Measures of Cell Turnover (Proliferation and Apoptosis) and Their Association with Survival in Breast Cancer[1]

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ABSTRACT

Our objective was to investigate the prognostic significance of cell turnover (apoptosis and proliferation) in breast cancer patients. Apoptosis was microscopically quantitated on histological sections from 791 breast cancer patients with long-term follow-up (median, 16.3 years). Apoptotic counts were also compared with proliferation data (mitotic counts and MIB-1 labeling); apoptosis data derived from terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay; and pathobiological variables, including p53, erbB-2, and estrogen receptor (ER). High apoptotic counts were associated with increased cellular proliferation, ER negativity, immunopositivity of erbB-2 and p53 ($P < 0.0001$), and shortened disease-specific survival (DSS; $P = 0.0009$) and disease-free survival (DFS; $P = 0.0006$). Other factors associated with shortened DFS and DSS by univariate analysis were high tumor grade, nodal metastases, and large tumor size ($P < 0.0001$ for each). Multivariate analysis of data for all of the patients demonstrated that tumor size, nodal status, ER, histological grade, and erbB-2 showed independent prognostic value. In node-negative patients, tumor size and mitotic rate per 1000 cells independently predicted DFS ($P = 0.0055$). Tumor grade was the only independent predictor of DSS. For node-positive patients, tumor size, nodal status, ER, and erbB-2 were independent prognostic factors. The number of mitoses per 1000 was independently associated with DFS ($P = 0.043$) but not with DSS. Apoptosis data did not provide independent prognostic value in any, node-positive or node-negative, breast cancer patients.

INTRODUCTION

Apoptosis, or programmed cell death, is an active process controlled by inducers and repressors (1, 2). The balance between these stimuli determines whether the cell enters proliferation or apoptotic pathways (1). An imbalance between cell proliferation and apoptosis may contribute to tumorigenesis and tumor progression (3, 4). Apoptosis is morphologically characterized by nuclear condensation and cytoplasmic shrinkage. Nuclear fragments and cell-surface protuberances then separate to produce apoptotic bodies that are visible microscopically. The distinct morphological features of apoptosis allow semiquantitative apoptotic body assessment using routinely processed and stained histology slides (5–7). This assessment is similar to mitotic figure counting, which is routinely performed in breast cancer evaluation. In this analysis, we also compare visual apoptotic body counting with TUNEL[3] assays.

Analyses of cell turnover (programmed cell death and proliferation) have been reported to provide insight into tumor doubling time, prognosis, and treatment response (8–15). High apoptotic counts have been associated with high histological grade, a high risk of lymph node metastasis (14), and shortened DFS in limited studies of breast cancer patients (13, 16). The independent prognostic value of apoptosis assessment on long-term survival for breast cancer patients, however, has not been well defined (7, 14).

The process of apoptosis is highly complex and regulated, at least in part, by molecular markers often associated with breast carcinogenesis. For example, p53 is a multifunctional protein that is involved in the regulation of growth of nearly all mammalian cell types. Wild-type p53 protein suppresses tumor cell growth via apoptosis induced by DNA damage and other signals. It further controls proliferation via checkpoint protein signaling (17–19). p53 mutations (or a surrogate, protein overexpression) have been found in about one-third of breast cancers and these portend a worse prognosis (2, 20–22). The *erbB-2* gene encodes a receptor with tyrosine kinase activity, which is overexpressed/amplified in nearly one-third of breast cancers (20, 23–26). Amplification or overexpression of erbB-2 leads to increased cell proliferation (20, 27) and a worse prognosis in node-positive cases (independent of therapy), and may portend response or lack thereof to specific therapeutics (26, 28).

This study explores the prognostic significance of apoptosis in a large group of breast cancer patients with long-term follow-up and extensive marker data using both univariate and multivariate statistical tools. Our underlying hypothesis was that

apoptosis data would have independent prognostic value. This study was not designed to test the predictive value of apoptosis; patients were not controlled for treatment or disease stage.

