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Increased MCL-1 Expression Is Associated with Poor Prognosis in Ovarian Carcinomas

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To investigate the potential role of the *BCL-2* gene family (*BAX*, *BCL-2*, *MCL-1*, and *BCL-XL*) in ovarian cancer development and progression, mRNA expression levels of these genes were measured using semi-quantitative PCR in epithelial ovarian tumor tissues and normal ovaries. The immunohistochemical expression of MCL-1 in ovarian tumors was also examined. The expression levels of BAX and MCL-1 mRNA were significantly higher in ovarian cancers and in adenomas than in normal ovaries ($P<0.05$). In contrast, the BCL-2 mRNA expression level in ovarian cancers was significantly lower than in ovarian adenomas and in normal ovaries ($P<0.05$). Expression of BCL-XL mRNA was no different between normal ovaries and ovarian tumors. Log-rank testing showed that low BAX mRNA expression and high MCL-1 mRNA expression significantly correlate with poor survival for patients with stage III ovarian carcinomas (BAX, $P=0.05$; MCL-1, $P=0.02$). Immunohistochemical analysis showed that diffuse-positive expression of MCL-1 protein in mucinous carcinomas was significantly higher than in mucinous low malignant potential (LMP) tumors ($P=0.03$). In ovarian cancer cases, diffuse-positive expression of MCL-1 protein significantly correlates with advanced clinical stage, high histologic grade, and poor survival (stage, $P<0.01$; grade, $P=0.01$; survival, $P=0.01$). These results suggest that increased MCL-1 expression may play an important role in replacing the functions of increased BAX and decreased BCL-2 in ovarian carcinoma cells, thereby promoting cell survival, and resulting in a poor prognosis for patients with ovarian cancer.

Key words: MCL-1 — Semi-quantitative PCR — Immunohistochemistry — Ovarian cancer — Prognosis

As ovarian cancer is often asymptomatic in its early stages, the majority of ovarian cancer patients are diagnosed at an advanced clinical stage. Despite recent advances in surgical treatment and chemotherapy, the 5-year survival rate for ovarian cancer patients has remained at less than 50% for the past 30 years.[1)] It is therefore important to elucidate the mechanisms involved in the development and progression of ovarian cancer. While the majority of genetic alterations involved in ovarian carcinomas remain largely unknown, decreased cell death is believed to be a major contributor to pathologic cell accumulation in ovarian neoplasms.

In recent years, many of the genes involved in apoptosis have been identified and cloned.[2, 3)] Some of the most important regulators of apoptosis are BCL-2 and related proteins, MCL-1, BCL-XL, and BAX. BCL-2, MCL-1, and BCL-XL all function as inhibitors of cell death while BAX functions as a cell death promoter. BCL-2 family members appear to regulate apoptosis by forming either homodimers or heterodimers with each other. In light of its antiapoptotic function, high BCL-2 expression levels in cancer cells might be expected to prolong cell survival, allowing the development and progression of tumor cells, and ultimately resulting in poor patient survival rates. In fact, BCL-2 expression is reported to be a poor prognostic marker in certain tumor types, including prostate cancer, leukemia, and high-grade lymphoma.[4–6)] However, several earlier studies have shown that high BCL-2 expression levels actually correlate with improved survival in lung, breast, and ovarian cancer patients, suggesting that other death checkpoint regulators, such as BAX, MCL-1, and BCL-XL, may interfere with BCL-2 functioning in certain tumor types, including ovarian cancer.[7–10)] Regulation of apoptosis in ovarian tumor cells therefore appears to be a highly complicated process involving a cascade of events requiring both proapoptotic and antiapoptotic factors.

The aim of this study was to examine the potential role of BCL-2 family members in the development and progression of ovarian cancer. mRNA expression levels of the proapoptotic factor BAX and antiapoptotic factors, BCL-2, MCL-1, and BCL-XL, were compared in ovarian tumors and normal ovaries using semi-quantitative PCR experi-

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ments. The expression of MCL-1 protein in ovarian tumors was also investigated immunohistochemically. The results were analyzed in terms of tumor type, clinical stage, histologic grade, and histologic type, and the prognostic significance of the expression levels of each factor in ovarian cancer patients was assessed.

MATERIALS AND METHODS

Tissue samples For the semi-quantitative PCR analysis, fresh surgical specimens of 36 ovarian tumors (6 adenomas and 30 carcinomas) were collected. The specimens were obtained immediately following the surgical procedure and were cut in half. One half was processed for histological examination in order to determine the percentage of tumor cells in the samples, which was never lower than 80%. The other half was used for mRNA preparation. In addition, eight normal ovaries were obtained from patients before undergoing surgery for benign gynecologic disease. Tissues were frozen in liquid nitrogen and stored at −80°C prior to mRNA isolation. Informed consent was obtained from each subject according to the institutional guidelines. All cancer patients were surgically treated, histologically diagnosed, and received follow-up care in the Department of Obstetrics and Gynecology at Hiroshima University Hospital. Clinical staging was determined in accordance with the criteria of the International Federation of Gynecology and Obstetrics (FIGO). The mean follow-up time was 36 months (range 15−60 months); 82% of the patients were followed for more than 2 years.

For immunohistochemical analysis, samples of epithelial ovarian tumor tissue were collected from 93 patients (21 low malignant potential (LMP) tumors and 72 adenocarcinomas) who gave informed consent according to the institutional guidelines. All patients were surgically treated between 1989 to 1997, histologically diagnosed, and received follow-up care in the Department of Obstetrics and Gynecology at Hiroshima University Hospital. Clinical staging was determined in accordance with the criteria of the FIGO staging system. Most of the patients with stage Ic, stage II, stage III, or stage IV ovarian cancer had received cisplatin-containing chemotherapy after surgery. The mean follow up time was 48 months (range 21−140 months).

mRNA extraction and cDNA synthesis Extraction of mRNA from the tissue specimens and cDNA synthesis were carried out by methods described previously.[11−15] mRNA was isolated using a RiboSep mRNA isolation kit (Becton Dickinson Labware, Bedford, MA). The amount of mRNA recovered was measured by UV spectrophotometry. cDNA was synthesized with 2.0 μg of mRNA by random hexamer priming using a 1st strand cDNA synthesis kit (Clontech, Palo Alto, CA). The efficiency of cDNA synthesis was estimated using glucose 3-phosphate dehydrogenase (G3PDH) amplimers (Clontech) as a positive control.

