INTRODUCTION

In Korea, breast cancer ranks the second most common cancer after stomach cancer in women, and its rate is gradually increasing (1). Despite having similar histological findings, breast cancer cases have distinctly different biological behaviors, so that it is difficult to evaluate the prognosis of breast cancer and to predict its response to treatment and the likelihood of recurrence. Traditionally, prognostic factors of breast cancer include axillary nodal status, tumor size, histological grade, pathologic subtype, cancer cell proliferation, and hormonal receptor status (2). The biological markers, for example c-erbB2, p53, Bcl-2, Ki-67 labeling index etc., have been intensively investigated as new prognostic or predictive markers, and are currently applied in the management of breast cancer patients in many hospitals.

Apoptosis is the process of active cell death that is observed in the generation of multinuclear creatures and the preservation of individuals (3). Recently, a great deal of interest has been devoted to the role of apoptosis in tumorigenesis. Bcl-2 was first identified as repressor of apoptosis and Bcl-2-related genes are involved in the regulation of apoptosis that is implicated in a variety of cellular activities, including the transformation of a normal cell into a cancer cell (4). At present, the reported Bcl-2-related genes are Bax, Bcl-x, mcl-1, A1, Bak, cat-9, Bfl-1, and BRAG-1. Among these, the Bfl-1 gene, which is isolated from human fetal liver, is a member of the Bcl-2 gene family and was only recently described, inhibits p53-induced apoptosis and exhibits a potent cooperative transforming activity (5, 6). Bfl-1 was also shown to be induced by inflammatory cytokines, TNF-α or IL-1β, suggesting that it may play a protective role during inflammation (8). It has been reported that over-expression of the Bfl-1 homologous genes, Bcl-2, Bax, and Bcl-x has a relationship with prognosis of breast cancer, but there has been no reports on the prognostic relevance of the Bfl-1 gene in literature.

The purpose of the present study was to compare the expression of the Bfl-1 gene by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis with other prognostic factors, such as the status of hormonal receptors, p53, c-erbB2, Bcl-2, and tumor infiltrating lymphocytes (TIL) and the most important clinicopathologic parameters.

PATIENTS AND METHODS

The study was performed on 30 breast cancer tissue sam-
ples. All the patients were enrolled at the Department of Surgery of Asan Medical Center in Korea. None of the breast cancer patients received preoperative radiation treatment or chemotherapy. Patients were treated by mastectomy or breast-conserving surgery. The median age of the patients was 47.5 yr (range, 32-83 yr). Nineteen (63.3%) patients were premenopausal and 11 (36.7%) were postmenopausal. During surgery, after the tumor had been cut in half, about 1 g of tumor tissue was frozen in liquid nitrogen immediately after surgical resection and stored at -80°C until being processed. The rest of the tissue was processed for histopathological examination, and immunohistochemical analysis was performed for the expression of the estrogen and progesterone receptors, p53, and c-erbB2, Bcl-2 using the antibody for individual proteins by routine ABC method. All the patients were staged according to the AJCC/UICC classification system, and each tumor was graded by the Bloom and Richardson grading system.

Semiquantitative RT-PCR analysis

Total RNA was isolated from tissue samples using Tri-reagent (Sigma, T9424) after removal of contaminating chromosomal DNA with DNase I treatment, and then was reverse transcribed by the Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Gaintnersberg, MD, U.S.A.) and oligo dT primers (Promega, Madison, WI, U.S.A.) to synthesize the first-strand complementary DNA (cDNA). Three microliters of cDNA were then amplified by polymerase chain reaction (PCR). The β-globin gene served as internal control. The primer used were: Bfl-1 upstream primer: 5' -AGCTCAAGACTTGTCTCCACCA-3'; Bfl-1 downstream primer: 5' -TGAGTGCCTTGTCTGAGCATTAAGG-3 ; β-globin upstream primer: 5' -GACA-CAACGTTCATAG-3 ; β-globin downstream primer: 5' -AGGGTAGACACCAGC-3. PCR was performed in a 30 μl reaction volume with the following parameters: 94°C for 5 min; 94°C for 45 sec; 60°C for 45 sec; 72°C for 30 sec for 28 cycles; and 72°C for 10 min. PCR products were electrophoresed on 2% agarose gels and visualized by staining with ethidium bromide (9). The gel was scanned, and the band intensity was measured by a computerized densitometric analysis device. We calculated the ratio of the intensity of the Bfl-1 bands to the intensity of the β-globin bands. We considered any ratio greater than 5 to indicate expression of the Bfl-1 gene.