MATERIALS AND METHODS

Patients

Archival invasive breast cancers ($n = 791$) from the Department of Pathology, Massachusetts General Hospital, Boston, MA, followed for a median of 16.3 years (mean, 15.6 years; range, 8.9–36.5 years) were used for this study. Tumors were classified according to the WHO schema (29) and graded using the modified Nottingham combined histological grading scheme, which includes proliferation rate and architectural and nuclear features to stratify tumors into three groups (grade 1, 2, or 3; 30). The following information was also available on each case: patient age at diagnosis, location of primary tumor, time to recurrence or metastasis, therapeutic interventions, time to last follow-up, cause of death, tumor size, and number of positive lymph nodes. For 781 patients, the ER data were obtained from medical records (derived from either charcoal dextran or immunohistochemical assay). Of the 666 patients whose node status was known, 335 cases were LN+ and 331 cases were LN−. Tumor staging was according to the criteria of the American Joint Committee on Cancer (31). The follow-up intervals were calculated from the date of biopsy/lumpectomy/mastectomy date to the last recorded follow-up. Local recurrence and distant metastasis intervals were calculated from biopsy/lumpectomy/ mastectomy date to first documented failure date (surgical, clinical, or radiological). Among the 331 lymph node-negative patients, 21 were known to have been treated with adjuvant chemotherapy. Of these, only four received anthracycline-containing therapies (doxorubicin). Among the 335 lymph node-positive patients, 247 received some form of documented chemotherapy. Of these, 42 were treated with anthracycline therapies (doxorubicin).

Specimens

Four-μm serial sections were cut and mounted on microscope slides. H&E-stained slides were reviewed to confirm tumor histological type (ductal or lobular as well as special subtypes), tumor grade, and regional metastasis as reported previously (19).

Counting Methods

Mitotic and apoptotic figures were counted on 791 four-μm H&E-stained slides using a conventional light microscope (Nikon; Labphot-2, Tokyo) equipped with a 5×5 squares grid on the eyepiece. Actual counts of mitotic and apoptotic figures were used. Ten viewing fields of invasive carcinoma (starting at the location with the highest density of mitotic and apoptotic figures) were analyzed for both. Areas with the least amount of stroma were selected for cell counting. The slide was randomly moved until 10 adjacent fields were counted. Apoptotic bodies and mitotic figures were counted according to the morphological criteria proposed by Kerr *et al.* (32) and van Diest *et al.* (33), respectively.

Our methods used estimates for cellularity. Highly cellular regions without necrosis were evaluated for estimates of cellu-

larity. Counts were performed using $\times 40$ objective and $\times 10$ ocular, numerical aperture of 0.75. An estimation of tumor cells for each field (ECE) was made by counting the nuclei touched by a cross-line at the viewing field (representing two $\times$ the diameter). The total number of nuclei counted was divided by 4 to estimate the radius. The squared radius was then multiplied by 3.14 (π) to calculate the area of the circle (viewing field). In summary, the following formula was used to estimate the cellularity viewing within the field area:

$$ECE = \pi \cdot \left(\frac{\text{Total nuclei touched by cross-line}}{4} \right)^2$$

The estimated cell count was then used as the denominator to calculate the apoptotic and mitotic counts per 1000 cells using the following formula:

Variable apoptosis or mitoses per 1000 cells

$$= \left(\frac{\text{Variable count per 10 HPFs}}{\text{Estimated cell count per 10 fields}} \right) \times 1000$$

Morphologic Criteria

Apoptosis. Apoptotic bodies present in normal human tissues and cancers were identified by well-established criteria (13, 32, 34). These included: (a) cell shrinkage (smaller in size, eosinophilic cytoplasm with round and smooth margin separating from neighboring cells); (b) chromatin condensation (hyperbasophilic in color and irregular in shape); (c) nuclear fragmentation (one or more chromatin pieces, round in shape and variable in size); and (d) non-inflamed field. Apoptotic bodies existed either singly or in clusters amongst the invasive tumor cells without inflammatory cells. A typical apoptotic body was separated from adjacent tumor cells by a clear "halo." The condensed chromatin was highly basophilic, irregularly distributed, and fragmented into one or more pieces. The number of the nuclear fragments varied from one to more than five per cell. Apoptotic data using this system was recorded as apoptotic counts per 1000 invasive tumor cells (see above).