Semi-quantitative PCR mRNA expression levels of BAX, BCL-2, MCL-1, and BCL-XL were determined by PCR, which was performed according to a previously described method with some modifications.[11−15] The oligonucleotide primer sequences used for PCR were as follows: BAX sense primer, 5′-AGCTGAGCGAGTGTCTCAAG-3′; BAX antisense primer, 5′-TCTTCCAGATGGTGAGCGAG-3′; BCL-2 sense primer, 5′-TGCCACCTGTGGTCCACCTG-3′; BCL-2 antisense primer 5′-TGTGGCCTCAGCCCAGACTCAC-3′; MCL-1 sense primer, 5′-TCTCTCGGTACCTTCGGG-3′; MCL-1 antisense primer, 5′-GCACTTACAGTAAGGCTATC-3′; BCL-XL sense primer, 5′-CATTCAGTGACCTGACATCC-3′; BCL-XL antisense primer, 5′-TTCTCCTGGATCCAAGGCTC-3′. β-Tubulin cDNA amplification was used as the internal PCR control. The β-tubulin sense primer was 5′-TGCATTGACAACGAGGC-3′, and the antisense primer was 5′-CTGTCTTGACATTGTTG-3′. The predicted sizes of the amplified genes were 396 bp for *BAX*, 330 bp for *BCL-2*, 504 bp for *MCL-1*, 204 bp for *BCL-XL*, and 454 bp for *β-tubulin*. The PCR reaction mixture consisted of cDNA derived from 50 ng of mRNA, 5 pmol of sense and antisense primers, 200 μmol of dNTPs, and 0.625 U of *Ampli Taq* DNA polymerase with reaction buffer (Perkin Elmer, Foster City, CA) in a final volume of 25 μl. Twenty-eight cycles of PCR were carried out in a Thermal Cycler (Perkin Elmer, Foster City, CA). Each PCR cycle involved 30 s of denaturation at 94°C, 60 s of primer annealing at 55°C for MCL-1 and BCL-XL, 60 s at 60°C for BAX, or 60 s at 62°C for BCL-2 and β-tubulin, and 30 s of extension at 72°C. We have confirmed that amplification under these conditions results in a linear production of each product. Tubes containing all ingredients except templates were included in all runs as a negative control. The PCR products were separated on 2.0% agarose gels, and the density of each PCR product was determined using a Printgraph-Densitograph system (ATTO Corp., Tokyo). In the present study, we used the expression ratio (target gene: *β-tubulin*) as measured by densitometry to evaluate gene expression. The results are expressed as the mean±SD. The differences in the mean value of the expression ratio of target genes between groups were assessed with respect to tumor type, clinical stage, histologic grade, and histologic type. In addition, in ovarian cancer cases, the cases were classified into the following two groups according to the expression ratio of each of the target genes: low-expression cases, <median value; high-expression cases, >median value. A Kaplan-Meier survival curve of ovarian cancer patients was categorized according to the high or low expression of BAX, BCL-2, MCL-1, and BCL-XL.

Immunohistochemistry Formalin-fixed and paraffin-embedded sections were cut and mounted on aminopro-

pyltriethoxysilane-treated slides. The slides were routinely deparaffinized with xylene and rehydrated with a series of ethanol washes. Nonenzymatic antigen retrieval was performed using a 500 W microwave heat treatment in 0.01 *M* sodium citrate buffer (pH 6.0) for 3 min×7. Immunohistochemical staining was performed by using the avidin-biotin peroxidase complex technique (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) as previously described.[11–13, 15] Anti-MCL-1 (S-19) rabbit polyclonal antibody (sc-819, Santa Cruz Biotechnology, Santa Cruz, CA) was used as a primary antibody. The final products were visualized by using the 3-amino-9-ethylcarbazole (AEC) substrate system (DAKO Corp., Carpinteria, CA), and sections were counterstained with Mayer hematoxylin for 20 s before mounting. Positive and negative controls were used for each section. As the positive control, we included normal endometrium, according to the literature.[16] Slides incubated with normal rabbit serum instead of the primary antibody served as a negative control. All experiments were duplicated. The percentage of positive tumor cells was scored as follows. When no positive tumor cell stain could be identified or when there were less than 5% focally distributed positive tumor cells, the staining was considered negative. When 5% to 50% of the tumor cells showed positive staining, staining was considered partial-positive. When more than 50% of the tumor cells showed positive staining, staining was considered diffuse-positive. All cases were scored independently by two of the authors without knowledge of patient information. Increased MCL-1 protein expression was defined as a diffuse-positive staining of MCL-1. In ovarian cancer cases, the cases were classified into two groups according to the immunohistochemical expression of MCL-1. A Kaplan-Meier survival curve of ovarian cancer patients was categorized according to the negative, partial-positive, or diffuse-positive expression of MCL-1.

Statistical methods For the statistical analysis, an unpaired *t* test was used to assess the differences in the mean value of the target gene:β-tubulin mRNA expression ratios between groups. The χ^2 test of significance and Fisher's exact probability were used to analyze the distribution of diffuse-positive MCL-1 expression cases according to clinicopathologic characteristics. Statistical significance of differences in survival rates was tested by log-rank. Significance was defined as $P<0.05$. The Statview software package (Abacus Concepts, Berkeley, CA) was used for the statistical tests.

