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IN SITU LABELING APOPTOSIS IN BREAST CANCER AS RELATED TO PROGNOSIS

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Objective: This study was undertaken to determine the expression of apoptosis in breast cancer and to evaluate its significance as a prognostic marker. Methods: A series of 91 invasive breast cancer was analysed for the expression of apoptosis by using the 3-end-labeling method of DNA in tissue sections. The apoptotic indexes were the percentages of apoptotic cells among tumor cells. Results: The end-labeling method allowed a precise evaluation of the expression of apoptosis. Apoptosis occurred in 91.1% of breast cancer patients, and apoptotic indexes were divided into two groups, 0–0.21 and 0.28–0.62. Low apoptotic index was related to axillary lymph node metastasis ($P<0.01$). In survival analysis, higher apoptotic index was related to disease free survival ($P=0.0095$) and overall survival ($P=0.0348$) in the entire cohort. Cox's analysis showed that apoptotic index had no independent prognostic value. Conclusion: The apoptosis was a spontaneous phenomenon in breast cancer tissue, and the expression was different from each other. Further analysis was needed to clarify the relationship between apoptosis and prognosis, especially the response to adjuvant therapy.

Key words: Apoptosis, Breast neoplasm, Prognosis.

Apoptosis (Programmed Cell Death, PCD) and necrosis are two forms of cell death. The molecular mechanism of apoptosis is the specific DNA breakage at internucleosomal sites.[1] Using *in situ* DNA end labeling method on paraffin embedded sections, and counting the apoptotic index (AI), we analysed the effect of apoptosis in prognosis of breast cancer.

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MATERIALS AND METHODS

Ninety-one breast cancer patients were treated in our department during 1981–1990. They were all pathologically diagnosed to be infiltrating breast cancer. Clinical data were demonstrated in Table 1. The follow-up of all patients was conducted ranging 3–13 years. 10 patients died of breast cancer, 14 patients were alive with tumor, 67 patients survived free of tumor.

Methods

Tissue Samples

Ninety-one surgical specimens were routinely formalin fixed and paraffin embedded. Then, paraffin sections were cut at 5 μm, mounted on silane-coated slide.

Apoptosis Assay (TUNEL)

In situ 3' end labeling of DNA breakage was performed by using an ApopTag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD) following the instructions with a few modifications. Briefly, after dewaxing and rehydration, endogenous peroxidase

activity was quenched in 3% hydrogen peroxide. Terminal transferase enzyme was used to catalyze the action of digoxigenin-labeled dUTP to the 3'-OH ends of the fragmented DNA. The incubation took 1 h under 37 ℃, and then washed in PBS 3 times. Thereafter, antidigoxigenin-peroxidase solution was applied on the slides. Diaminobenzidine hydrogen peroxide was used to develop the color reaction, which was stopped till we saw positive cells microscopically. Specimens were counterstained with methyl green. Slides were dehydrated and mounted.

Determination of Apoptotic Index (AI)

. The percent of apoptosis cells was determined by counting apoptotic cells in 500 tumor cells in 5 different HPFs (× 400), which were the most positive areas in each slide.

Table 1. Clinical and laboratory data of 91 breast cancer patients

Variable	Number	Percentage	Range	Median
Age	91		26–74	50
Tumor diameter (cm)				
<2	18	19.8		
2–5	65	71.4		
>5	8	8.8		
Axillary lymph node				
Negative	45	49.5		
Positive	46	50.5	1–12	
Receptor status				
ER positive	40	66.7		
ER negative	20	33.3		
Apoptotic index (AI)				
0–0.21	56	61.5	0.05± 0.05	
0.28–0.62	35	38.5	0.41± 0.08	

Statistical Analysis

AI was divided into two groups by using cluster analysis. The significance of the association between axillary lymph node status and AI was determined by using the x^2 test. Univariate and multivariate analyses of survival data were undertaken by using survival curves and applying Kaplan-Meier method with log rank analysis and the Cox regression model respectively.

RESULTS

The Morphological Features of Apoptotic Breast Cancer Cells

In situ DNA 3' end labeling could give a distinction among apoptotic cells, apoptotic bodies and peripheral cells. Positive cells, with brown stained nuclei, showed intensive labeling underneath the nucleus envelope or the nuclear staining was more uniform. Besides, apoptotic bodies were detected frequently in tissue sections.

