The expression profile of apoptosis-related genes in the chicken as a human epithelial ovarian cancer model

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Abstract. The purpose of our study was to examine the expression pattern of apoptosis-related genes in normal and cancerous ovaries of the hen. Localization of apoptosis-related gene mRNA was investigated in cancerous ovaries using *in situ* hybridization. The expression patterns of apoptosis-related genes were confirmed with RT-PCR in normal and cancerous ovaries. Differences of expression level between normal ovaries and ovarian cancers were analyzed using quantitative RT-PCR. In both normal and cancerous chicken ovaries, the expression of *CASP1*, *CASP2*, *CASP3*, *CASP6*, *CASP8* and *CASP9* were detected through RT-PCR analysis. The expression of *BCL2*, *BCL2L1* and *BID* were confirmed in normal and cancerous ovaries of the hen. Quantitative RT-PCR showed that *CASP1* expression was significantly increased in cancerous ovaries compared with normal ovaries, whereas *BID* expression was decreased. Our results showed a resistance to removal of abnormal cells via apoptosis in cancerous ovaries of the hen. Collectively, this phenomenon is closely associated with the dysregulation of *CASP1* and *BID* expression in chicken ovarian cancer.

Introduction

Until recently, epithelial ovarian cancer presented a major risk among gynecologic cancers due to its high lethality (1). The survival rate of patients with epithelial ovarian cancer can sharply increase if it is diagnosed at an early stage. For diagnosis of ovarian cancer in the early stages, a moderate biomarker is insufficient; the specificity and sensitivity of cancer antigen 125 (CA125), a representative biomarker for ovarian cancer, are insufficient for early detection (2,3). Although the cause of ovarian cancer is not clear, it is assumed that most human ovarian cancers originate from the epithelial surface of the ovary (4). It is also believed that accumulated damage during periodic ovulations is associated with the development of ovarian cancer (4). Despite many studies, the molecular and genetic factors linked to ovarian cancer are not clearly understood.

To discover appropriate chemopreventive or chemotherapeutic agents to conquer human ovarian cancer, the development of a suitable animal model is essential. Because ovarian cancers are rarely spontaneously developed from the ovarian epithelial surface in rodent models, several genetic modifications have been applied to advance ovarian cancer research in mice (5). However, ovarian cancers of these transgenic mice do not closely resemble human ovarian cancer (6-8). On the other hand, chicken epithelial ovarian cancers spontaneously occur at a high rate. Like human ovarian cancer, the incidence of chicken ovarian adenocarcinoma spontaneously increases in aged hens that no longer produce or produce fewer eggs after 2 years of age (9). It has been recently noted that chicken and human ovarian cancers share the same immunohistochemical biomarkers, such as CA125 and the erbB2 antibody (10,11).

Programmed cell death or apoptosis has a vital role in various biological events. The elimination of unwanted or damaged cells by apoptosis is indispensable for embryogenesis, maintaining cellular homeostasis, and normal regulation of the immune system (12). Because impaired apoptosis induces abnormal cell proliferation, the dysregulation of cell death mechanisms is a major hallmark of cancer development (13). Programmed cell death is activated by intracellular stresses and developmental cues. Well-known representative intrinsic regulators, the extended BCL2 family proteins play crucial roles in cell death regulation and are able to regulate several cell death mechanisms, including apoptosis, necrosis and autophagy (14-16). BCL2 family proteins contain at least one of four BCL2 homology (BH) domains (i.e., BH1, BH2, BH3 or BH4), and the number of BH domains included in proteins is associated with its apoptotic functions (17). Antiapoptotic BCL2 family members (e.g., BCL2, BCL2L1, MCL1 and BCL2A1) have all four BH domains. Proapoptotic BCL2 family members are categorized into two groups: the multiple-BH domain, which includes members BAX, BAK1 and BOK, and a single BH-3 group that includes members BID, BIM, BAD and NOXA.