RESULTS

mRNA expression of *BCL-2* family genes To evaluate the mRNA expression of BAX, BCL-2, MCL-1, and BCL-XL in ovarian tumors and normal ovaries, we performed semi-quantitative PCR. As shown in Fig. 1A, BAX transcript expression relative to the β-tubulin was more elevated in the cancer samples than in normal ovaries. The ratios of BAX mRNA expression relative to those of β-tubulin in ovarian tumors and normal ovaries are shown in Fig. 2A. The mean and SD of the BAX, BCL-2, MCL-1, and BCL-XL expression ratios determined for the various tumor subtypes are shown in Table I. The BAX:β-tubulin expression ratio (mean±SD) was 0.72±0.29 for normal ovaries, 1.20±0.19 for ovarian adenomas, and 1.12±0.32 for adenocarcinomas. The mean value of the relative BAX expression ratio was significantly higher in both ovarian cancer samples (P=0.005) and adenoma samples (P=0.005) than in normal ovary samples. As shown in Figs. 1B and 2B, the relative expression of BCL-2 was lower in some cancer samples than in normal ovaries. The BCL-2:β-tubulin expression ratio was 0.81±0.12 for normal ovaries, 0.72±0.20 for ovarian adenomas, and 0.34±0.33 for adenocarcinomas. The mean relative BCL-2 expression ratio was significantly lower in ovarian cancers than in either ovarian adenomas (P=0.010) or normal ovaries (P=0.0003). The PCR results shown in Fig. 1C indicate that higher levels of MCL-1 transcript relative to β-tubulin are expressed in some carcinomas and lower levels are expressed in other carcinomas. As shown in Fig. 2C and Table I, the MCL-1:β-tubulin expression ratio was 1.57±0.05 for normal ovaries, 1.79±0.11 for ovarian adenomas, and 1.72±0.17 for adenocarcinomas. MCL-1

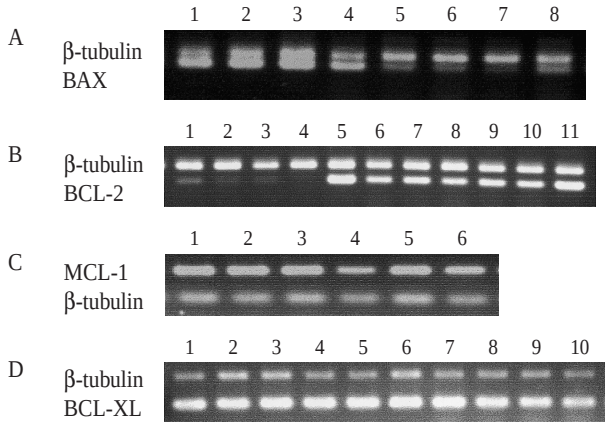

Fig. 1. A, Lanes 1–4 are ovarian cancer samples. Lanes 5–8 are normal ovaries. BAX expression levels are elevated in these cancer samples compared to normal ovaries. B, Lanes 1–4 are ovarian cancers with low BCL-2 expression. Lanes 5 and 6 are ovarian cancers with high BCL-2 expression. Lanes 7–11 are normal ovaries with high BCL-2 expression. C, Lanes 1, 2, 3, and 5 are ovarian cancer samples with high MCL-1 expression. Lanes 4 and 6 are ovarian cancer samples with low MCL-1 expression. D, Lanes 1–5 are ovarian cancers and lanes 6–10 are normal ovaries. There is no difference in BCL-XL expression levels in these tissues.

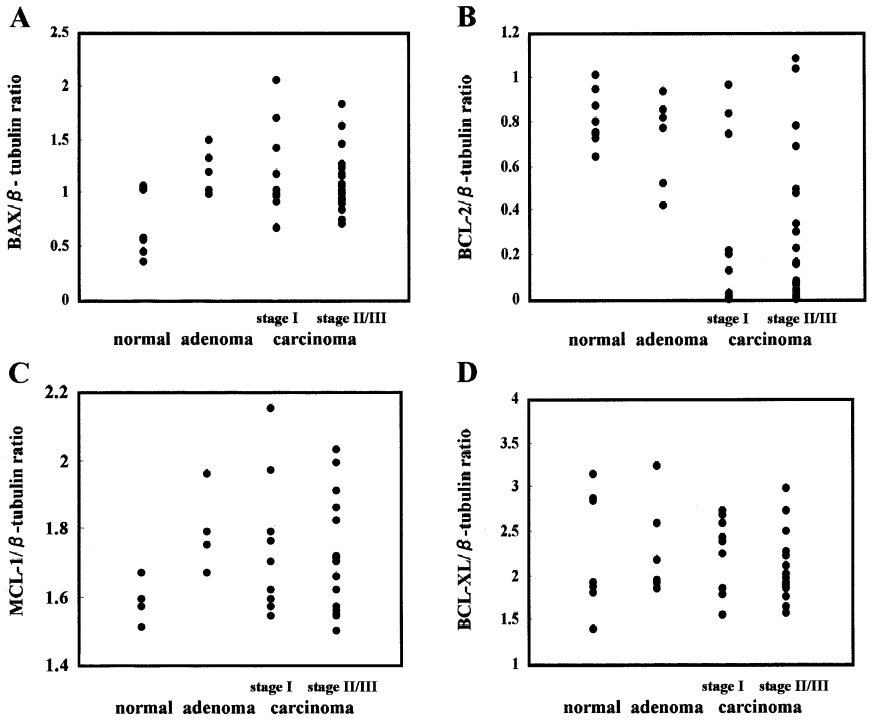

Fig. 2. A, BAX:β-tubulin ratios determined by semi-quantitative PCR. B, BCL-2:β-tubulin ratios determined by semi-quantitative PCR. C, MCL-1:β-tubulin ratios determined by semi-quantitative PCR. D, BCL-XL:β-tubulin ratios determined by semi-quantitative PCR.