Mitosis. The morphologic criteria for mitosis counting proposed by van Diest *et al.* (35) were strictly followed. In brief: (*a*) the nuclear membrane was absent (post-prophase); (*b*) condensed chromosomes showed hair-like extensions of nuclear material; (*c*) no central clear zone was present; and (*d*) the cytoplasm was basophilic. Mitotic data have been previously reported on all of these cases ($n = 808$; Ref. 36). In that prior publication, we compared proliferation methodologies but did not include the apoptotic data. Mitotic figure counts were calculated and expressed either as mitoses per 1000 invasive cancer cells or mitoses per 10 HPFs (mitoses/10 HPFs). Equivocal mitotic figures were not counted.

In situ Apoptosis Assay (TUNEL)

In situ detection of cleaved, apoptotic DNA fragments was also performed on 234 cases using the TdT-FragEL Detection kit (Oncogene Science Inc, Uniondale, NY) according to the manufacturer's instructions. In this assay, TdT binds to exposed 3′-OH ends of DNA fragments. This catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides at these sites.
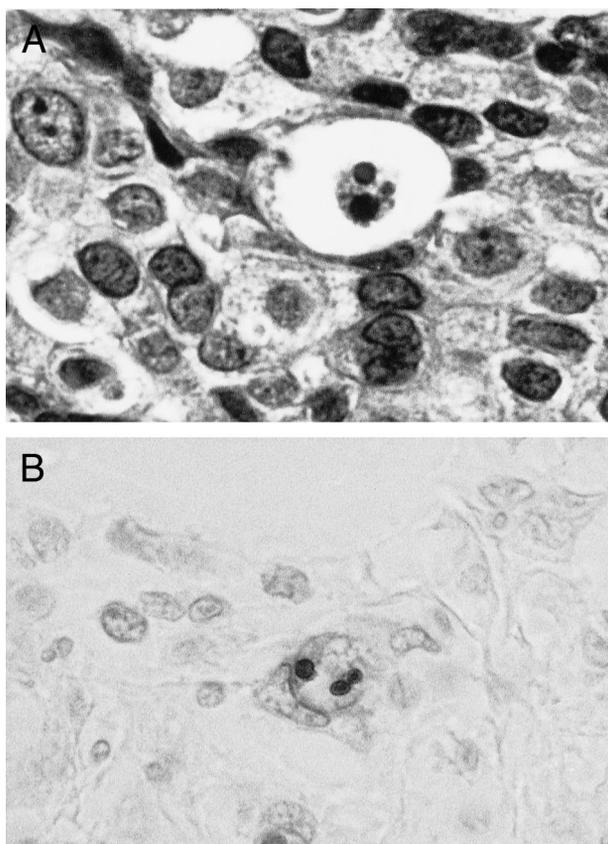
Fig. 1 A, H&E-stained representative breast cancer (×60). An apoptotic cell is present (*center*), surrounded by a clear halo. Condensed chromatin is basophilic and irregularly distributed, with nuclear fragmentation. *B,* TUNEL assay on representative breast cancer, counterstained with methyl green (×60). The apoptotic cell demonstrates nuclear fragmentation and fragment labeling at sites of DNA cleavage (*arrow*).

Table 1 Apoptosis and proliferation data (medians) for breast cancer patients

Markers	All patients (*n* = 791)	LN− (*n* = 331)	LN+ (*n* = 331)	*P*[a]
Apoptosis[b]	3.7	3.4	4.1	NS[c]
Mitosis[d]	2.3	1.7	3.1	0.0051
A:M ratio	1.50	1.56	1.60	NS
Mitoses/10 HPFs	6	5	8	NS
MIB-1 (%)[e]	17.8	16.7	19.5	0.0066

[a] Represents value of statistical difference between node-negative and node-positive subsets for each variable.
[b] Apoptotic count per 1000.
[c] NS, not significant.
[d] Mitotic count per 1000.
[e] Percentage of cells that are MIB-1-positive.

Other Markers

MIB-1 immunostaining sections were quantitatively evaluated similar to the method described for TdT *in situ* apoptosis labeling. p53, erbB-2, and ER were scored using a semiquantitative system as described previously elsewhere (27, 37, 38).