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The antiapoptotic BCL2 family prevents endoplasmic reticulum (ER)-mediated cell death by reducing basal Ca^{2+} concentrations in the ER for regulation of cell survival (18-20), whereas proapoptotic family members like BAX and BAK induce ER-mediated apoptosis by inhibiting the effect of antiapoptotic members or by opposing effects on ER Ca^{2+} concentrations for regulation of cell death (17). Proapoptotic BCL2 family proteins, such as BAX and BID, stimulate mitochondrial outer membrane permeabilization (MOMP) that induces the release of regulators or activators (such as caspase activator) and other cell-death mediators. In contrast, antiapoptotic family members, such as BCL2 and BCL2L1, guard the outer membrane and conserve its integrity by the action of BAX and BAK (17). There is a close association between tumor development, initiation and resistance to chemotherapy and the dysfunction of BCL2 family proteins (12).

Cysteine aspartate-specific proteases (CASPs or caspases) serve as intrinsic initiators of apoptosis by cleaving substrates at aspirate residues (21). The caspase protein family currently consists of 13 and 11 isoenzymes in mammals and humans, respectively (22). The function of caspases is closely connected to initiation and execution of apoptosis, with caspases categorized into either initiator or effector caspases. CASP2, CASP8, CASP9 and CASP10 are initiator caspases, and CASP3, CASP6 and CASP7 are effector caspases (23). Known as interleukin 1ß converting enzyme, CASP1 plays an important role in both apoptosis and inflammation (24). Apoptosis is regulated by stepwise activation of caspases for the processing or cleaving of other caspases (25). Therefore, caspases are proapoptotic proteins that are likely to serve as tumor suppressors, and their dysregulation is involved in the initiation and development of tumors.

Chicken ovarian cancer is an excellent animal disease model for the development, treatment, or prevention of human epithelial ovarian cancer. Thus, we have investigated the apoptotic status of chicken ovarian adenocarcinoma and then analyzed the expression pattern of antiapoptotic or proapoptotic genes. The expression level of *BID* was decreased, whereas the expression level of *CASP1* was increased in chicken ovarian cancer compared with levels in normal ovaries. Although the expression level of *CASP1* was increased to initiate apoptosis of cancer cells, it was impaired by the downregulation of the proapoptotic gene *BID*. This dysregulation of the apoptosis pathway is believed to be closely linked to the occurrence of chicken ovarian cancer. Our study has provided insight into the study of apoptotic status using a chicken ovarian cancer model and can be applied for the discovery of therapeutic agents or the prevention of human ovarian cancer.

Materials and methods

General information. The care and experimental use of White Leghorns were approved by the Institute of Laboratory Animal Resources at Seoul National University (SNU-070823-5). White Leghorns were maintained in a standard management program at the University Animal Farm of Seoul National University in Korea. The procedures used for animal management, reproduction and embryo manipulation followed the standard operating protocols of our laboratory.

TUNEL assay. Normal and cancerous ovary were cryosectioned and fixed in a 4% paraformaldehyde solution for 20 min, and tissues were incubated in a permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate in PBS). Apoptotic cells were detected using the *In Situ* Cell Death Detection Kit with the fluorescein-labeled cell marker TMR red (Roche Applied Science, Penzberg, Germany). Then, the tissues were counterstained with DAPI, mounted and analyzed under a confocal laser microscope (Carl Zeiss, Oberkochen, Germany).

RNA isolation and RT-PCR. cDNA was synthesized from total RNA (3 μg) using the Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The cDNA was serially diluted 10-fold and was quantitatively equalized for PCR amplification. PCR reactions had a total volume of 20 μl and contained 100 ng of genomic DNA, 2 μl 10X PCR buffer (CoreBio, Seoul, Korea), 1.6 μl of dNTPs (10 mM each), 2 pmol of each gene-specific primer (Table I), and 0.25 U of Taq polymerase (CoreBio). PCR conditions were 94˚C for 3 min, followed by 35 cycles at 94˚C for 30 sec, 60˚C for 40 sec and 72˚C for 1 min. PCR products were analyzed using a 1% agarose gel with ethidium bromide.