Measurement of Tumor Infiltrating Lymphocytes (TIL)

The 5-μm thick sections of the formalin-fixed, paraffin-embedded blocks were used for hematoxylin-eosin stains. Using a microscope, a total of three areas of tumor with infiltration by TILs were selected for the counting of TILs in the following order: (a) TIL1, the area of the maximal number of TILs; (b) TIL2, the area of tumor with a median number of TILs in each case; and (c) TIL3, the area with the lowest number of TILs as judged by examination at low-power magnification (× 100). For the counting of TILs, a high-powered magnification (× 200) image was captured by Image Pro Plus (version 4.0, Media Cybernetics), and printed out on a developing paper. The TILs in the entire image were counted using grids, and we calculated the mean number of the three areas. The infiltration of TILs was divided into 3 groups: patients with a low number of TIL infiltration (<100 cells/high-power field), a moderate number (100-<300), and a high number (>300).

Statistical Analysis

The Chi-squared test, Fisher’s exact test, the Mann-Whitney nonparametric test, or Pearson’s correlation test was used to analyze the expression of the Bfl-1 levels according to the clinicopathological characteristics, other prognostic factors and TIL, as appropriate. Those with p<0.05 were considered significant.

RESULTS

The median relative ratio was 3.0 (range, 0.12-26.83) and the Bfl-1 gene expression rate was 36.7% (11/30) (Fig. 1). No statistically significant relationship was found between the clinicopathologic parameters of patients: menopausal status, tumor size, axillary nodal status, stage, histologic grade, lympho-vascular invasion of tumor, and the relative ratio numerical values of gene expression. However, the median value was 2.14 (range, 0.83-3.34) in the group with stage 0 and stage I, but the value was 4.09 (range; 0.12-26.83) in the group with stage II or over. Therefore, a lower level of expression of the Bfl-1 gene was shown in cases of the early stage of breast cancer. Compared with other cases, higher intensity of expression was displayed in cases with larger tumors and those with metastasis to axillary lymph nodes showed high-

![Fig. 1. RT-PCR analysis of Bfl-1 mRNA in breast cancer tissues. lanes 1-8, patient number; lane 9, positive control (B-cell lymphoma); lane 10, negative control (liver tissue).](image-url)
er intensity of expression (1.86 versus 4.06, 2.61 versus 4.57). These observations showed that the Bfl-1 gene was highly expressed in more advanced breast cancers than in early cancers. There was no significant relationship between expression values and currently acknowledged prognostic factors, that is estrogen and progesterone receptors, p53, and c-erbB2, but a higher expression pattern was noticed in the groups of positive hormone receptors and negative p53 and negative c-erbB2, albeit without statistical significance. Regarding the gene expression rate, none of the group of 7 patients with tumors smaller than 2 cm showed expression, while 52.2% (11/23) of those in the group with tumors larger than 2 cm ($p=0.029$) did. This trend could also be applied to patients with breast cancer of stage 0 or I and of stage II: the former group was markedly different from the latter ($p=0.02$). In the group with only positive estrogen receptor among other prognostic factors, 9 out of 16 cases showed expression (56.3%) compared to 14.3% (2/14) of the negative cases ($p=0.017$) (Table 1). In the group with positive expression of the Bcl-2