Statistics

The unpaired Fisher *t* test was used to compare the medians of the various markers between node-negative and node-positive patients. The Pearson product moment correlation was used to calculate the relationship between various factors using continuous variables. The Cox proportional hazards model (39) was used to test the significance of apoptosis/1000, mitosis/1000, mitosis/10 HPFs, MIB-1, and p53, erbB-2, ER, and various clinical and pathological markers as continuous variables over time with respect to time-to-failure, death, or censure at the last follow-up (univariate and multivariate analysis). Because the Cox model assumes that factors are linear (constant) over time and multiplicative when multiple factors are studied, the number of positive lymph nodes was transformed into a log function (log number of positive nodes plus 1) because it provides a more linear measure of nodal status. The relationships calculated were DFS, which included both local and distant failures, and overall DSS, which included only those patients dead of documented breast cancer. The calculation of DSS was used in place of overall survival because of the long follow-up time and the number of non-cancer-related deaths that have occurred in this patient group.

The goal of the multivariate analysis was to evaluate the associations between apoptotic and mitotic counts, molecular markers or other factors in relation to patient outcome. Using the Cox proportional hazards model, standard factors are removed from the model until only statistically significant variables remain ($P < 0.05$); those variables were used as the base. Multivariate analyses were also evaluated using the Wald statistic (40). This allowed marker data to be added to the base (significant variables identified by the Cox model) to determine added statistical value (if any). The numerous models were evaluated but not shown because of partial colinearity of some variables. This resulted in some variables being significant in some models but not in others. Numerous additional models were also tested to determine the impact on significance levels of various marker

Biotinylated nucleotides are then detected using a streptavidin-horseradish peroxidase conjugate. Diaminobenzidine reacts with the labeled sample to generate an insoluble colored substrate at the site of DNA fragmentation.

Control slides, BT 20 cell line (kindly provided by Nancy Davidson, Johns Hopkins Hospital, Baltimore, MD) treated with Actinomycin D. and human colon mucosa processed similarly to the actual cases were included in each TdT assay. To check the intra- and interassay consistency, the apoptotic bodies in colonic mucosa, stained as the first and the last slide of each assay, were assessed. The assays were considered adequate only when the frequency of apoptosis on the two sections of colonic mucosa were similar. Each TdT *in situ*-labeled section was quantitatively evaluated for reactivity using a ×40 objective with an eyepiece grid as described above. The frequency of labeled cells was calculated by counting at least 1000 cells in areas with the highest number of TdT-labeled nuclei, avoiding areas of necrosis. Nuclei with any detectable staining above background were scored as positive (Fig. 1). The ratio of nuclei labeled with TdT to the total invasive cancer cells counted was expressed as apoptoses per 1000 cells.

Table 2 Correlation between apoptotic counts per 1000 and other markers

Markers	All patients				LN−				LN+			
	n	r	95% CI[a]	P	n	r	95% CI	P	n	r	95% CI	P
Age	791	−0.16	(−0.23−−0.10)	<0.0001	331	−0.15	(−0.26−−0.04)	0.0057	335	−0.11	(−0.21−−0.00)	0.0472
Elston grade	755	0.40	(0.33−0.45)	<0.0001	314	0.45	(−0.35−0.53)	<0.0001	320	0.37	(0.27−0.46)	<0.0001
Tumor size	790	0.08	(0.01−0.15)	0.0304	331	0.06	(−0.04−0.17)	NS	334	0.09	(−0.01−0.20)	NS
LN mets	666	−0.001	(−0.08−0.08)	NS−					335	−0.026	(−0.13−0.08)	NS
ER	781	−0.29	(−0.35−−0.22)	<0.0001	328	−0.34	(−0.43−−0.24)	<0.0001	333	−0.25	(−0.35−−0.15)	<0.0001
MIB-1	791	0.36	(0.30−0.42)	<0.0001	331	0.36	(0.26−0.45)	<0.0001	335	0.32	(0.22−0.42)	<0.0001
Mits/1000	791	0.46	(0.41−0.52)	<0.0001	331	0.54	(0.46−0.61)	<0.0001	335	0.39	(0.30−0.48)	<0.0001
Mits/10 HPFs	791	0.32	(0.25−0.38)	<0.0001	331	0.33	(0.23−0.42)	<0.0001	335	0.28	(0.18−0.38)	<0.0001
p53	788	0.22	(0.15−0.29)	<0.0001	330	0.30	(0.20−0.39)	<0.0001	334	0.14	(0.04−0.25)	0.0095
erbB-2	787	0.20	(0.13−0.26)	<0.0001	330	0.15	(0.04−0.25)	0.0071	335	0.25	(0.15−0.35)	<0.0001
Apoptosis by TUNEL	232	1.15	(0.02−0.27)	0.0238	104	0.08	(−0.11−0.27)	NS	91	0.23	(0.03−0.42)	0.0260

[a] CI, confidence interval; NS, not significant; mets, metastases; Mits, mitoses.

combinations. These also are not shown but are described when relevant.

