Downregulation of *KLF4* and the *Bcl-2/Bax* ratio in advanced epithelial ovarian cancer

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Abstract. Kruppel-like factor 4 (*KLF4*) is a key transcriptional regulator of cell differentiation and proliferation and an altered expression of *KLF4* has been reported in a number of human malignancies. In the present study, we investigated *KLF4* expression and its role in cell proliferation in advanced epithelial ovarian cancer (EOC). We compared *KLF4*, *Bcl-2* and *Bax* transcript levels in ovaries isolated from advanced EOC and normal control ovaries. In addition, the *KLF4* gene was transduced into ovarian cancer cells and transcript levels of *Bcl-2* and *Bax* and cell proliferation were analyzed by real-time RT-PCR and MTT assays, respectively. Ovarian *KLF4* expression and *Bcl-2/Bax* ratios were downregulated in most cases of advanced EOC. In addition, *KLF4* overexpression in ovarian cancer cells increased the *Bcl-2/Bax* ratio. However, MTT analysis indicated that the overexpression of *KLF4* had no effect on the proliferation of ovarian cancer cells. The inactivation of *KLF4* is frequently observed in ovarian cancers and a reduced expression of *KLF4* in the ovarian cancers may lead to a reduction in the *Bcl-2/Bax* ratio. The latter has a role in predicting cancer grade, although its exact role in ovarian carcinogenesis requires clarification.

Introduction

Epithelial ovarian cancer (EOC) is the most lethal of all gynecologic cancers and most women are diagnosed at an advanced stage (1). Overall mortality rates have remained relatively constant over the past several decades and the cause of EOC is largely unknown.

The Kruppel-like factor 4 (*KLF4*) gene encodes an epithelial cell-enriched, zinc finger-containing transcription factor that has been shown to play important roles in cell proliferation and differentiation (2,3). In total, 70% of breast carcinomas have elevated *KLF4* mRNA levels, which are associated with a more aggressive phenotype (4,5). By contrast, *KLF4* expression is frequently lost in various human cancers, including gastrointestinal (6), bladder (7) and advanced prostate cancer, and *KLF4* has been found to exert tumor-suppressive effects (8). The dual and opposing roles of *KLF4* in tumorigenesis suggest that *KLF4* is one of the molecular elements that define the tissue-specific epithelial carcinogenesis pathway.

Conversely, the ultimate vulnerability of cells to apoptosis is determined by the relative ratio of various pro-apoptotic and anti-apoptotic members of the *Bcl-2* family (9). The expression of *Bcl-2* and *Bax* has been reported to be a prognostic factor in ovarian cancer (10,11). In addition, *KLF4* overexpression in a leukemia cell line affected the transcriptional regulation of *Bcl-2* and *Bax*, with effects on apoptosis and cell growth (12).

The expression of *KLF4* and its role in the development of ovarian cancer has not previously been studied. Therefore, to investigate the possible role of *KLF4* in ovarian carcinogenesis, we examined its expression in human ovarian cancer tissues and measured *Bcl-2* and *Bax* mRNA levels in the same specimens. We also transduced the *KLF4* gene into ovarian cancer cells to investigate the changes in *Bcl-2* and *Bax* gene expression and the consequences in terms of cell proliferation.

Materials and methods

Patients. This study was approved by the institutional review board of Hanyang University Hospital (HYUHIRB-2009-R-50) and written informed consent was obtained from each patient. The patients included were women with surgically determined primary advanced stage (III-IV) EOC who received debulking surgery at our institution. Normal control samples obtained at the time of salpingo-oophorectomy for benign indications were used for comparative purposes. All tumor samples were snap frozen at the time of surgery and stored at -70˚C until use.

Real-time RT-PCR analysis. RNA from EOC, normal tissues and ovarian cancer cells was isolated using an RNeasy extraction kit (Qiagen Inc., Valencia, CA, USA). Following quantification of RNA and verification of its integrity, 1 µg samples were reverse transcribed with an Advantage RT for PCR kit (BD Biosciences, Clontech, Palo Alto, CA, USA). Primers were designed with the Primers Express program (PE Applied Biosystems, Carlsbad, CA, USA): *KLF4* forward, 5'-ATCAGATGCAGCCGCAAGTCCC-3' and reverse, 5'-TCT