Quantitative real-time RT-PCR. To confirm mRNA expression levels of apoptosis-related genes in normal ovaries and ovarian cancers of chickens, quantitative RT-PCR was performed. Based on RT-PCR analysis of apoptosis-related genes, we selected genes and designed primers using Primer 3 software (26) and sequences from the GenBank database (Table II). Quantitative RT-PCR was performed using the Real-time PCR Detection System (Applied Biosystems, Foster City, CA) with SYBR® Green (Sigma, St. Louis, MO). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was simultaneously run as a control and used for normalization. Each test sample was run in triplicate. Following the standard curve method, expression quantities of target genes were determined using standard curves and Ct values and were normalized using *GAPDH* expression quantities. PCR conditions were 94˚C for 3 min, followed by 40 cycles at 94˚C for 20 sec, 60˚C for 40 sec and 72˚C for 1 min using a melting curve program (increased temperature from 55 to 95˚C with a heating rate of 0.5˚C per 10 sec). Continuous fluorescence measurement and ROX dye (Invitrogen) was used as a negative control of fluorescence measurement, and sequence-specific products were identified by generating a melting curve. With the Ct value representing the cycle number at which a fluorescent signal rises statistically above background, relative quantification of gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method (27) and was normalized to the Ct of the normal ovary control group.

Hybridization probes. To determine the exact localization of apoptosis-related genes, expression of target genes was examined using *in situ* hybridization as previously described (28). The cDNA from chicken ovarian cancer tissues was amplified with the appropriate forward and reverse primers. The amplified fragment of apoptosis-related genes was loaded onto a 1% agarose gel and separated by electrophoresis at 5 V/cm for ~30 min. The DNA from each cancer-related gene

Table I. Information of primers used for RT-PCR analysis.

Gene	GenBank accession no.	Sequence (forward and reverse)
BCL2	NM_205339.1	TTCCTCTCCCCCTCCCAC
		GACCCAGTTGACCCCATC
MCL1	XM_001233734.1	TTGTGGTTGTGGGTTTTTGG
		GCCTTTGCTTTTTGTGTCGT
BCL2L1	NM_001025304.1	ATGATGGTGTGAACTGGGGG
		AGGGGCTACGACAAAAGGAG
BCL2A1	NM_204866.1	ATCTCGGACCAGCCCAAAC
		ACTCTCTGAACAAGGAAAGAACA
BID	NM_204552.2	TCAGCCAACACTTCCACAAC
		CCCCCAAAACCCCCTCCT
NOXA	XM_001233389.1	CCTGAACCTCATCACGAAAC
		CCACTTCCCATCCAGCAGA
CASP1	NM_204924.1	CGGATGGCTGGAGATGTGT
		CACGAATGGGGAGATGTTTG
CASP2	XM_416524.2	ATGGCACTGATGGCAAACTC
		CTGGGGCATAACCTTCTCGT
CASP3	NM_204725.1	GAAGGAACACGCCAGGAAAC
		GCAAAGTGAAATGTAGCACCAA
CASP6	NM_204726.1	GCAAACCTACACCAACCACC
		TGGCACTTGTCTCCTCTGAAC
CASP7	XM_421764.2	AGCACAAGGCAGAACAGCAA
		GGCAAAACAAGCAGCATCAC
CASP8	NM_204592.2	GTGGGGGACCTGTTCTTCTT
		GCTCCTGTGCTGCTTCTTTG
CASP9	XM_424580.2	GCCCGAGTTTGAGAGGAAAA
		CACAGCCAGACCAGGAACAC
CASP10	XM_421936.2	GAAACCATCTTCCAAAGCGA
		CTCACATCCAGACCAAGCCA
CASP18	NM_001044689.1	CCTCGGTGCCATTTCTAACC
		GCTGATACCTTTGCCTTCCC
GAPDH	NM_204305.1	AGCAACATCAAATGGGCAGA
		GGCAGGTCAGGTCAACAACA