RESULTS

Apoptosis and Cell Proliferation. The median value of apoptotic and mitotic counts per 1000 cells for the 791 breast cancers was 3.68 and 2.33, respectively (Table 1). When expressed per 10 HPFs, the median mitotic count data were 6 (per 10 HPFs). The median of MIB-1 labeling data for all of the patients was higher (as is usually reported by others) at 17.8%. Stepwise increases in both apoptosis and proliferation rates were noted in LN− and LN+ breast cancer cases. Differences in cell turnover data between the node-negative and -positive subgroups were significant for mitoses/1000 and MIB-1 only (Table 1). Morphological apoptotic counts were compared with apoptosis data derived from an *in situ* apoptotic detection assay (TUNEL: see Fig. 1). Apoptotic counts (using either method) were significantly correlated (*r*, 0.188; *P* = 0.0037).

Relationships between Apoptosis, Proliferation, and Other Markers. For all of the patients, high apoptotic counts were significantly correlated with increased cell proliferation [measured by MIB-1, mitoses per 1000, and mitoses per 10 HPFs (*P* < 0.0001 for each)], erbB-2, and p53 protein overexpression (*P* < 0.0001 for each). Higher apoptotic rates were also significantly associated with other measures of tumor aggressiveness, including high tumor grade (*P* < 0.0001), larger tumor size (*P* = 0.03), negative ER status (*P* < 0.0001), and younger patient age (*P* < 0.0001; Table 2). Apoptotic counts were not significantly correlated with the number of lymph node metastases. Similar correlations were observed among the LN− and LN+ subsets with the exception of the loss of correlation between apoptosis and tumor size (see Table 2).

Univariate Analysis of Markers and Patient Survival. Univariate analyses were performed for all of the patients as well as the LN− and LN+ patient subsets for both DFS and DSS. Nearly 100 patients died of causes not directly related to breast cancer because of the long follow-up (median, 16.3 years); these were censored at date of death. Apoptotic counts were significantly associated with both DFS and DSS (*P* = 0.0009 and *P* = 0.0006, respectively) using a cut point of either

3.68 or 5.0 [reported by Vakkala (16)] apoptoses/1000 cells (*P* < 0.0001 for DFS and *P* < 0.0001 for DSS). Other factors univariately associated with outcome for all of the patients included tumor grade, tumor size, lymph nodes metastasis, ER, MIB-1, mitoses per 1000 cells and per 10 HPFs, erbB-2, and p53 (DSS only; Table 3).

Among LN− patients, the apoptotic count was significantly associated only with DFS using a cut point of 5.0 per 1000 (RR, 1.72; *P* = 0.0058) but not the median cut point of 3.68 per 1000 (RR, 1.41; *P* = 0.08). Proliferation by MIB-1 was more strongly related to prognosis, both for DFS (RR, 2.08; *P* = 0.0003) and DSS (RR, 2.01; *P* = 0.0038). Mitotic count per 10 HPFs was strongly associated with DFS (RR, 1.78; *P* = 0.0036) and DSS (RR, 1.93; *P* = 0.0057), as was the mitotic count per 1000 cells, DFS (RR, 1.67; *P* = 0.0092) and DSS (RR, 1.69; *P* = 0.0263). Histological grade remained significant in predicting both DFS and DSS. Tumor size, patient age, and p53 were also significantly associated with DFS but not with DSS (data not shown).

For LN+ patients, the apoptotic count was significantly associated with DFS (RR, 1.61; *P* = 0.0024) and marginally associated with DFS (RR, 1.32; *P* = 0.051) using the 3.68 cut point. It was also significantly associated with DSS (RR, 1.62; *P* = 0.0016) but not with DFS using 5.0 apoptoses/1000 as the cut point. MIB-1 was associated with both DFS and DSS (RR, 1.38; *P* = 0.0221; and RR, 1.56; *P* = 0.0046, respectively), whereas mitoses per 1000 cells and mitoses per 10 HPFs were associated only with DSS (RR, 1.53; *P* = 0.0076; and RR, 1.49; *P* = 0.014, respectively). Other factors that predicted both DFS and DSS were the number of lymph node metastases, erbB-2 overexpression, and ER status. Histological tumor grade and p53 protein accumulation were significant predictors of DSS only (data not shown).