### Table 1. Clinical data of patients and result of Bfl-1 gene expression

|                          | No. of patients | Ratio to internal control | Expression (%) |
|--------------------------|-----------------|---------------------------|----------------|
|                          |                 | Median | Range | Present (n=11) |
| Menopausal status        |                 |        |       |               |
| premenopause             | 19              | 3.20   | 0.5-26.83 | 7 (26.8) |
| postmenopause            | 11              | 2.79   | 0.12-26.76 | 4 (26.4) |
| Tumor size               |                 |        |       |               |
| < 2 cm                   | 7               | 1.86   | 1.50-3.34 | 0 (0)$^*$ |
| > 2 cm                   | 23              | 4.09   | 0.12-26.83 | 11 (52.2) |
| Stage                    |                 |        |       |               |
| 0, I                     | 7               | 2.14   | 0.83-3.34 | 0 (0)$^*$ |
| II, III                  | 23              | 4.09   | 0.12-26.83 | 11 (52.2) |
| Node                     |                 |        |       |               |
| negative                 | 14              | 2.61   | 0.50-6.56 | 3 (21.4) |
| positive                 | 16              | 4.57   | 1.11-26.83 | 8 (50.0) |
| Histologic grade         |                 |        |       |               |
| 1, 2                     | 13              | 2.64   | 0.12-26.76 | 6 (46.2) |
| 3                        | 16              | 3.27   | 0.50-26.83 | 5 (31.2) |
| Lympho-vascular tumor emboli |             |        |       |               |
| No                       | 20              | 2.99   | 0.12-26.76 | 6 (30.0) |
| Yes                      | 9               | 2.64   | 0.50-12.63 | 4 (44.4) |
| Extramodal extension     |                 |        |       |               |
| negative                 | 16              | 2.61   | 0.12-26.76 | 4 (25.0) |
| positive                 | 13              | 5.20   | 1.25-26.83 | 7 (53.8) |
| Estrogen receptor        |                 |        |       |               |
| negative                 | 14              | 2.54   | 0.50-26.83 | 2 (14.3)$^*$ |
| positive                 | 16              | 5.35   | 0.12-26.76 | 9 (56.2) |
| Progesterone receptor    |                 |        |       |               |
| negative                 | 14              | 2.72   | 0.50-26.83 | 3 (21.4) |
| positive                 | 16              | 4.72   | 0.12-26.76 | 8 (50.0) |
| P53                      |                 |        |       |               |
| negative                 | 19              | 3.93   | 0.12-26.76 | 8 (42.1) |
| positive                 | 11              | 2.79   | 1.07-2.92 | 3 (27.3) |
| c-erbB2                  |                 |        |       |               |
| negative                 | 13              | 4.09   | 1.34-26.83 | 6 (46.2) |
| positive                 | 17              | 2.44   | 0.12-11.67 | 5 (29.4) |

*: The difference between subgroup, as determined by the Mann-Whitney U test, was not significant ($p>0.05$); $^*$: Statistically significant ($p<0.05$).

Fig. 2. Box and whiskers plots showing the distribution of Bfl-1 values, expressed as relative ratio in the category of Bcl-2 protein expression-the median values are indicated as a line across each box.
protein, the expression value of the Bfl-1 gene ranged between 0.83 and 26.76 (median value, 5.93), and the gene expression rate was 64.3% (9/14). On the other hand, in the group with negative Bcl-2 expression, the value ranged from 0.12 to 26.83 (median value, 2.29) and 12.5% (2/16). This observation suggests that there would be a significant relationship between the two genes (p<0.04) (Fig. 2). Infiltration of TILs was present in the tumor stroma in all examined cases. The distribution of TILs was uneven with the heaviest infiltration at the periphery of the tumor in most cases. The TIL infiltration ranged from 0 cells per high-power field to 530 cells per high-power field in cancerous tissue, with a mean number ranging between 1 and 502 (total mean number, 150.4). For the grade of TIL density, the rates of the low, moderate, high density groups were 46.7%, 30.0%, and 23.3%, respectively. However, a correlation between the density difference of tumor infiltrating lymphocytes and the expression of the Bfl-1 gene was not found.