Table I. Relative Expression Levels of BAX, BCL-2, MCL-1, and BCL-XL mRNA in Normal Ovaries and Ovarian Tumors

	N	BAX Mean±SD	BCL-2 Mean±SD	MCL-1 Mean±SD	BCL-XL Mean±SD
Normal ovary	8	0.72±0.29	0.81±0.12	1.57±0.05	2.26±0.68
Ovarian adenoma	6	1.20±0.19[a)]	0.72±0.20	1.79±0.11[d)]	2.18±0.29
Ovarian adenocarcinoma	30	1.12±0.32[b)]	0.34±0.33[c)]	1.72±0.17[e)]	2.22±0.59
Clinical stage					
Stage I	11	1.17±0.40	0.35±0.35	1.74±0.20	2.21±0.40
Stage II/III	19	1.10±0.29	0.33±0.32	1.71±0.15	2.23±0.68
Histologic grade					
Grade 1	15	1.16±0.35	0.35±0.35	1.73±0.19	2.21±0.39
Grade 2/3	15	1.08±0.31	0.33±0.31	1.71±0.15	2.23±0.69
Histologic type					
Serous	14	1.13±0.37	0.38±0.38	1.73±0.16	2.20±0.38
Mucinous	7	1.17±0.34	0.25±0.25	1.69±0.14	2.43±0.98
Endometrioid	6	1.06±0.10	0.30±0.21	1.70±0.24	1.95±0.45
Clear cell	3	1.10±0.53	0.42±0.41	1.77±0.20	2.38±0.24

a) Adenoma vs. normal, $P=0.005$, unpaired *t* test.
b) Adenocarcinoma vs. normal, $P=0.005$, unpaired *t* test.
c) Adenocarcinoma vs. normal, $P=0.0003$; adenocarcinoma vs. adenoma, $P=0.010$; unpaired *t* test.
d) Adenoma vs. normal, $P=0.007$, unpaired *t* test.
e) Adenocarcinoma vs. normal, $P=0.028$, unpaired *t* test.

expression was significantly higher in both ovarian cancer (P=0.028) and adenoma samples (P=0.007) than in normal ovary samples. Figs. 1D, 2D, and Table I show that there was no significant difference in BCL-XL expression levels in ovarian cancer versus normal ovaries. The BCL-XL:β-tubulin expression ratio was 2.26±0.68 for normal ovaries, 2.18±0.29 for ovarian adenomas, and 2.22±0.59 for adenocarcinomas. Finally, there was no statistically significant difference between the BAX, BCL-2, MCL-1, and BCL-XL mRNA expression levels in terms of clinical stage, histologic grade, or histologic type.

Correlation between BAX, BCL-2, MCL-1, and BCL-XL mRNA expression in ovarian cancers and patient survival rates The Kaplan-Meier survival curves for 30 ovarian cancer patients were compared with the tumor tissue expression levels of BAX, BCL-2, MCL-1, and BCL-XL. Analysis of all four target genes failed to reveal an association between expression levels and survival prognosis in all cases studied. However, when the analysis was restricted to 18 patients with FIGO stage III disease, log-rank testing revealed that high BAX mRNA expression was significantly correlated with better survival in stage III ovarian carcinomas (P=0.0498) (Fig. 3A). The most significant association was found between high MCL-1 mRNA expression levels and poor prognosis in stage III ovarian cancers (P=0.0187) (Fig. 3B). No significant correlations were found between BCL-2 or BCL-XL mRNA expression levels and survival in stage III carcinomas.

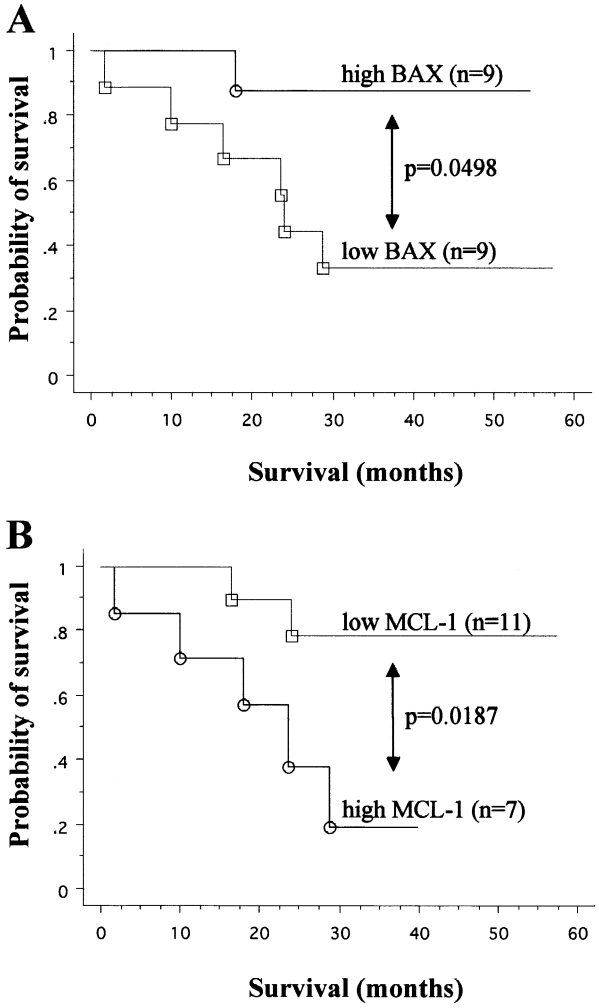

Fig. 3. A, Log-rank testing revealed that high BAX mRNA expression is significantly correlated with better patient survival in stage III ovarian carcinomas (P=0.0498). B, A significant association was found between high MCL-1 mRNA expression and poor prognosis in stage III ovarian cancers (P=0.0187).

