SUMMARY

In order to identify the prognostic factors that significantly influence the disease-free survival rate after surgical resection of primary breast cancers, we determined tumour and lymph node grades, and immunohistochemical staining for estrogen and progesterone receptors (ER and PR), c-erbB-2, p53, bcl-2, bax and PCNA in 76 patients. Univariate analysis showed that increased grade of tumour and lymph nodes, negative immunostaining for ER, positive immunostaining for c-erbB-2, and a high PCNA index (≥30%) negatively influenced the disease-free survival rate, but PR, p53, bcl-2 and bax had no predictive value. Although p53 was not an independent prognostic factor by itself, the combination of p53, bcl-2, and bax proved to correlate with the disease-free survival, with the best prognosis noted in tumours negative for p53 and positive for both bcl-2 and bax, intermediate prognosis in tumours negative for p53 and positive for either bcl-2 or bax and worst prognosis in tumors negative for p53 as well as bcl-2 and bax. Tumour grade correlated positively with PCNA index, while positive staining for ER correlated negatively with tumour grade as well as with PCNA index, although this was statistically insignificant. Immunostaining of breast cancers for Bcl-2 correlated negatively with tumour grade and PCNA index. Immunostaining for c-erbB-2 correlated positively with PCNA but not with tumour grade. Immunostaining for p53 tended to correlate positively with PCNA, but not with tumour grade. Immunostaining for PR and bax did not correlate with tumour grade and PCNA index. These results suggest that in addition to tumour size and lymph node involvement, immunostaining for ER, c-erbB-2, and a high PCNA index are important prognostic factors in human breast cancer. Wild-type p53 with preserved bcl-2 and bax gene products is also a favorable prognostic factor indicating breast cancer at an early stage of cancer progression.

INTRODUCTION

Several studies investigating the long-term survival of patient with operable breast carcinoma have confirmed the beneficial effects of both combination chemotherapy and hormone replacement therapy, although the results so far can not be considered optimal (Early Breast Cancer Trialists: Collaborative Group, 1992). In addition to identifying the predictors of sensitivity or resistance of
breast cancers to cytotoxic or hormonal therapies, reliable prognostic information provided by many clinicopathologic and biologic factors are essential for an accurate prediction of the clinical outcome. Among these prognostic factors, tumour size and lymph node involvement, estrogen and progesterone receptors (ER and PR), various growth factors, oncogenes, tumour suppressor genes, tumour angiogenesis factors and apoptosis-related factors have so far been extensively investigated.

Absence of ER in breast cancer is associated with a significant increase in early recurrence independent of other prognostic factors such as tumour size and axillary lymph node status (Knight et al., 1977). Furthermore, a critical level of ER or PR is an important determinant prognostic factor for both disease-free survival (DFS) and overall survival (Chevallier et al., 1988). However, Caldarola et al. (1986) reported that hormone receptor status was not an independent prognostic factor, but a favorable effect for adjuvant hormonal therapy, tamoxifen, was related to ER level. Recently, a general variant, lacking exon 5 of the hormone-binding domain, was reported (Fuqua et al., 1991). The resultant truncated receptors are constitutively active and are unresponsive to endocrine therapies directed at ER hormone-binding site, such as estrogen deprivation from ovariectomy or aromatase inhibitors or antiestrogen treatment (Fuqua et al., 1991). Gallacchi et al. (1998) found increased expression of the ER exon-5-deletion variant in relapse to clonal selection of cells resistant to anti-estrogen treatment.

King et al. (1985) first reported that a v-erbB-related gene was highly amplified in a breast cancer cell line. Slamon et al. (1987) later demonstrated that amplification of the c-erbB-2 gene was a significant predictor of both overall survival and time to relapse in node-positive patients with breast cancer. In contrast, however, Ali et al. (1988) found no significant association between the increased copy number of c-erbB-2 proto-oncogene in breast cancers and time to relapse, and survival period. Concordance between Western blot technique and immunohistochemistry was found in breast cancers in detecting overexpression of c-erbB-2 protein (Molina et al., 1992). Recent studies have shown that the amplification of c-erbB-2 in patients with breast cancer is associated with poor prognosis (Press et al., 1993; Quenel et al., 1995; O’Malley et al., 1996; Charpin et al., 1997; Andrilis et al., 1998) as well as a lower response rate and duration of response to hormonal therapy at relapse (Berns et al., 1995; Houston et al., 1999). However, other reports showed no significant relationship between overexpression of c-erbB-2 and response to treatment, and concluded that this gene plays no role in therapeutic decision-making in advanced breast cancer (Archer et al., 1995; Elledge et al., 1998).