The Incidence of Apoptosis in Breast Cancer

This study showed that different levels of apoptosis were detected in 83 breast cancer of all 91 patients. No apoptosis was found in only 8 breast cancer. The incidence of apoptosis was 91.2%.

The Relationship between AI and Axillary Lymph Node Metastasis

AI was correlated with axillary lymph node metastasis. In 56 patients whose AI was 0–0.21, 35 patients had axillary lymph node metastasis. While in 35 patients with AI 0.28 to 0.62, only 11 patients were found to have axillary metastasis. The difference

was significant (x^2=8.32, P<0.01).

AI in Relation to Patient Survival

The AI distribution of 91 patients was shown in Table 1. By univariate analysis, AI, tumor diameter and axillary lymph node status had effect on disease free survival (DFS) and overall survival (OS), the difference was significant (Table 2). Kaplan-Meier survival line showed that disease free survival and overall survival of higher AI patients were better than that of lower AI patients. While in multivariate analysis, AI was not an independent prognostic marker.

Table 2. Univariate analysis of prognostic factors in breast cancer patients (log-rank test)

Variable	*P* value	
	DFS	OS
AI	0.0095	0.0348
ER	0.1709	0.0040
Lymph node status	0.0000	0.0004
Tumor diameter	0.0011	0.0931

DISCUSSION

Programmed cell death (apoptosis) is a spontaneous phenomenon in malignant tumors.[1] Breast is a target organ of estrogen. In the late stage of menstrual cycle, normal mammary tissue shows significant apoptosis,[2] and it is also usually happened during involution of mammary tissue. Therefore, it is demonstrated that apoptosis in breast tissue is related to endocrinal factors. The method for studying apoptosis was originally morphological, now molecular biological methods have developed.[3] On the knowledge of molecular mechanism of apoptosis, and being connected with histochemical method, *in situ* 3'-OH DNA end labeling has precise orientation, and is advantageous to detect the early DNA breakage in apoptosis.[4] In our study, under light microscope, some cells showed intensive labeling just underneath the nuclear envelope, indicating a condensation of chromatin at the early stage of apoptosis. In cells that had proceeded further on the pathway of apoptosis, the nuclear labeling was more uniform, with the fragmented chromatin being distributed throughout the nucleus. After apoptotic bodies had been released, the apoptotic cells usually had small nuclei and condensed cytoplasm. Thereafter, by comparing with the morphological criteria, TUNEL method could detect more apoptotic cells, AI was correspondingly increased, which could be used to evaluate the level of apoptosis more accurately.

It was theoretically demonstrated that higher incidence of apoptosis was correlated with slower tumor growth and better prognosis. Our study showed that breast cancer patients with higher AI had better DFS and OS. By using TUNEL method, Gestblom had studied apoptosis in neuroblastoma,[5] and also found the same relationship between AI and prognosis. Nevertheless, there were totally different opinions. By using morphological criteria,[6,7] Lipponen et al. had detected the AI in bladder and breast cancer, and found that patients with higher AI had poorer prognosis; and AI was positively related to proliferating markers. Lipponen explained that there was a balance between cell gain and cell loss when the tumor growth had arrived a certain level. Some faster proliferating, progressing tumor cells had a higher level of apoptosis. We considered that TUNEL method could evaluate apoptosis more accurately than morphological standards. However, either TUNEL method or morphological standards could only detect apoptosis happened at one moment. Currently, there are no established techniques to determine the duration of apoptosis in tumor tissues. When studying apoptosis in NSCLC,[8] Tormanen et al. had tried to count the number of apoptotic cells and bodies, and demonstrated that the number of apoptotic bodies could represent different stage of apoptosis.

Since our samples were not large enough, and some patients' follow-up was not long enough, multivariate analysis showed that AI wasn't an independent prognostic factor.

Endocrinotherapy, chemotherapy and radiotherapy can induce apoptosis, and contribute to break the balance between apoptosis and proliferation. Therefore, besides the effect of apoptosis on prognosis, the biological regulation of apoptosis and apoptosis induction in breast cancer treatment deserve further investment.

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