MCL-1 immunohistochemistry To confirm the presence of MCL-1 protein in ovarian tumor cells, we performed immunohistochemical staining of 21 LMP tumors and 72 adenocarcinomas. As shown in Fig. 4, MCL-1 was immunolocalized to the cytoplasm of cancer cells, but not in the underlying stromal cells. The total amount of positive MCL-1 protein expression, including both partial-positive and diffuse-positive cases, was 90% (19/21) for ovarian LMP tumors and 86% (62/72) for carcinomas. Table II shows the percentage of diffuse-positive MCL-1-expressing tissues as determined by immunohistochemical analysis: 57% (12/21) of ovarian LMP tumors and 72% (52/72) of carcinomas were diffuse-positive for MCL-1 expression. The percentage of diffuse-positive MCL-1-expressing mucinous carcinomas was significantly higher than that of mucinous LMP tumors (P=0.032). In terms of histologic types, there was a significant higher percentage of diffuse-positive MCL-1-expressing serous and mucinous carcinomas compared to clear cell carcinomas (serous vs. clear cell, P=0.009; mucinous vs. clear cell, P=0.013). The percentage of diffuse-positive MCL-1-expressing tissues significantly correlated with advanced clinical stage and high histologic grade (stage I/II vs. stage III/IV, P=0.0004; grade 1 vs. grade 2/3, P=0.009). The Kaplan-Meier survival curve of 52 ovarian cancer patients was categorized according to the immunohistochemical expression levels of MCL-1. Log-rank testing revealed that increased MCL-1 protein expression was significantly correlated with poor survival in ovarian carcinomas (P=0.0097) (Fig. 5).

DISCUSSION

This study revealed a striking association between increased MCL-1 expression levels and a poor prognosis in ovarian cancers. Lomo *et al.*[17] previously observed a correlation between cell survival and MCL-1 expression in peripheral blood B cells. BCL-2 expression levels were unaltered in this system, suggesting the possible involvement of MCL-1 rather than BCL-2 in the regulation of apoptosis in these cells. Rieger *et al.*[18] reported that MCL-

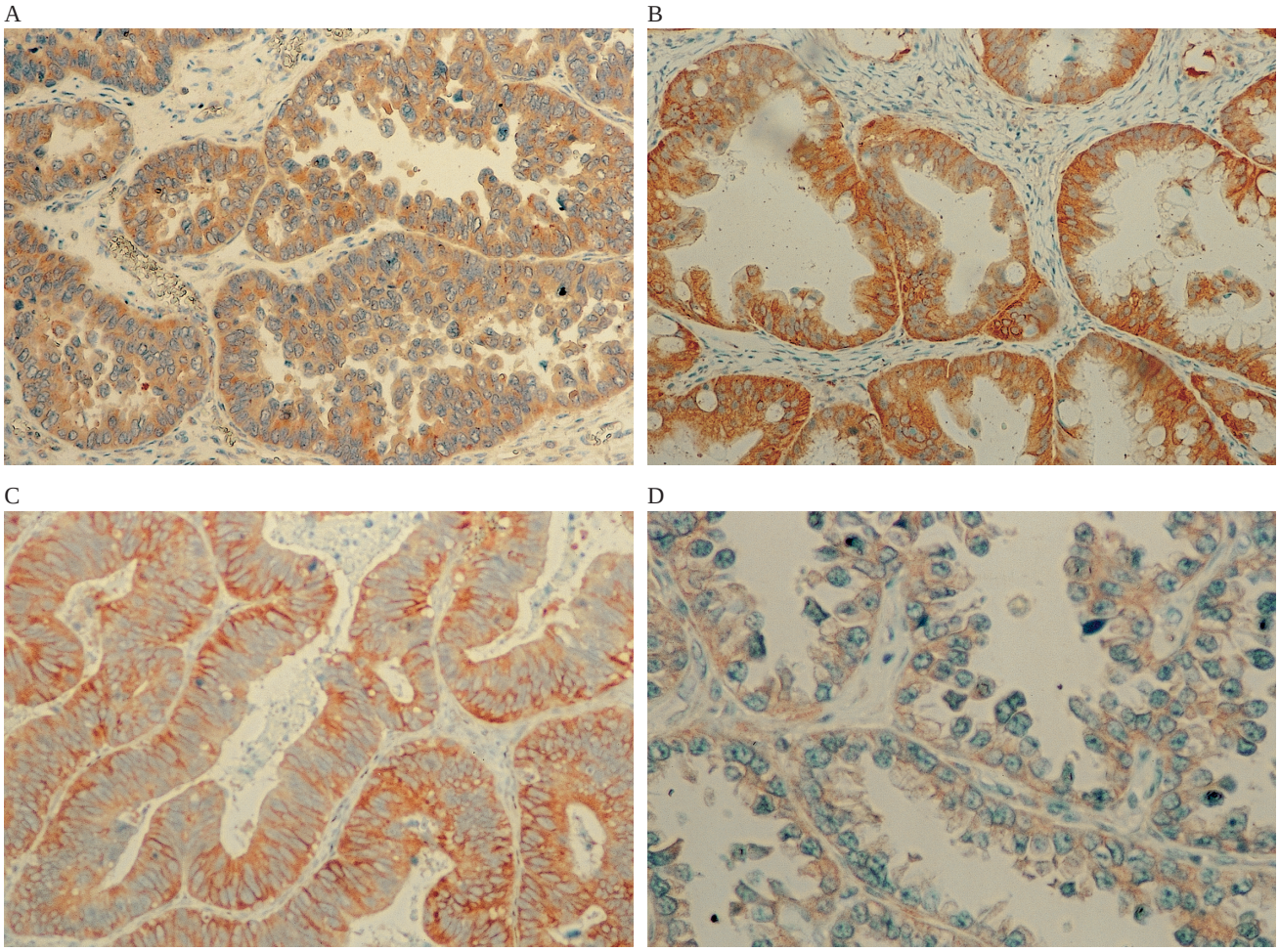

Fig. 4. Immunohistochemistry. Cytoplasmic expression of MCL-1 protein was observed in serous (A, ×50), mucinous (B, ×50), endometrioid (C, ×50), and clear cell (D, ×100) subtypes of ovarian adenocarcinoma cells.