DISCUSSION

Despite the gradual increase in the incidence of breast cancer worldwide, there has been only substantial improvement in the management of breast cancer that has contributed to the substantial fall in its mortality since 1990. A major contributor to this decrease is clearly the widespread application of hormonal and adjuvant chemotherapy after surgery. However, it is essential to understand the characteristics of breast cancer, including the carcinogenic process itself and its pathogenesis (2). In recent years, there have been important clinical researches on the genes, c-erbB2, p53, Bcl-2, etc., and their association with breast cancer, including their roles as prognostic or predictive factors (10-16). Bcl-2 has been studied as a cell survival-promoting protein in a variety of human tumors with contradictory results. For instance, in accordance with its anti-apoptotic nature, Bcl-2 has been shown to be associated with a poorer prognosis in prostate and colon cancers, and neuroblastoma (17). In breast cancer, on the other hand, Bcl-2 expression has been reported to be associated with better outcomes and improved disease-free survival, although Bcl-2 was not an independent prognostic variable (19). Complex interactions among the various Bcl-2 family members determine the outcome in terms of cell progression toward either apoptotic cell death or survival (20, 21). The Bfl-1 gene is one of the Bcl-2 related genes recently identified by computer analysis of the expressed sequenced tag databases constructed by random cDNA clones from a human fetal liver (5, 23). The Bfl-1 gene, as a member of the Bcl-2 family, was reported to have 72% homology to the murine A1 gene, one of the Bcl-2-related genes. The Bfl-1 gene contains the BH1 and BH2 domains, which are known to be important regulators for apoptosis in Bcl-2-related proteins (22). Recently, some investigators demonstrated that the Bfl-1 is a direct transcriptional target of NF-κB, and thus the activation of Bfl-1 may be the means by which NF-κB promotes oncogenesis and cell resistance to anticancer therapy. The expression patterns of other Bcl-2 related genes are very distinctive (6). The Bfl-1 gene was highly expressed in bone marrow and was also present in hematopoietic cell lines, such as Raji and HL60, and in some normal adult tissues including the lung, spleen, and esophagus. It was not, however, detected in the heart, testis, thyroid, or brain (7). Conflicting data have been reported on the expression of the Bfl-1 gene in different tumor tissues: a high rate of expression in gastric (86%) and colon cancer (93%) specimens, a low rate of expression in breast cancer (33%), bone and soft tissue sarcoma, and ovarian cancer specimens, and a very low rate in some human cancer cell lines (7). We performed a study on the Bfl-1 expression in a series breast cancer samples to analyze and correlate the expression with the clinicopathological features of the patients and other prognostic factors, including Bcl-2 and tumor infiltrating lymphocytes. The latter is considered to reflect the host’s effort to resist tumor growth and has predictive values also (26). In our study, Bfl-1 was expressed in 36.7% as a mRNA band on sensitive RT-PCR. These data were in agreement with the previous observation by Park et al. who demonstrated the Bfl-1 gene was expressed at a low rate (33.3%) in breast cancer (7).