Recent studies investigating the mechanisms underlying the biological activity of p53 indicate that the protein is involved in gene transcription, DNA synthesis and repair, genomic plasticity, and programmed cell death (Greenblatt et al., 1994). Alterations of p53 protein due to missense mutations and loss of p53 protein by nonsense or frameshift mutations provide a selective advantage for clonal expansion of preneoplastic and neoplastic cells (Vogelstein and Kinzler, 1992). The presence of elevated levels of mutant p53 was identified as an important prognostic factor in human breast cancer (Davidoff et al., 1991; Lipponen et al., 1993; MacGrogan et al., 1995), although some studies found no significant association between overexpression of mutant p53 and clinical prognosis (Ostrowski et al., 1991; Hanzal et al., 1992; Haerslev et al., 1995). Furthermore, overexpression of p53 was found to induce down-regulation of bcl-2 protein, which is known to inhibit apoptosis (Haldar et al., 1994; Miyashita et al., 1994), and also up-regulation of bax which inhibits bcl-2 (Miyashita and Reed, 1995).

Tumour growth rate should be determined not only by cell loss due to apoptosis and necrosis but also by cell proliferation rate. In fact, several investigators reported that the proliferation rate or activity is a major prognostic factor in breast cancer (Betta et al., 1993; Veronese et al., 1993; Silvestrini et al., 1997), which enhances tumour progression (Pavelic et al., 1992).

In the present study, we examined all the above factors in 76 unselected invasive breast cancer cases in order to identify reliable prognostic factors, and to define the relationship between these different factors.

MATERIALS AND METHODS

Patients

Breast cancer tissues and regional lymph nodes were surgically resected from 76 female patients...
(20-80 years old, mean age 54.9 years) by radical or modified radical mastectomy in the First Department of Surgery, Fukui Medical University. None of these patients had received any pre-operative adjuvant chemotherapy or radiation therapy. Post-operative adjuvant therapies were administered, especially to patients with tumours classified as T2 and T3, or lymph node grade pN1 based on TNM classification (International Union Against Cancer, 1997). Specifically, 62 patients received hormonal adjuvant therapy (tamoxifen antiestrogen, 20 mg daily for ≥ 2 years), 61 and 7 patients received oral and systemic chemotherapy, 5-FU (150 mg daily for ≥ 2 years) and CAF (2 cycles), respectively, and 7 patients received radiotherapy. Follow-up periods ranged from 7 to 146 months (mean, 54.5 months, median 42.0 months). Resected tumor tissues and lymph nodes were fixed with 10% buffered formalin for 1 week at room temperature, and embedded with paraffin. Sections (4-µm thick) were prepared and stained with haematoxylin and eosin, and histologically examined.