was recovered with a Wizard SV Gel and PCR Clean-up System (Promega, WI, USA) and cloned into a pGEM-T plasmid vector (Promega). After verification of sequences, the recombinant plasmids for each sequence were amplified with T7- and SP6-specific primers (T7, 5'-TGTAATACGAC TCACTATAGGG-3'; SP6, 5'-CTATTTAGGTGACACTAT AGAAT-3') to prepare the template for labeling cRNA probes. Digoxigenin (DIG)-labeled sense and antisense cRNA probes (promoted by T7 or SP6 RNA polymerase according to the cloning directions) were transcribed *in vitro* with a DIG RNA labeling kit (Roche).

In situ hybridization. Chicken ovarian cancer tissue was cut into 8- to 10-mm pieces and frozen in liquid nitrogen. Frozen sections (10 μm) were mounted on 3-aminopropyltriethoxysilane-pretreated slides (Sigma), dried on a 50˚C slide warmer and fixed in freshly prepared 4% paraformaldehyde in 1X phosphate-buffered saline (PBS; 1.4 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4 and 18 mM KH_2PO_4, pH 7.4) for 1 h at room temperature. The sections were washed twice in 1X PBS, treated in 1% Triton X-100 for 20 min, and washed three times in 1X PBS. The sections were incubated with a prehybridization mixture containing 50% formamide and 5X standard saline citrate (SSC; 150 mM NaCl and 15 mM sodium citrate, pH 7.0) for 15 min at room temperature. After prehybridization, the sections were incubated with a hybridization mixture containing 50% formamide, 5X SSC, 10% dextran sulfate sodium salt, 0.02% bovine serum albumin,

Table II. Information of primers used for quantitative RT-PCR analysis.

Gene	GenBank accession no.	Sequence (forward and reverse)
BCL2	NM_205339.1	GCTTTATCCTCCTGCCCCTC
		CCTTTTTCCTCCACCCTGTT
MCL1	XM_001233734.1	CACCTGAAAAGCATCAACCA
		GAAGTCAACAAAGCCCTCCC
BCL2L1	NM_001025304.1	TCACACATCCACCACGCA
		AGAGGGGCTACGACAAAAGG
BID	NM_204552.2	CTGTGAAAGGGAAGGCAGAG
		GCTACCAAAAAGGAGAGGGAA
CASP1	NM_204924.1	CGGATGGCTGGAGATGTGT
		CACGAATGGGGAGATGTTTG
CASP3	NM_204725.1	TGGTATTGAAGCAGACAGTGGA
		GGAGTAGTAGCCTGGAGCAGTAGA
CASP6	NM_204726.1	AAACCTACACCAACCACCACA
		TTCTGTCTGCCAAAGTCCCA
CASP8	NM_204592.2	ATTTGGCTGGCATCATCTGT
		ACTGCTTCCCTGGCTTTTG
GAPDH	NM_204305.1	ACACAGAAGACGGTGGATGG
		GGCAGGTCAGGTCAACAACA

250 μg/ml yeast tRNA and denatured DIG-labeled sense or antisense cRNA probes for target gene for 18 h in a 55˚C incubation chamber. The sections were washed for stringency in a series of solutions containing formamide and SSC. After non-specific binding was blocked with a 1% blocking reagent (Roche) in buffer I (1 M Tris-HCl at pH 7.5 and 2.5 M NaCl), the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche). The signal was visualized with a visualization solution containing 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.4 mM nitroblue tetrazolium and 2 mM levamisole (Sigma) in buffer III (1 M Tris-HCl at pH 9.5, 2.5 M NaCl and 0.5 M $MgCl_2$). All sections were counterstained with 1% (w/v) methyl green (Sigma), and photographs were taken with a Zeiss Axiophot light microscope equipped with an Axiocam HR-camera (Carl Zeiss).