There have been reports that the function of Bfl-1 is distinct from that of Bcl-2 because Bfl-1 permits cell proliferation by its structural variation (6), while the two molecules have a similar function, inhibition of apoptosis. Bcl-2 expression has been reported to be associated with better outcomes and factors that have favorable prognosis, although Bcl-2 was not an independent prognostic variable (19). In the present study, expression of the Bcl-2 protein on immunohistochemistry has a significant association with the hormonal receptors and c-erbB2 (data not shown). Indeed, these patterns were similar to the results for Bfl-1. In the patient groups with the higher level of Bfl-1 expression intensity, the tumors were positive for hormonal receptors and negative for p53 or c-erbB2, which are known as good prognostic markers. A good correlation was found between the expression of the Bfl-1 gene and the estrogen receptor only. When the intensities of Bfl-1 gene expression were compared with the characteristics, including existing prognostic factors, there were no statistically significant parameters. But considering the trend of higher expression in the cancer tissues of patients with larger tumors, auxillary nodal metastasis, or extranodal tumor ex-tension, this observation suggests that Bfl-1 could possibly play a role in advanced cases of breast cancer. Interestingly, there were no significant association of the expression of the Bcl-2 protein with the tumor size or staging as showing a significant difference on the expression of the Bfl-1 gene. Breast cancer cells are often surrounded by inflammatory cell infiltrates as a sign of tumor-host interaction and the presence of lymphocyte infiltrates...
is considered to reflect the host's effort to resist tumor growth (26). Some authors have related the presence of tumor infiltrating lymphocytes to favorable prognosis (27). A study in (26). Some authors have related the presence of tumor infiltrating lymphocytes, including neutrophils and eosinophils. In the present study, we examined whether the expression of the Bfl-1 gene has a correlation with the infiltration of lymphocytes in breast cancer as preliminary research. We found no association between the expression of Bfl-1 and the densities of tumor infiltrating lymphocytes, although further studies are needed before drawing a definite conclusion.

In conclusion, it seems that the increased expression of the Bfl-1 gene serves as a contributory factor in breast cancer, in the same way that another group of genes, the Bcl-2 family, contribute to apoptosis. The Bfl-1 gene was related to more advanced breast cancer and to factors that have favorable prognosis. However in order to know if the Bfl-1 gene may be influential in the clinical manifestation mechanism and outcomes of breast cancer, long-term follow-up of patients with further research on the role of the gene is needed.