1 protein expression was associated with early tumor recurrence and shorter survival in malignant glioma patients, whereas there was no prominent association with the expression of p53, RB, BCL-2, BCL-X, or BAX. In this study, we used semi-quantitative PCR to show that high MCL-1 mRNA expression significantly correlates with poor survival for patients with stage III ovarian carcinomas. Furthermore, we used immunohistochemistry to demonstrate that increased MCL-1 protein expression significantly correlates with advanced clinical stage, high histologic grade, and poor survival for ovarian cancer patients. Baekeland *et al.*[19] also reported that immunohistochemical expression of MCL-1 was significantly associated with poorer prognosis for stage III ovarian cancer patients. These results suggest that MCL-1 may be involved in the regulation of apoptosis in ovarian cancer cells and that it may play an important role in taking over the functions of BAX and BCL-2. MCL-1 expression levels have the potential to become a useful prognostic marker in ovarian cancer patients. Molecular mechanisms underlying high MCL-1 expression levels should be elucidated to clarify the cause of resistance to chemotherapeutic agents. It has been reported that MCL-1 is induced after stimulation by various growth factors or cytokines.[20–22] Recently, Wei *et al.*[23] reported that interleukin-6 regulated MCL-1 expression through a PI3K/Akt-dependent pathway in human cervical cancer; that might facilitate the oncogenesis of cervical cancer by modulating the apoptotic threshold. Since we have reported that vascular endothelial growth factor (VEGF) induces MCL-1 expression and inhibits apoptotic death in hematopoietic cells,[24] we also examined the expression levels of VEGF mRNA in our ovarian cancer samples. There was, however, no correlation between VEGF expression levels and those of

Table II. Immunohistochemical Expression of MCL-1 in Ovarian Tumors

Tumor type	*N*	Diffuse-positive expression of MCL-1[a] (%)
Ovarian LMP tumor	21	12 (57)
Histologic type		
Serous	7	5 (71)
Mucinous	14	7 (50)[b]
Ovarian carcinoma	72	52 (72)
Histologic type		
Serous	26	22 (85)[c]
Mucinous	16	14 (88)[b,d]
Endometrioid	16	10 (63)
Clear cell	14	6 (43)[c,d]
Clinical stage		
Stage I/II	41	23 (56)[e]
Stage III/IV	31	29 (94)[e]
Histologic grade		
Grade 1	48	30 (63)[f]
Grade 2/3	24	22 (92)[f]

a) Diffuse-positive=more than 50% positive tumor cells.
b) Mucinous LMP tumor vs. mucinous carcinoma, $P=0.032$, Fisher's exact test.
c) Serous carcinoma vs. clear cell carcinoma, $P=0.009$, Fisher's exact test.
d) Mucinous carcinoma vs. clear cell carcinoma, $P=0.013$, Fisher's exact test.
e) Stage I/II vs. stage III/IV, $P=0.0004$, χ^2 test.
f) Grade 1 vs. grade 2/3, $P=0.009$, χ^2 test.

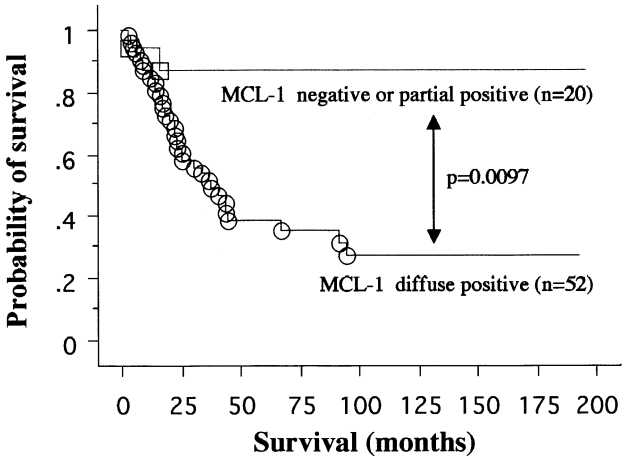

Fig. 5. Log-rank testing showed that increased MCL-1 protein expression is significantly correlated with poor prognosis in ovarian cancers ($P=0.0097$).

MCL-1 (data not shown), indicating that signal transduction pathways stimulated by other growth factors or cytokines may be involved in transcriptional regulation of the *MCL-1* gene in ovarian cancers.

BCL-2 family members form homo- or hetero-dimers with each other, and these complex protein-protein associations direct cells toward either survival or death, although the precise mechanisms of these processes are unclear.[2, 3] Marone *et al.*[25] reported that BCL-2, BAX, and BCL-XL were present to a variable degree in both normal and neoplastic ovarian tissues and that their mRNA and protein levels were directly correlated. Several earlier studies have suggested that BCL-2 mRNA and/or protein expression levels were higher in normal ovaries than in ovarian carcinomas, whereas both BAX and BCL-XL expression was higher in carcinomas.[25, 26] In the present study, we also observed decreased BCL-2 and increased BAX mRNA expression levels in ovarian cancers compared with those in normal ovaries, although we did not find any significant difference between the level of BCL-XL mRNA expression in normal ovaries vs. ovarian cancer samples. Increased BAX expression and decreased BCL-2 expression in ovarian cancer cells seem to be paradoxical, given the proposed pro-apoptotic function of BAX and anti-apoptotic function of BCL-2. It is likely that increased BAX and decreased BCL-2 expression in ovarian cancer cells may be counteracted by other anti-apoptotic proteins which bind to BAX and/or BCL-2. The present study suggests the possibility that significantly elevated MCL-1 expression might replace the functioning of BAX and BCL-2 in ovarian cancer cells.