Statistical analysis. Difference in variance between normal and cancerous ovaries was analyzed using the F-test, and the difference of means was analyzed using the Student's t-test. A p-value of <0.05 was considered statistically significant. Microsoft Office Excel was used for statistical analysis.

Results

Apoptotic status of chicken ovarian cancer. For pathological analysis of chicken ovarian cancer in hens >2 years of age, paraffin-embedded normal ovaries and cancerous ovaries were stained with hematoxylin and eosin (H&E). The general stromal region was observed in normal ovaries; small follicles were detected, and abnormal proliferated cells were not developed (Fig. 1A, B). The abnormal neoplasia region was observed in cancerous ovaries; many tubular structures were infiltrated into the stromal region (Fig. 1C, D). To investigate the apoptotic status of chicken ovarian cancer, a TUNEL assay for detecting DNA fragmentation via labeling the terminal end of nucleic acids was conducted. Stained cells near the epithelial region were present in normal ovaries, whereas many unstained tubular structures were present in cancerous ovaries (Fig. 2).

The expression pattern of apoptosis-related genes in normal and cancerous ovaries. To elucidate the difference in apoptotic status between normal and cancerous ovaries, the expression pattern of apoptosis-related genes was examined by RT-PCR (Fig. 3). We first investigated the expression of the *BCL-2* family (*BCL2*, *BCL2L1*, *MCL1*, *BCL2A1*, *BID* and *NOXA*). In both normal and cancerous ovaries, we observed expression of *BCL2*, *BCL2L1* and *BID*, significant weak expression of *MCL1*, and no expression of *BCL2A1* and *NOXA*. The expression pattern of the caspase gene family was confirmed, with *CASP1*, *CASP3*, *CASP6*, *CASP8* and *CASP9* expressed in normal and cancerous ovaries. Both tissues showed weak expression of *CASP7* and no expression of *CASP2*, *CASP10* and *CASP18*.

Quantification of expression level of apoptosis-related genes. Using quantitative RT-PCR, the expression levels of apoptosis-related genes in normal and cancerous ovaries were investigated (Fig. 4). No significant difference was found between the expression levels of *BCL2* (p=0.11) and *BCL2L1* (p=0.16) in normal and cancerous ovaries. A dramatically decreased expression of *BID* was observed in cancerous ovaries compared with that in normal ovaries (p=0.0003). No significant

