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
mRNA from the tissue specimens and cDNA synthesis. Extraction of mRNA was carried out by methods described previously.11 mRNA from the tissue specimens and cDNA synthesis were carried out by methods described previously.11 mRNA extraction and cDNA synthesis were carried out by methods described previously.11 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'-AGCTGAGCGAGTGCTCAAG-3'; BAX antisense primer, 5'-TCTTCCAGATGGTGAGCGAG-3'; BCL-2 sense primer, 5'-TGCCACCTGTGGTCCACCTG-3'; BCL-2 antisense primer 5'-TGTGGCCTCAAGCCAGACTC-3'; MCL-1 sense primer, 5'-TCTTCTCGGTACCTTGCGG-3'; MCL-1 antisense primer, 5'-GCACCTTACGTAAGGCTATC-3'; BCL-XL sense primer, 5'-CATTCAGTGACCTTGACATCC-3'; BCL-XL antisense primer, 5'-TTCTCTGGATCAAAGGCTC-3'. β-Tubulin cDNA amplification was used as the internal PCR control. The β-tubulin sense primer was 5'-TGCTTACAGGTACCTTG-3', and the antisense primer was 5'-CTGTCTTGACATCAGCTC-3'. The predicted sizes of the amplified genes were 396 bp for BAX, 300 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-
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

[Diagram Fig. 1]
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.

**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-
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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

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.
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. Marone et al. 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. 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. However, in the present study using semiquantitative PCR, low BCL-2 expression was not associated with a survival advantage. Diebold et al. 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. p53 is known to be a direct transcriptional activator of the BAX gene, 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. It has been reported that a cisplatin-resistant ovarian cancer cell line was found to have reduced BAX mRNA levels, which is consistent

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

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, \( P = 0.0004 \), \( \chi^2 \) test.

f) Grade 1 vs. grade 2/3, \( P = 0.009 \), \( \chi^2 \) test.

Fig. 5. Log-rank testing showed that increased MCL-1 protein expression is significantly correlated with poor prognosis in ovarian cancers (\( P = 0.0097 \)).
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with the loss of the ability of p53 to transactivate BAX as a consequence of p53 mutation.31 Tai et al.30 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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