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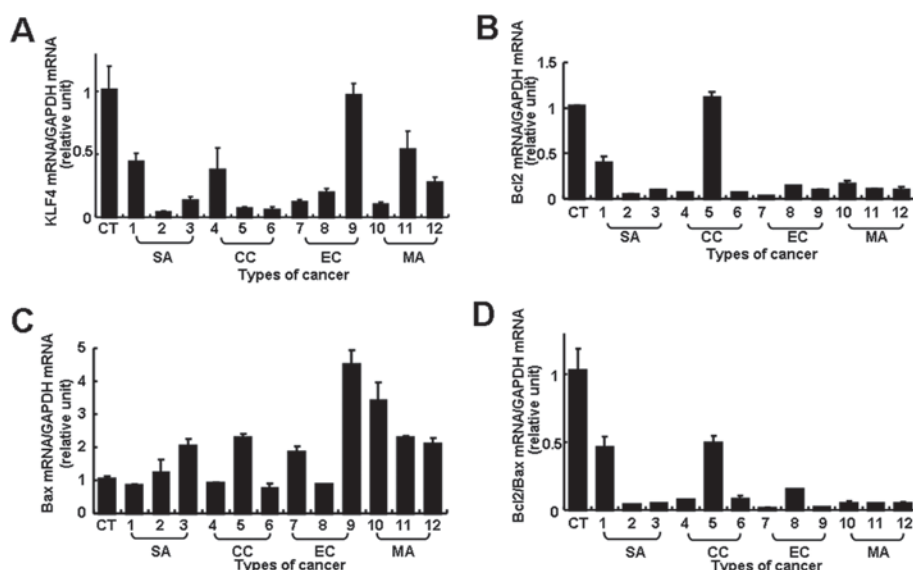E-mail: rohjaesook@hanyang.ac.kr

Figure 1. Real-time RT-PCR analysis of *KLF4*, *Bcl-2* and *Bax* expression in ovarian cancer tissues. Transcript levels of (A) *KLF4*, (B) *Bcl-2*, (C) *Bax* and (D) *Bcl-2/Bax* ratios were determined in normal ovarian tissues (n=3) and advanced primary epithelial ovarian cancer tissues (n=12). CT, normal ovarian tissues; SA (1-3), serous cystadenocarcinoma; CC (4-6), clear cell carcinoma; EC (7-9), endometrioid carcinoma; MA (10-12), mucinous adenocarcinoma. GAPDH mRNA was used as an internal control. Mean values were compared with the normal control value to calculate relative amounts of transcripts. Data are represented as the means ± SD of the triplicate assays.
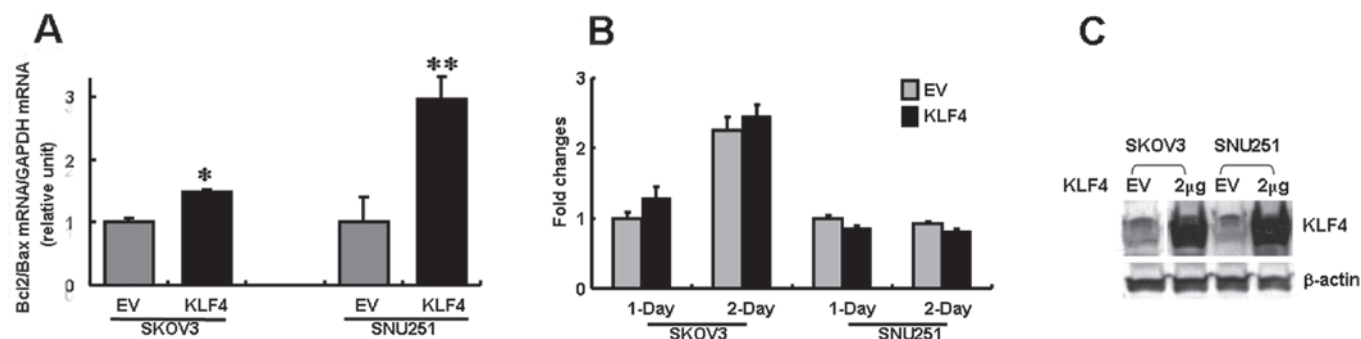

Figure 2. Effect of *KLF4* on *Bcl-2/Bax* expression and cell proliferation. (A) Real-time RT-PCR analysis of *Bcl-2/Bax* expression in *KLF4*-expressing and control vector-only SKOV3 and SNU251 cells. GAPDH mRNA was used as an internal control. Mean values were compared with the value of empty vector-transfected cells to calculate relative amounts of transcripts; (B) MTT cell proliferation assays of *KLF4*-expressing and vector-only SKOV3 and SNU251 cells from 1 and 2-day cultures. Each bar represents the fold change compared to the value for empty vector-transfected cells cultured for 24 h; (C) Immunoblot analysis of *KLF4* protein in transfected cells. β-actin was used as an internal control. EV, empty vector; *KLF4*, pCMV3xFLAG-*KLF4* plasmid (2 µg). Data are the means ± SD of 3 separate experiments. P-values were compared to EVs using the Student's t-test (*P=0.024; **P=0.014).