Kaplan-Meier Survival Curves. The relationship between apoptotic data and outcome is demonstrated using Kaplan-Meier survival curves (Fig. 2). Using the log-rank Cox-Mantel test, breast cancer patients whose tumors had high apoptotic counts ≥ 3.68 per 1000 cells had significantly shorter DFS (*P* = 0.0008) and DSS (*P* = 0.0006) survival, both for all patients (Fig. 2) and for the LN+ subset (*P* = 0.0484 for DSF and *P* = 0.0021 for DSS, figure not shown). Significant asso-

Table 3 Factors associated with outcomes of breast cancer patients using Cox proportional hazard model (univariate analysis for all patients)

Factors	*n*	DFS			DSS		
		r	95% CI	*P*	*r*	95% CI	*P*
Age							
<50	184	1			1		
≥50	589	0.862	(0.69–1.08)	0.19	0.903	(0.70–1.16)	0.42
Tumor grade							
1	72	1			1		
2	346	1.524	(1.02–2.28)	<0.0001	1.439	(0.91–2.29)	<0.0001
3	322	2.296	(1.54–3.43)		2.474	(1.57–3.91)	<0.0001
Tumor size							
≤2 cm	346	1			1		
>2 cm	427	1.509	(1.23–1.85)	<0.0001	1.367	(1.09–1.71)	0.0067
Lymph node							
Negative	322	1			1		
Positive	329	2.724	(2.15–3.45)	<0.0001	3.05	(2.32–4.01)	<0.0001
Lymph node's							
0	322	1			1		
1–3	180	1.964	(1.49–2.59)	<0.0001	2.057	(1.49–2.84)	<0.0001
4+	149	4.094	(3.13–5.36)		4.848	(3.58–6.57)	
ER							
Negative	330	1			1		
Positive	433	0.707	(0.58–0.86)	0.0006	0.652	(0.52–0.81)	0.0002
erbB-2							
<40%	616	1			1		
≥40%	153	1.37	(1.08–1.74)	0.0091	1.474	(1.14–1.91)	0.0034
p53							
Negative	546	1			1		
Positive	224	1.169	(0.94–1.45)	0.15	0.275	(1.01–1.61)	0.0428
MIB-1							
<17.8%	384	1			1		
≥17.8%	389	1.697	(1.39–2.07)	<0.0001	1.765	(1.41–2.21)	<0.0001
Mitosis[a]							
<2.33	380	1			1		
≥2.33	393	1.54	(1.26–1.88)	<0.0001	1.782	(1.42–2.23)	<0.0001
Mitoses/10 HPFs							
<6	364	1			1		
≥6	409	1.529	(1.25–1.87)	<0.0001	1.758	(1.40–2.21)	<0.0001
Apoptosis[b]							
<3.68	379	1			1		
≥3.68	394	1.397	(1.15–1.70)	0.0009	1.474	(1.18–1.84)	0.0006
<5.0	464	1			1		
≥5.0	309	1.487	(1.22–1.81)	<0.0001	1.583	(1.27–1.98)	<0.0001

[a] Mitotic count per 1000.

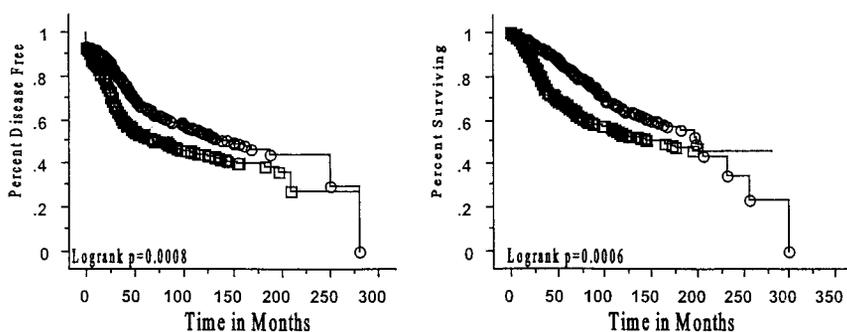[b] Apoptotic count per 1000.

