are two opposite roles of AIF. Urbano et al. [10] observed that AIF maintained the transformed state of colon cancer cells through its NADH oxidase activity. Additionally, AIF knockout colon cancer cells failed to form tumors in athymic mice and showed enhanced apoptosis sensitivity, suggesting that the expression of AIF in colon cancer could function as an oncogenic protein [10].

The present study shows that the staining levels of Bax and AIF in adenomas and carcinomas were significantly higher than in normal tissues, and there was also a significant difference between HPs and APs.

This study also indicates that the level of Bax and AIF in carcinomas is higher than in adenomas. Generally, Bax and AIF staining were at the same level, and there was no significant difference between Bax and AIF. For Bax only, the difference between HPs and APs was more significant than the difference between APs and carcinomas.

It may be of interest in future research to confirm whether the changed expression of Bax and/or AIF between benign tumors and malignant tumors provides valuable information for determining the clinicopathologic characteristics of colon and other tumors.

Disclosure of conflict of interest

None.

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