TCATGTGTAAGGCGAGGTGGTCC-3' (GenBank accession no. NM_004235.4); *Bcl-2* forward, 5'-ATGTGTGTG GAGAGCGTCAA-3' and reverse, 5'-ACAGTTCCACAA AGGCATCC-3' (GenBank accession no. NM_000633.2); and *Bax* forward, 5'-GGGGACGAACTGGACAGTAA-3' and reverse, 5'-CAGTTGAAGTTGCCGTCAGA-3' (GenBank accession no. NM_004324.3). Amplification of glyceralde-hyde-3-phosphate dehydrogenase (GAPDH) (forward, 5'-CAGCCTCAAGATCATCAGCA-3' and reverse, 5'-TGT GGTCATGAGTCCTTCCA-3'; GenBank accession no. NM_002046.3) was used to normalize each reaction (amplification product sizes 369, 136, 122 and 106 bps for *KLF4*, *Bcl-2*, *Bax* and GAPDH, respectively). Real-time PCR reactions were carried out in total volumes of 25 µl using SYBR-Green Supermix (Bio-Rad, Hercules, CA, USA) with an iCycler™

Thermal Cycler (Bio-Rad). PCR conditions were 10 min at 95°C, 35 cycles of 95°C for 15 sec, 60°C for 45 sec and 72°C for 1 min. Samples were run in triplicate in 96-well optical plates (Bio-Rad) and the mean values were compared with normal controls to obtain relative transcript levels.

Cell line transfection. Human ovarian cancer cell lines (SKOV3 and SNU251) were purchased from the Korean Cell Line Bank (Seoul, Korea) and transiently transfected with pCMV3xFLAG-*KLF4* made by subcloning the PCR-amplified coding region of *KLF4*. The cells were plated in 6-well plates and incubated with 2 µg aliquots of plasmid or empty vector in the presence of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Following incubation for 24-48 h, the cells were harvested for the analysis of RNA, protein and cell numbers.
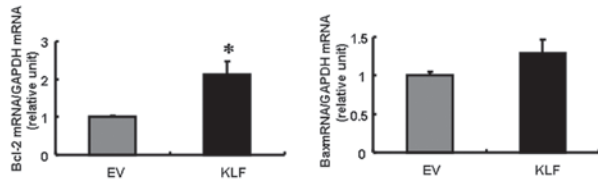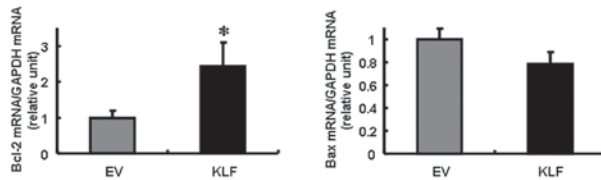
Figure 3. Real-time RT-PCR analysis of *Bcl-2* and *Bax* mRNA expression in (A) SKOV3 and (B) SNU251 cells overexpressing *KLF4*. GAPDH mRNA was used as an internal control. Mean values were compared with the normal control value (empty vector-transfected cells) to calculate relative amounts of transcripts. Data are represented as the means ± SD of the triplicate assays. P-values were compared to empty vectors using the Student's t-test (*P<0.05).

Cell proliferation assay. For assays examining FLAG-*KLF4* abundance and turnover, cell pellets were lysed in Laemmli buffer containing β-mercaptoethanol (Bio-Rad). Samples were resolved by 4-12% NuPAGE gel electrophoresis (Invitrogen), transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) and immunoblotted with anti-FLAG M2 antibody (1:1000) (F3165, Sigma, St. Louis, MO, USA). Promega horseradish peroxidase-conjugated anti-mouse immunoglobin G (W402B) antibody was used as a secondary antibody. To ensure that lysates were loaded equally, the blots were stripped and incubated with an anti-β-actin antibody (1:1000; Sigma).

For the cell proliferation assays, cells were transferred to 96-well microplates 24 or 48 h after transfection and seeded at a density of approximately $1x10^5$ cells per well before the assay. Cell viability was subsequently determined using an MTT cell proliferation assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). Absorbance was measured at 570 nm with a microplate reader. The experiment was repeated 3 times and the data were expressed as fold changes relative to empty vector-transfected cells cultured for 24 h.

Statistical analysis. All data were analyzed using the Student's t-test, with P<0.05 considered to indicate a statistically significant result. Data are expressed as the means ± standard deviation (SD) of triplicate measurements.

Results

Using real-time RT-PCR, we analyzed mRNA expression in pathology specimens from 3 normal ovaries and 12 cases of advanced EOC, including 3 cases each of serous, clear cell and endometrioid carcinoma and 3 mucinous cystadeno-carcinomas.