REFERENCES

1. Ministry of Health and Welfare, Republic of Korea. Annual Report of the Central Cancer Registry in Korea 2002 (2000. 1.-2000. 12.).
2. Page DL, Jensen RA, Simpson JF. Routinely available indicators of prognosis in breast cancer. Breast Cancer Res Treat 1998; 51: 195-208.
3. White E. Life, death, and the pursuit of apoptosis. Genes Dev 1996; 10: 1-15.
4. Kapranos N, Karaisfidi H, Valavanis K, Kouri E, Vasilaros S. Prognostic significance of apoptosis related proteins Bcl-2 and Bax in node-negative breast cancer patients. Anticancer Res 1997; 17: 2499-505.
5. Choi SS, Park IC, Yun JW, Sung YC, Hong SI, Shin HS. A novel Bcl-2 related gene, Bfl-1, is overexpressed in stomach cancer and preferentially expressed in bone marrow. Oncogene 1995; 11: 1693-8.
6. D'Sa-Eipper C, Subramanian T, Chinnadurai G. Bfl-1, a Bcl-2 homologue, suppresses p53-induced apoptosis and exhibits potent cooperative transforming activity. Cancer Res 1996; 56: 3879-82.
7. Park IC, Lee SH, Whang DY, Hong WS, Choi SS, Shin HS, Choe TB, Hong SI. Expression of a novel Bcl-2 related gene, Bfl-1, in various human cancers and cancer cell lines. Anticancer Res 1997; 17: 4619-22.
8. Ha HJ, Kim D, Lee SB, Hong SI, Park SY, Huh JR, Kim CW, Kim SS, Lee Y, Choi SS, Shin HS. Expression of Bfl-1 in normal and tumor tissues: Bfl-1 overexpression in cancer is attributable to its preferential expression in infiltrating inflammatory cells. Hum Pathol 1998; 29: 723-8.
9. Shim YH, Byun EK, Lee MJ, Huh JR, Kim CW. Anti-apoptotic role of Bfl-1 in staurosporine-treated B-lymphoblastic cells. Int J Hematol 2000; 72: 484-90.
10. Korean Breast Cancer Society. Clinical characteristics of Korean breast cancer patients in 1998. J Korean Med Sci 2000; 15: 569-79.
11. Hamilton A, Piccart M. The contribution of molecular markers to the prediction of response in the treatment of breast cancer: a review of the literature on HER-2, p53 and Bcl-2. Ann Oncol 2000; 11: 647-63.
12. Bottini A, Berruti A, Bersiga A, Brizzi MP, Brunelli A, Gorzegno G. p53 but not Bcl-2 immunostaining is predictive of poor clinical complete response to primary chemotherapy in breast cancer patients. Clin Cancer Res 2000; 6: 2751-8.
13. Daidone MG, Luisi A, Veneroni S, Benini E, Silvestrini R. Clinical studies of Bcl-2 and treatment benefit in breast cancer patients. Endocr Relat Cancer 1999; 6: 61-8.
14. Nakopoulou L, Michalopoulou A, Giannopoulou I, Tzonou A, Keramopoulos A, Lazaris AC. Bcl-2 protein expression is associated with a prognostically favorable phenotype in breast cancer irrespective of p53 immunostaining. Histopathology 1999; 34: 310-9.
15. Raiolo M, Nordling S, von Vogtswalinsky K, Leitonen M, Kyllo nen L, von Smitten K. Prognostic value of Ki-67 immunolabelling in primary operable breast cancer. Br J Cancer 1993; 68: 579-83.
16. Kobayashi S, Iwase H, Ito Y, Yamashita H, Iwata H, Yamashita T. Clinical significance of Bcl-2 gene expression in human breast cancer tissues. Breast Cancer Res Treat 1997; 42: 173-81.
17. Reed JC. Bcl-2 and the regulation of programmed cell death. J Cell Biol 1994; 124: 1-6.
18. Doglioni C, Del Tos AP, Laurino L, Chiarelli C, Barbareisci M, Viale G. The prevalence of BCL-2 immunoactivity in breast carcinomas and its clinicopathological correlates, with particular reference to oestrogen receptor status. Virchows Arch 1994; 424: 47-51.
19. Silvestrini R, Benini E, Veneroni S, Daidone MG, Tomasic G, Salvadori B, p53 and Bcl-2 expression correlates with clinical outcome in a series of node positive breast cancer patients. J Clin Oncol 1996; 14: 1604-10.
20. Krajewski S, Thor AD, Edgerton SM, Moore DH, Reed JC. Immunohistological analysis of Bax and Bcl-2 in p53-immunopositive breast cancer. Clin Cancer Res 1997; 3: 199-208.
21. Reed JC, Miyashita T, Takayama S, Wang HG, Sato T, Krajewski S, Aime-Sempe C. Bcl-2 family proteins: regulator of cell death involved in the pathogenesis of cancer and resistance to therapy. J Cell Biochem 1996; 60: 23-32.
22. D’Sa-Eipper C, Chinnadurai G. Functional dissection of Bfl-1, a Bcl-2 homology: anti-apoptosis, oncogene-cooperation and cell proliferation activities. Oncogene 1998; 16: 3105-14.
23. Choi SS, Park SH, Kim UJ, Shin HS. Bfl-1, a Bcl-2-related gene, is the human homolog of the murine A1, and maps to chromosome 15q 24.3. Mamm Genome 1997; 8: 781-2.
24. Cheng Q, Lee HH, Li Y, Parks TP, Cheng G. Upregulation of Bcl-x and Bfl-1 as a potential mechanism of chemoresistance, which can be overcome by NF-kappa B inhibition. Oncogene 2000; 19: 4936-40.
25. Zong WX, Edelstein LC, Chen C, Bash J, Gelinas C. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-κappa B that blocks TNFalpha-induced apoptosis. Genes Dev 1999; 13: 382-7.

26. Aaltomaa S, Lipponen P, Eskelinen M, Kosma V.-M, Marin S, Alhava E. Lymphocyte infiltration as a prognostic variable in female breast cancer. Eur J Cancer 1992; 28A: 859-64.

27. Yasunaga M, Tabira Y, Nakano K, Iida S, Ichimaru N, Nakamato N. Accelerated growth signal and tumor infiltrating lymphocyte level predict poor outcome in T4 esophageal squamous cell carcinoma. Ann Thorac Surg 2000; 70: 1634-40.