Immunohistochemistry

Serial 4-µm thick sections were prepared from the same tissue specimens, deparaffinized with xylene then treated with ethanol. After washing with water, intrinsic peroxidase was blocked with 3% H$_2$O$_2$ solution dissolved in absolute methanol at 20°C for 20 min and washed with PBS (pH 7.4). For ER, PR, p53, bcl-2, and bax immunohistochemistry, sections were incubated in 10 mM citrate buffer (10 mM citrate monohydrate in distilled water, pH 6.0) and heated in an autoclave (HICLAVE™, HA-240M II, Hirayama, Tokyo, Japan) at 121°C for 5 min, followed by cooling at room temperature for 10 min and washed with PBS (pH 7.4). Then, sections were mounted with 2% skim milk (Yukijirushi, Sapporo, Japan) dissolved in PBS (pH 7.4) at 37°C for 20 min to block background adsorption of antiserum, and washed with PBS (pH 7.4). For ER, PR, c-erbB-2, p53, bcl-2 and PCNA, sections were incubated with blocking agent (HISTOFINE SAB-PO(M) Kit, Nichirei, Tokyo) containing 10% normal rabbit serum, at 20°C for 20 min. For bax, the sections were incubated with the blocking agent (HISTOFINE SAB-PO(R) Kit, Nichirei, Tokyo) containing 10% normal goat serum, at 20°C for 20 min. Sections were followed by reaction with the following primary antibodies, respectively, at 4°C overnight, after washing with PBS (pH 7.4): monoclonal anti-ER antiserum (Clone 1D5, DAKO A/S, Glostrup, Denmark, 50 µl dissolved in 2.5 ml PBS, pH 7.4), monoclonal anti-PR antiserum (Clone 1A6, DAKO, 50 µl dissolved in 2.5 ml PBS, pH 7.4), monoclonal anti-c-erbB-2 antiserum (Clone CB11, Novocastra Laboratories, Newcastle, UK, 50 µl dissolved in 2 ml PBS, pH 7.4), monoclonal anti-p53 antiserum (Ab-6, Oncogene Science, New York, 50 µl dissolved in 2.5 ml PBS, pH 7.4), monoclonal anti-bcl-2 antiserum (Clone 124, DAKO, 50 dissolved in 2 ml PBS, pH 7.4), monoclonal anti-p53 antiserum (PC10, Novocastra Laboratories, 50 µl dissolved in 2.5 ml PBS, pH 7.4), and polyclonal anti-bax antiserum (Ab-1, Oncogene Research Product, Cambridge, MA, 50 µl dissolved in 1.0 ml PBS, pH 7.4). After washing with PBS (pH 7.4), sections were incubated with the secondary antiserum at 20°C for 40 min and rewashed in PBS (pH 7.4). For ER, PR, c-erbB-2, p53, bcl-2, and PCNA, biotinylated rabbit anti-mouse immunoglobulin (HISTOFINE SAB-PO(M) Kit, Nichirei) was used and for bax, biotinylated goat anti-rabbit immunoglobulin (HISTOFINE SAB-PO(R) Kit, Nichirei) was applied. In the next step, all slides were allowed to react with streptavidin solution (streptavidin conjugated to horseradish peroxidase, HISTOFINE SAB-(M)(R) Kit, Nichirei) at 20°C for 40 min and rinsed with PBS (pH 7.4). In order to visualize the peroxidase color reaction, sections were incubated with DAB solution (DAB, CB090, Dojin, Kumamoto, Japan, 20 mg dissolved in 100 ml of 0.05 M Tris-HCl buffer, pH 7.4), containing 0.05% H$_2$O$_2$, at 20°C for 5 min. Nuclear counterstaining was carried out with haematoxylin in all slides.

Evaluation of immunohistochemical staining

In each specimen, over 500 cells were counted in randomly selected fields. Staining of the nucleus represented a positive expression for ER, PR, p53 and PCNA, while staining of the cell membrane was considered positive for the expression of c-erbB-2, and staining of the cytoplasm represented a positive expression of bcl-2 and bax. Specimens in which positive cancer cells constituted equal to or greater than 20% of the total cells were classified as positive for ER, PR, c-erbB-2, p53, bcl-2, or bax.
Statistical analysis
Statistical analyses were carried out using the SPSS statistical package (SPSS Inc., Chicago, IL). Postoperative DFS curves were generated according to the Kaplan-Meier method. Patients who died without recurrence were treated as censored cases. Differences in the DFS rates of groups classified by various background factors were analyzed with the log-rank test. Analyses of inter-group differences in clinicopathological factors (categorical variables) were performed using \( \chi^2 \) test. PCNA index data did not show a normal distribution, and the Mann-Whitney U test was used to compare these data to those of other immunohistochemical staining (two independent negative or positive groups). The relationships between PCNA data and those of bcl-2 and bax were analyzed first by Kruskal-Wallis test. When the result of the Kruskal-Wallis test was found to be significant, the data of the two groups were analyzed using Mann-Whitney U test. Statistical significance was defined as \( p<0.05 \).