KLF4 transcript levels were substantially lower in most of the EOC samples (11 of 12 specimens) than in the normal

controls, although the extent of the difference varied between tumors (Fig. 1A). The level of expression in most samples was less than half the normal level and it was particularly low (less than 0.3-fold of normal level) in 7 EOC samples. *Bcl-2* or *Bax* mRNA expression also varied between tumors and there was no correlation with histological type (Fig. 1B and C). However, an analysis of the *Bcl-2/Bax* ratio showed a significantly reduced ratio, more than 2-fold lower than normal in all 12 cases and even lower in 10 cases (more than 5-fold less than normal) (Fig. 1D). Thus, both reduced *KLF4* transcript levels and lower *Bcl-2/Bax* ratios were found in most of the EOC samples, but the extents of the changes were not always correlated.

In order to examine the biological effects of *KLF4* expression in ovarian cancer cells, SKOV3 and SNU251 cells were transfected with FLAG-*KLF4*. An immunoblot analysis confirmed marked expression of *KLF4* in the transfected cells (Fig. 2C). Notably, *Bcl-2* expression was upregulated in both cell lines, whereas *Bax* was downregulated in SNU251 cells and slightly upregulated in SKOV3 cells (Fig. 3). The differential regulation of *Bcl-2* and *Bax* expression resulted in a decreased *Bcl-2/Bax* ratio in both cell lines, but the decrease was particularly significant in the SNU251 cells (P<0.03; Fig. 2A). This result suggests that the decreased expression of *KLF4* in EOC is correlated with the reduced *Bcl-2/Bax* ratio, which is a known prognostic factor in EOC.

To evaluate the effect of *KLF4* on cell proliferation, we used the MTT assay for quantitative analysis. In contrast to the known inhibition of cell growth and induction of apoptosis by *KLF4* in a number of non-gonadal cancer cells, the *in vitro* assays indicated that cell proliferation following *KLF4* gene transfection was unaffected at both 24 or 48 h of culture (P>0.05; Fig. 2B).

Discussion

This study is the first to suggest that *KLF4* may play an important role in the development and progression of ovarian cancer. We found that both the expression of *KLF4* and the *Bcl-2/Bax* ratio were downregulated in many advanced EOC cases, and that *KLF4* overexpression in ovarian cancer cells resulted in an increased *Bcl-2/Bax* ratio, which is known to indicate a favorable prognosis in ovarian cancer.

Several lines of evidence indicate that *KLF4* has variable effects on cell cycle arrest and inhibition of apoptosis depending on the cellular context (7,8,12,13). Although the expression of *KLF4* was found to be downregulated in ovarian cancers in this study, which is consistent with the results for many human cancers, we observed no change in cell proliferation due to *KLF4* overexpression (Fig. 2B). Given that the multiplication of human Sertoli cells is not affected by a lack of *KLF4* (14), it is likely that the different effects of *KLF4* on cell proliferation depend on unidentified cellular factors.

In this study we found that *Bax* and *Bcl-2* expression levels varied among tumors, as demonstrated in other studies (10,11). Thus, it has been suggested that the ratio of *Bcl-2* to *Bax*, rather than the absolute concentration of either, is predictive of cell fate (9), and their relative expression has been reported to be a better predictor of outcome for both

progression-free survival and overall survival (10,11). The transcription of *Bcl-2* and *Bax* in leukemia cells is affected by *KLF4* overexpression (12). We also demonstrated in this study that the transgenic expression of *KLF4* in ovarian cancer cells modulates *Bcl-2/Bax* gene expression (Fig. 2A). Previously, a microarray study showed that decreased *KLF4* expression was associated with chemotherapy resistance in ovarian cancers (15). In principle, chemotherapeutics act on rapidly proliferating cells and promote cell cycle arrest or apoptosis. Thus, lower expression of *KLF4* linked to a lower *Bcl-2/Bax* ratio may possibly contribute to resistance or relapse in advanced EOC. This increases the possibility that a crucial function of *KLF4* is to increase the *Bcl-2/Bax* ratio, at least in ovarian cells, and that this is essential for a favorable prognosis in ovarian cancer.

Although further research should be devoted to understanding why *KLF4* regulates cell growth and apoptosis in many other cancer cells (6-8) but not in ovarian cancer cells, our data indicate that the downregulation of *KLF4* may be a frequent step in ovarian carcinogenesis. The decreased expression of *KLF4* in ovarian cancer may modulate *Bcl-2/Bax* expression, a known prognostic factor for cancer grade, although its exact role in ovarian carcinogenesis needs to be clarified.

Acknowledgements

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