Several earlier studies have shown that immunohistochemical expression of BCL-2 is associated with improved survival.[9, 10] However, in the present study using semi-quantitative PCR, low BCL-2 expression was not associated with a survival advantage. Diebold *et al.*[27] have reported that BCL-2 expression failed to reveal a correlation with the prognosis in the total study population of ovarian carcinoma patients. Therefore, the relationship between BCL-2 expression and prognosis in ovarian cancer patients is still controversial. However, decreased BCL-2 expression in ovarian cancer cells compared to that in ovarian adenomas and normal ovaries suggests that BCL-2 regulation may play an important role in the development of ovarian cancer.

In the present study, we found that high BAX mRNA expression was significantly correlated with a better survival in stage III ovarian carcinomas. It has been reported that immunohistochemical expression of BAX is also significantly associated with improved prognosis of ovarian cancer patients.[19, 28] p53 is known to be a direct transcriptional activator of the *BAX* gene,[29] and p53 accumulation in tumor cells, which presumably is correlated with the presence of p53 missense mutations, is associated with a poor prognosis in ovarian cancer.[30] It has been reported that a cisplatin-resistant ovarian cancer cell line was found to have reduced BAX mRNA levels, which is consistent

with the loss of the ability of p53 to transactivate BAX as a consequence of p53 mutation.[31] Tai *et al.*[32] have demonstrated that reduced expression of BAX was related to lower response rates in chemotherapy-treated ovarian cancer patients. Therefore, ovarian cancer tissues expressing low levels of BAX mRNA may be partly related to the loss of p53-dependent transcription in at least some patients and may be expected to be associated with poorer survival rates.

Although these preliminary observations will have to be confirmed with larger samples of ovarian cancer patients, determination of a selected apoptosis-related protein such as MCL-1 or BAX would be a useful prognostic molecular marker for ovarian carcinomas. An extension of these findings would argue for the use of apoptosis-related proteins as therapeutic agents in the treatment and prevention of ovarian cancers.

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REFERENCES

1) Parker, S. L., Tong, T., Bolden, S. and Wingo, P. A. Cancer statistics. *CA Cancer J. Clin.*, **46**, 5–27 (1996).
2) Kroemer, G. The proto-oncogene Bcl-2 and its role in regulating apoptosis. *Nat. Med.*, **3**, 614–620 (1997).
3) Korsmeyer, S. J. *BCL*-2 gene family and the regulation of programmed cell death. *Cancer Res.*, **59** (Suppl.), 1693s–1700s (1999).
4) McDonnell, T. J., Troncoso, P., Brisbay, S. M., Logothetis, C., Chung, L. W. K., Hsieh, J.-T., Tu, S.-M. and Campbell, M. L. Expression of the protooncogene *bcl*-2 in the prostate and its association with emergence of androgen-independent prostate cancer. *Cancer Res.*, **52**, 6940–6944 (1992).
5) Campos, L., Rouault, J.-P., Sabido, O., Oriol, P., Roubi, N., Vasselon, C., Archimbaud, E., Magaud, J.-P. and Guyotat, D. High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. *Blood*, **81**, 3091–3096 (1993).
6) Yunis, J. J., Mayer, M. G., Arnesen, M. A., Aeppli, D. P., Oken, M. M. and Frizzera, G. *bcl*-2 and other genomic alterations in the prognosis of large-cell lymphoma. *N. Engl. J. Med.*, **320**, 1047–1054 (1989).
7) Pezzella, F., Turley, H., Kuzu, I., Tungekar, M. F., Dunnill, M. S., Pierce, C. B., Harris, A., Gatter, K. C. and Mason, D. Y. *bcl*-2 protein in non-small-cell lung carcinoma. *N. Engl. J. Med.*, **329**, 690–694 (1993).
8) Leek, R. D., Kaklamanis, L., Pezzella, F., Gatter, K. C. and Harris, A. L. *bcl*-2 in normal human breast and carcinoma, association with oestrogen receptor-positive, epidermal growth factor receptor-negative tumours and *in situ* cancer. *Br. J. Cancer*, **69**, 135–139 (1994).
9) Henriksen, R., Wilander, E. and Oberg, K. Expression and prognostic significance of Bcl-2 in ovarian tumours. *Br. J. Cancer*, **72**, 1324–1329 (1995).
10) Herod, J. J. O., Eliopoulos, A. G., Warwick, J., Niedobitek, G., Young, L. S. and Kerr, D. J. The prognostic significance of Bcl-2 and p53 expression in ovarian carcinoma. *Cancer Res.*, **56**, 2178–2184 (1996).
11) Shigemasa, K., Hu, C., West, C. M., Clarke, J., Parham, G. P., Parmley, T. H., Korourian, S., Baker, V. V. and O'Brien, T. J. p16 overexpression: a potential early indicator of transformation in ovarian carcinoma. *J. Soc. Gynecol. Invest.*, **4**, 95–102 (1997).
12) Shigemasa, K., Hu, C., West, C. M., Moon, S. H., Parham, G. P., Parmley, T. H., Korourian, S., Baker, V. V. and O'Brien, T. J. p21: a monitor of p53 dysfunction in ovarian neoplasia. *Int. J. Gynecol. Cancer*, **7**, 296–303 (1997).
13) Shigemasa, K., Tanimoto, H., Parham, G. P., Parmley, T. H., Ohama, K. and O'Brien, T. J. Cyclin D1 overexpression and p53 mutation status in epithelial ovarian cancer. *J. Soc. Gynecol. Invest.*, **6**, 102–108 (1999).
14) Shigemasa, K., Underwood, L. J., Beard, J., Tanimoto, H., Ohama, K., Parmley, T. H. and O'Brien, T. J. Overexpression of testisin, a serine protease expressed by testicular germ cells, in epithelial ovarian tumor cells. *J. Soc. Gynecol. Invest.*, **7**, 11–18 (2000).
15) Shigemasa, K., Shiroyama, Y., Sawasaki, T., Fujii, T., Nagai, N., Parmley, T. H., O'Brien, T. J. and Ohama, K. Underexpression of cyclin-dependent kinase inhibitor p27 is associated with poor prognosis in serous ovarian carcinomas. *Int. J. Oncol.*, **18**, 953–958 (2001).
16) Krajewski, S., Bodrug, S., Krajewska, M., Shabaik, A., Gascoyne, R., Berean, K. and Reed, J. C. Immunohistochemical analysis of Mcl-1 protein in human tissues. Differential regulation of Mcl-1 and Bcl-2 protein production suggests a unique role for Mcl-1 in control of programmed cell death *in vivo*. *Am. J. Pathol.*, **146**, 1309–1319 (1995).
17) Lomo, J., Smeland, E. B., Krajewski, S., Reed, J. C. and Blomhoff, K. Expression of the Bcl-2 homologue Mcl-1 correlates with survival of peripheral blood B lymphocytes. *Cancer Res.*, **56**, 40–43 (1996).
18) Rieger, L., Weller, M., Bornemann, A., Schabet, M., Dichgans, J. and Meyermann, R. BCL-2 family protein expression in human malignant glioma: a clinical-pathological correlative study. *J. Neurol. Sci.*, **155**, 68–75 (1998).
19) Baekeland, M., Holm, R., Nesland, J. M., Trope, C. G. and Kristensen, G. B. Expression of apoptosis-related proteins is an independent determinant of patient prognosis in