RESULTS
Clinicopathological findings
Histopathological examination showed that among 76 breast cancer cases, 71 (93.4%) were invasive ductal carcinomas, 4 (5.3%) were invasive lobular

<table>
<thead>
<tr>
<th>Table I</th>
<th>Univariate disease-free survival analyses of independent prognostic factors</th>
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<tr>
<td>Prognostic factors</td>
<td>Number of cases (%)</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td>Tumour grade</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>30 (39.5)</td>
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<tr>
<td>T2</td>
<td>27 (35.5)</td>
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<tr>
<td>T3</td>
<td>19 (25.0)</td>
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<tr>
<td>Lymph node grade</td>
<td></td>
</tr>
<tr>
<td>pN0</td>
<td>35 (46.1)</td>
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<tr>
<td>pN1</td>
<td>41 (53.9)</td>
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<tr>
<td>ER</td>
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<tr>
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<td>36 (47.4)</td>
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<tr>
<td>positive</td>
<td>40 (52.6)</td>
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<td>48 (63.2)</td>
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<tr>
<td>positive</td>
<td>28 (36.8)</td>
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<tr>
<td>c-erbB-2</td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>59 (77.6)</td>
</tr>
<tr>
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<td>17 (22.4)</td>
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<tr>
<td>p53</td>
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<td>53 (69.7)</td>
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<td>29 (38.2)</td>
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<td>positive</td>
<td>47 (61.8)</td>
</tr>
<tr>
<td>bax</td>
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<td>46 (60.5)</td>
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<tr>
<td>&lt;30</td>
<td>44 (57.9)</td>
</tr>
<tr>
<td>≥30</td>
<td>32 (42.1)</td>
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</table>
carcinomas, and 1 case (1.3%) was mucinous carcinoma. According to the TNM classification, the primary tumour grade was classified into T1 (30 cases, 39.5%), T2 (27 cases, 35.5%) and T3 (19 cases, 25.0%), and the lymph node grade was classified into pN0 (35 cases, 46.1%) and pN1 (41 cases, 53.9%). Recurrence was noted in 19 cases (25.0%), either locally, in lymph nodes or other organs.

a) ER, PR
Normal and cancer cells showed positive nuclear staining for ER and PR (Fig. 1a and b) but a small number of cancer cells also showed cytoplasmic staining. Forty cases (52.6%) and 28 cases (36.8%) were positive for ER and PR, respectively (Table I).

b) c-erbB-2
Cancer cell membranes were positively stained for c-erbB-2 (Fig. 1c), but some cancer cells showed cytoplasmic positive staining. In positive cases, almost all cancer cells were stained positive, and all cancer cells were negative in negative cases. Normal tissue elements were totally and completely negative. As shown in Table I, 17 cases (22.4%) were positively stained.

c) p53
Only cancer cells showed positive nuclear staining for p53 (Fig. 1e and f) and all normal tissue elements were completely negative. The percentage of positively stained cells varied widely in different cases and the distribution of positively stained cancer cells was also heterogeneous in the same tumour tissue. The number of positively stained cancer cells tended to be higher in the tumour periphery than in the center. Twenty-three cases (30.3%) were positive for p53 (Table I).

d) bcl-2, bax
Cytoplasmic positive staining for bcl-2 and bax was immunohistochemically detected in cancer cells (Fig. 1g and h). In positive cases, almost all cancer cells were homogeneously stained without any heterogeneity. Lymphocytes infiltrating the stromal tissue were largely stained positive for bcl-2 but only a small proportion of these cells stained positive for bax. The majority of background normal breast tissue, ductal cells and acinar cells, were positive for bax but only a small proportion of these cells were positive for bcl-2. Forty-seven cases (61.8%) and 46 cases (60.5%) were positive for bcl-2 and bax, respectively (Table I).

e) PCNA
Cancer cells showed positive nuclear staining for PCNA (Fig. 1d). The ratio of positively stained cells varied widely among cases, and the number of positively stained cells tended to be higher in the periphery of the tumour than in the center. A small number of normal ductal cells and acinar cells also showed positive nuclear staining. The PCNA index score ranged from 2 to 85% (median, 21%; 25th percentile, 8.25%; and 75th percentile, 55.75%, Table I).