Fig. 2 Kaplan-Meier disease-free (*A*) and disease-specific overall survival (*B*) curves for all of the patients. ○, cases with <3.68 apoptotic bodies per 1000 cells (*n* = 378). □, patients with ≥3.68 apoptotic bodies per 1000 cells (*n* = 393).

ciations between outcomes and apoptotic counts were not identified for LN− patients using this statistical test.

Multivariate Analysis of Outcomes. To determine the factors that independently predict DFS and DSS, multivariate analyses were performed using the Cox proportional hazards model. Base variables were identified by the Cox model. The statistical significance of additional prognostic values were determined using the Wald statistical method. Four factors,

Table 4 Multivariate survival analyses

Variables in model	No. of patients	No. of events	χ^2	$\Delta\chi^2$	P
I. All patients					
A. Base = LN$^+$ + Size+ER+erbB-2	645	312	143.22		
DFS					
Base + Apoptosis[a]	645	312	145.86	2.64	0.10
Base + Mitosis[b]	645	312	143.48	0.26	0.62
Base + Mits$^-$/10 HPFs	645	312	143.85	0.63	0.43
Base + MIB-1	645	312	146.22	3.53	0.06
B. Base = LN$^+$ + Size+Grade+ER+erbB-2	618	235	134.54		
DSS					
Base + Apoptosis	618	235	135.21	0.07	0.41[c]
Base + Mitosis	618	235	135.61	1.07	0.30
Base + Mits/10 HPFs	618	235	134.56	0.02	0.88[d]
Base + MIB-1	618	235	137.17	2.63	0.10[d]
II. LN$-$ patients					
A. Base = Size+Grade	307	101	19.92		
DFS					
Base + Apoptosis	307	101	21.37	1.45	0.23
Base + Mitosis	307	101	27.63	7.71	0.0055[c]
Base + Mits/10 HPFs	307	101	21.46	1.54	0.21
Base + MIB-1	307	101	22.10	2.17	0.14[c]
B. Base = Grade	308	72	8.57		
DSS					
Base + Apoptosis	308	72	8.67	0.10	0.76
Base + Mitosis	308	72	9.57	1.00	0.32[c]
Base + Mits/10 HPFs	308	72	9.61	1.04	0.20[c]
Base + MIB-1	308	72	10.21	1.64	0.020
III. LN+ patients					
A. Base = LN$^+$ + Size+ER+erbB-2	327	208	63.82		
DFS					
Base + Apoptosis	327	208	63.96	0.13	0.72
Base + Mitosis	327	208	67.94	4.11	0.043[e]
Base + Mits/10 HPFs	327	208	65.72	1.90	0.17
Base + MIB-1	327	208	64.38	0.55	0.46
B. Base = LN$^+$ + Age+ER+erbB-2	325	171	79.34		
DSS					
Base + Apoptosis	325	171	81.24	1.91	0.17
Base + Mitosis	325	171	81.05	1.72	0.19
Base + Mits/10 HPFs	325	171	79.44	0.10	0.75
Base + MIB-1	325	171	83.01	3.68	0.06

[a] Apoptotic count per 1000.
[b] Mitotic count per 1000.
[c] Grade was not significant.
[d] Grade and ER were not significant.
[e] Size was no longer significant.

including apoptotic count per 1000 cells, mitotic figures per 1000 cells, mitoses per 10 HPFs, and MIB-1 labeling (%) were added singly, or in combination, to the base to determine their independent prognostic value. None of these four factors were independently significant for all patients (shown in Table 4). Independent predictors of DFS were the number of positive lymph nodes, tumor size, ER status, and erbB-2 positivity. Neither proliferation, nor apoptosis, nor tumor grade were independently associated with DSS for all patients.

For LN$-$ patients, tumor size, grade, and cell proliferation using mitotic figures/1000 cells independently predicted DFS ($P = 0.0055$). Tumor grade was the only independent predictor of DSS. For DFS, the addition of proliferation data reduced the significance of grade such that it was no longer an independent variable. Apoptosis was not an independent predictor of outcome for these patients.

For LN+ patients, four independent factors, including the number of positive lymph nodes, tumor size, ER status, and erbB-2, predicted outcome (DFS and DSS). The addition of mitoses per 1000 cells to this base model improved predictability for DFS only. Apoptosis per 1000 cells independently predicted DSS ($P = 0.048$) only if erbB-2 was excluded from the model. Hence, with erbB-2 data included, apoptosis was not an independent prognostic factor in these cases.