advanced ovarian cancer. *J. Clin. Oncol.*, **18**, 3775–3781 (2000).
20) Chao, J. R., Wang, J. M., Lee, S. F., Peng, H. W., Lin, Y. H., Chou, C. H., Li, J. C., Huang, H. M., Chou, C. K., Kuo, M. L., Yen, J. J. Y. and Yang-Yen, H. F. mcl-1 is an immediate-early gene activated by the granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling pathway and is one component of the GM-CSF viability response. *Mol. Cell. Biol.*, **18**, 4883–4898 (1998).
21) Leu, C. M., Chang, C. and Hu, C. Epidermal growth factor (EGF) suppresses staurosporine-induced apoptosis by inducing mcl-1 via the mitogen-activated protein kinase pathway. *Oncogene*, **19**, 1665–1675 (2000).
22) Puthier, D., Bataille, R. and Amiot, M. IL-6 up-regulates mcl-1 in human myeloma cells through JAK/STAT rather than ras/MAP kinase pathway. *Eur. J. Immunol.*, **29**, 3945–3950 (1999).
23) Wei, L. H., Kuo, M. L., Chen, C. A., Chou, C. H., Cheng, W. F., Chang, M. C., Su, J. L. and Hsieh, C. Y. The anti-apoptotic role of interleukin-6 in human cervical cancer is mediated by up-regulation of Mcl-1 through a PI3K/Akt pathway. *Oncogene*, **20**, 5799–5809 (2001).
24) Katoh, O., Takahashi, T., Oguri, T., Kuramoto, K., Mihara, K., Kobayashi, M., Hirata, S. and Watanabe, H. Vascular endothelial growth factor inhibits apoptotic death in hematopoietic cells after exposure to chemotherapeutic drugs by inducing MCL-1 acting as an antiapoptotic factor. *Cancer Res.*, **58**, 5565–5569 (1998).
25) Marone, M., Scambia, G., Mozzetti, S., Ferrandina, G., Iacovella, S., Pasqua, A. D., Benedetti-Panici, P. and Mancuso, S. bcl-2, bax, bcl-x_L, and bcl-x_S expression in normal and neoplastic ovarian tissues. *Clin. Cancer Res.*, **4**, 517–524 (1998).
26) Witty, J. P., Jensen, R. A. and Johnson, A. L. Expression and localization of BCL-2 related proteins in human ovarian cancers. *Anticancer Res.*, **18**, 1223–1230 (1998).
27) Diebold, J., Baretton, G., Felchner, M., Meier, W., Dopfer, K., Schmidt, M. and Lohrs, U. *bcl*-2 expression, p53 accumulation, and apoptosis in ovarian carcinomas. *Anat. Pathol.*, **105**, 341–349 (1996).
28) Schuyer, M., van der Burg, M. E. L., Henzen-Logmans, S. C., Fieret, J. H., Klijn, J. G. M., Look, M. P., Foekens, J. A., Stoter, G. and Berns, E. M. J. J. Reduced expression of BAX is associated with poor prognosis in patients with epithelial ovarian cancer: a multifactorial analysis of TP53, p21, BAX and BCL-2. *Br. J. Cancer*, **85**, 1359–1367 (2001).
29) Miyashita, T. and Reed, J. C. Tumor suppressor p53 is a direct transcriptional activator of the human *bax* gene. *Cell*, **80**, 293–299 (1995).
30) Bosari, S., Viale, G., Radaelli, U., Bossi, P., Bonolidi, E. and Coggi, G. p53 accumulation in ovarian carcinomas and its prognostic implications. *Hum. Pathol.*, **24**, 1175–1179 (1993).
31) Perego, P., Giarola, M., Righetti, S. C., Supino, R., Caserini, C., Delia, D., Pierotti, M. A., Miyashita, T., Reed, J. C. and Zunino, F. Association between cisplatin resistance and mutation of p53 gene and reduced BAX expression in ovarian carcinoma cell systems. *Cancer Res.*, **56**, 556–562 (1996).
32) Tai, Y. T., Lee, S., Niloff, E., Weisman, C., Strobel, T. and Cannistra, S. A. BAX protein expression and clinical outcome in epithelial ovarian cancer. *J. Clin. Oncol.*, **16**, 2583–2590 (1998).