Statistical evaluations of different parameters as the prognostic factors for disease-free survival rate
Univariate analysis of all parameters showed that tumour grade and lymph node grade classified according to the TNM system, ER immunopositivity, PCNA index ≥30%, and c-erbB-2 immunonegativity were significant prognostic factors (Table I). Cases negative for p53 and positive for bcl-2 or bax tended to show better prognosis than those positive for p53 and negative for bcl-2 or bax, but the difference was not statistically significant.

Relationships between different p53, bcl-2 and bax immunohistochemical stainabilities and disease-free survival
Cases positive for p53 as well as bcl-2 or bax tended to show better prognosis than those positive for p53 but negative for the other two proteins, although the difference was not statistically significant. In p53 negative cases, however, those positive for both bcl-2 and bax showed the best prognosis while those negative for both bcl-2 and bax showed the worst prognosis, while prognosis of those positive for either bcl-2 or bax was between the two above groups. Differences between the latter three groups were statistically significant (Fig. 2).

Relationships between tumour status and other parameters
Tumour grade correlated closely and significantly with PCNA index, and negatively with staining for ER and bcl-2. On the other hand, however, tumour grade did not correlate with immunostaining for PR, c-erbB-2, p53 and bax (Table II).
Relationships between PCNA index and other parameters

PCNA index was significantly higher in bcl-2 negative tumours compared to tumours positive for this protein. The PCNA index was also significantly high in c-erbB-2 positive tumours compared to cases negative for the gene (Fig. 3). Tumours negative for ER tended to show a higher PCNA index than ER-positive tumours. Tumours positive for p53 tended to show a higher PCNA index than p53-negative tumours, but these differences were not significant (p = 0.052, 0.070). On the other hand, the staining pattern for PR or bax did not influence PCNA index (Fig. 3). Further analysis showed that tumours negative for p53 negative as well as bcl-2 and bax had a significantly higher PCNA index compared to those positive for bcl-2 and bax (Fig. 4).

DISCUSSION

In the present study, a high tumour and lymph node TNM grade, negative immunostaining for ER, positive immunostaining for c-erbB-2 and a high PCNA index (≥30) correlated negatively with DFS. The findings that tumour size and lymph node involvement are important prognostic factors are in agreement with previous reports (Chevallier et al., 1988; Carter et al., 1989; Clayton and Hopkins, 1993; Hawkins et al., 1996; Imamura et al., 1997; Chapman et al., 1999).

It has been suggested that estrogen participates not only in normal proliferation of mammary tissue but also in breast cancer progression and growth (Lemieux and Fuqua, 1996). However, several studies have reported that ER-immunonegative breast cancers exhibit a higher proliferative activity and poor differentiation compared to those positive for ER (McDivitt et al., 1986; Goodson et al., 1991; Bonetti et al., 1996). This discrepancy seems to be attributable to the presence of exon 5 ER deletion variant or hyper-sensitive ER variant, as demonstrated by Fuqua and coworkers (Fuqua et al., 1991; Lemieux and Fuqua, 1996). According to these investigators, the exon 5 ER deletion variant is transcriptionally active in the absence of estrogen, lacking a large portion of hormone binding domains (HBD), while the hyper-sensitive ER variant is functionally active in response to subphysiological concentrations of estrogen, having a point mutation in a conserved region of the HBD. These ER variants may appear during the course of tumour progression and may switch immunoreactivity to become negative, although the early stages of carcinogenesis and progression of breast cancer would require both genetic damage and proliferative stimuli through estrogen and intact ER (Lippman and Dickson, 1989). Cancer progression may also be associated with a complete loss of differentiated function of ER resulting in automatic cell proliferation. The above ER abnormalities may induce further activation of cell proliferation and cancer progression. Considered together, these results indicate that ER immunopositive breast cancer represent those at early stages of progressions with preserved normal ER function, exhibiting effective response to adjuvant hormonal therapy and good prognosis.
This interpretation lends support to our finding that ER immunonegativity correlated positively with PCNA index (Fig. 3). But the relatively low percentage of ER-positive tumours in the present study in comparison to that reported in the literature may be attributable to the rather limited num-