Multivariate analysis truncated at 5 years follow-up was also performed to evaluate which of the factors predicted early disease progression. Apoptosis was not significant. Proliferation was a strong early prognostic factor for all of the LN+ and LN$-$ cases in this analysis.

DISCUSSION

Cancerous cell populations exhibit abnormally high cell proliferation and enhanced apoptotic cell death (4, 7, 17, 34). Each of these processes is highly regulated and involves multiple regulatory genes and factors (1–2, 34, 41). The rate of tumor growth depends in part on an excess of proliferation over apoptosis. We hypothesized that the A:M ratio might reflect the clinical aggression of breast cancers, but our data fail to show an association between the A:M ratio and disease progression (not shown). Apoptotic counts were strongly associated with proliferation data (including MIB-1, mitoses per 10 HPFs and per 1000 cells) as well as other measures of tumor aggressiveness (tumor grade, tumor size, and lymph node metastasis). These findings are consistent with the observations of others who have studied apoptosis in breast cancers (13, 14, 16, 42) and other malignancies (9–11, 13).

In our 791 breast cancer patients with long-term follow-up, apoptotic counting was associated with DFS and DSS by univariate analysis. Using either a cut point at our median (3.68/1000) or an apoptoses rate at 0.5% (5/1000 cells) reported by Vakkala (16), stratification by outcome was observed. However, when other more typical measures of cell proliferation or aggressiveness were considered with multivariate statistics, apoptosis was not an independent prognostic marker of patient outcome. The statistical power of apoptotic index was weakened by cell proliferation, p53, and erbB2 consistent with biological interrelationships.

Our data support the theory that cell proliferation and apoptosis pathways may be tightly coupled (43, 44). High apoptosis was observed in highly proliferative, high-grade, and more aggressive breast cancers, although a stepwise increase in apoptotic count medians was observed (differences between groups failed to reach statistical significance: 3.4 in LN− cases, 4.1 in LN+ breast cancer). Two measures of proliferation (mitoses/1000 and MIB-1) were significantly different between node-negative and node-positive subgroups, with increased proliferation in node positive patients. In a practical sense, however, proliferation (rather than apoptosis) appears more important as a prognostic marker in breast cancer patients.

Wild-type *p53*, a tumor suppresser gene, protects DNA from cleavage by arresting cells at the G_1 stage and inducing apoptosis in normal human cells on DNA damage (1, 2, 40, 45). p53 mutation or protein expression in tissues is often associated with a high rate of proliferation, high tumor grade, lymph node positivity, erbB-2 overexpression, and ER negativity (19, 20, 22, 26, 46). erbB2 and steroid hormones have also been linked to apoptosis (47). In these 791 cases, p53 overexpression was significantly associated with both high apoptotic and high proliferation counts, perhaps because of the loss of master brake function.

Methods of apoptosis measurement have been discussed in detail elsewhere (6, 48). Apoptotic body counts can be performed using routine H&E-stained fixed tissues; this method is more easily adapted to the routine practice of anatomical pathology. A disadvantage is that the counting of 1000 cells is time consuming. The TUNEL assay developed by Garvrieli and colleagues in 1992 (49) is widely used for detecting apoptosis in experimental systems. This assay permits *in situ* visualization of DNA cleavage by inserting a marker at sites of DNA cleavage (3′ end). Disadvantages include lowered sensitivity and specificity associated with tissue fixation and processing (50, 51). Given this reported limitation, we maximized specificity and sensitivity by running the TUNEL assay on small numbers of slides at one time and by monitoring apoptotic count frequency using multiple controls in each assay. We found that slide-based TUNEL assays were more expensive, time consuming, and difficult to standardize on archived fixed tissues than visual apoptotic counting. The data using either assay were roughly comparable.

In summary, apoptotic data has limited independent prognostic value. Considered in the context of other markers, it failed to provide additional prognostic value in these patients. The presence of numerous apoptotic figures suggests that there are intact signaling mechanisms that direct cell execution. It is unclear from this analysis whether or not estimates of apoptotic turnover or proliferation would be useful in determining which patients might respond to chemo- or radiotherapy. However, it is possible that assays that are run after treatment may provide a quantitative estimate of treatment-associated cell death.

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