<table>
<thead>
<tr>
<th>Marker</th>
<th>Tumour grade</th>
<th>p value</th>
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<tr>
<td></td>
<td>T1</td>
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</tr>
<tr>
<td>ER</td>
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<td></td>
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<tr>
<td>c-erbB-2</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
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<td>13</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>PCNA index</td>
<td>Median (50th percentile, 75th percentile)</td>
<td>10</td>
</tr>
</tbody>
</table>

Fig. 3 - Relationship between PCNA index and expression of various histochemical markers. The PCNA index is expressed using the box plot. In these plots, the median value is represented by the horizontal line within the box, while the upper and lower ends of box represent the 75th and 25th percentile values.
number of cases examined and the heterogeneity of the post-operative treatments.

Previous studies confirmed the relationship between c-erbB-2 gene amplification and immunohistochemical detection of the oncogene product (Molina et al., 1992). In the present study, c-erbB-2 was an important prognostic factor showing positive correlation with PCNA index, in agreement with other studies demonstrating that the expression of c-erbB-2 oncoprotein affects cell proliferation in breast cancers (Ioachim et al., 1996). In a series of studies, Hirohashi and co-workers (Kanai et al., 1995; Shibata et al., 1996) demonstrated that tyrosine phosphorylation of β-catenin by c-erbB-2 gene product induces inactivation of the E-cadherin cell adhesion system resulting in dissociation, invasion and metastasis of cancer cells. These changes clearly explain the importance of c-erbB-2 as a significant prognostic factor in breast cancer.

Our study showed that PCNA index (a marker of tumour proliferative activity) lower than 30% was an important prognostic factor, while index values < 20% did not correlate with DFS. These findings indicate that the cutoff percentage value of positively stained cancer cells seriously influences the results of statistical analyses evaluating the importance of this index as a prognostic factor. Moreover, in order to minimize the difference in the results of different laboratories, other factors such as methodologies, probes and immunological reagents used, and the size and characteristics of the tumours surveyed including follow-up time, should be standardized (McGuire, 1991). In the present study, breast cancers containing ≥20% positively stained cells were evaluated as immunohistochemically positive cases for ER, PR, c-erbB-2, p53, bcl-2 and bax, but different critical values should be also examined in future studies.

Our study showed that bcl-2 immunonegativity was not an important prognostic factor however, it correlated positively with PCNA index (Fig. 3), suggesting its inverse correlation with cancer progression. In fact, several studies have reported that bcl-2 expression correlates negatively with the histological grade of breast cancer (Joensuu et al., 1994; Lapponen et al., 1995; Lee et al., 1996; van Slooten et al., 1998). Furthermore, expression of bcl-2 protein appears to be positively associated with tumor cell differentiation and inversely with tumor progression (Mikami et al., 1999; Yang et al., 1999). In other words, loss of bcl-2 protein positivity due to gene mutations during the course of cancer progression might induce the loss of differentiation of cancer cells, thus shifting these cells to a higher grade of malignancy. Considering these discussions, the expression of bcl-2 protein may represent an efficient modulator of carcinogenesis at an intercellular level, rather than acting on the transformation process itself (Jurgensmeier and Bauer, 1997). Furthermore, we demonstrated in the present study that among the p53 negative cases, those positive for both bcl-2 and bax showed the best prognosis, those negative for both bcl-2 and bax showed the worst prognosis, while prognosis in those positive for either bcl-2 or bax was between these two groups (Fig. 2). As far as we know, these findings are quite new and original and may be interpreted to be due to the preserved...
p53, bcl-2 and bax genes in early stages of cancer progression without increase in malignancy.

In the present study, other clinicopathological parameters such as age of the patients and the histological grade of the tumours were not entered in the analysis. Furthermore, statistical analysis of DFS did not take into consideration the effect of post-operative adjuvant therapies. These factors should be examined in future studies using larger series of cases.

In summary, in addition to tumour size and lymph node involvement, we demonstrated in the present study that ER, c-erbB-2, and PCNA are important prognostic factors in human breast cancer. Furthermore, the wild-type p53 with preserved bcl-2 and bax gene products is also a favorable prognostic factor indicative of early stages of cancer progression.

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