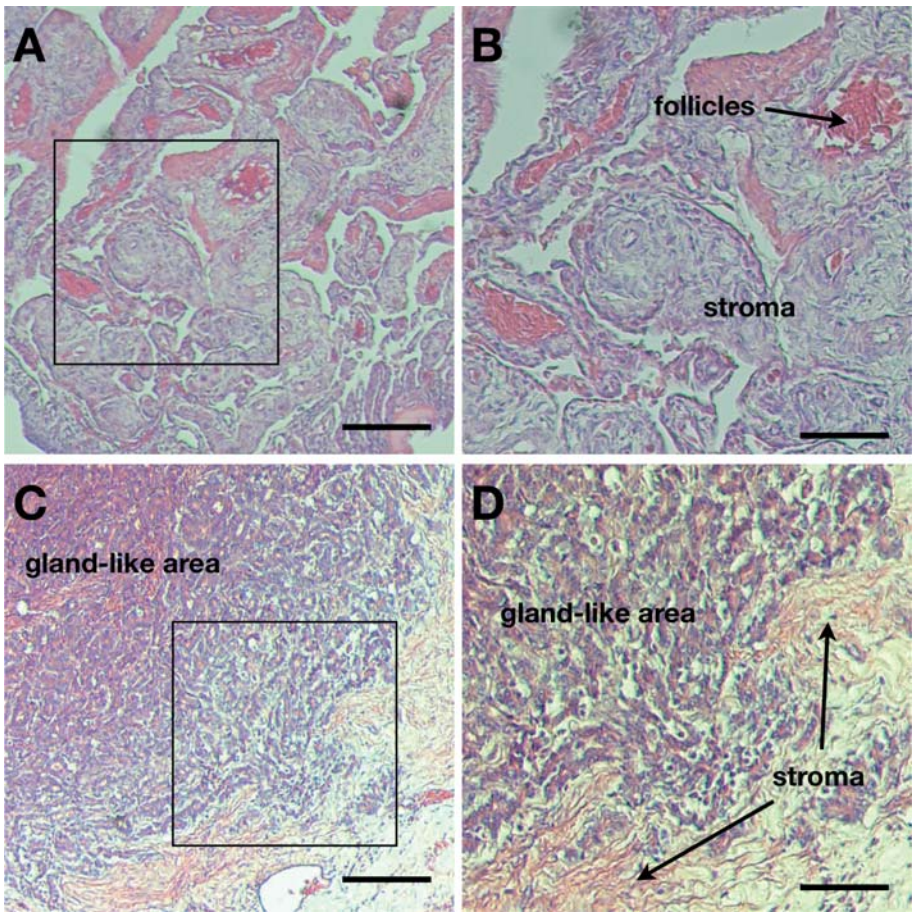

Figure 1. Histological analysis of chicken ovarian cancer. Cancerous and normal chicken ovaries were embedded with paraffin and then stained with hematoxylin and eosin. Normal ovary with normally growing follicles (A and B) and cancerous ovary with gland-like area (neoplasia region, C and D). Scale bar, 100 μm (A and C) and 50 μm (B and D).

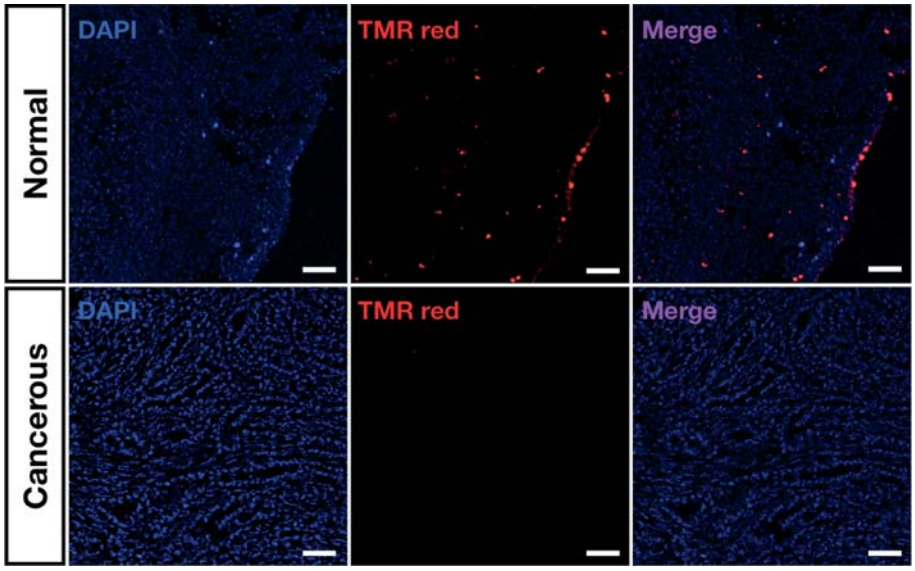

Figure 2. TUNEL assay of normal and cancerous chicken ovary. Cryosectioned tissues were stained with a TUNEL assay kit (red) and counterstained with DAPI (blue, nucleus). Scale bar, 50 μm.

difference in the expression levels of *CASP3* (p=0.38), *CASP6* (p=0.10) and *CASP8* (p=0.16) was found between normal and cancerous ovaries, whereas increased expression of *CASP1* (p=0.007) was observed in cancerous ovaries.

Localization of CASP mRNA in normal ovary and ovarian cancer. To determine where *CASP1* is expressed, *in situ* hybridization using a *CASP1*-specific RNA probe was performed. *CASP1* gene mRNA was not detected in normal

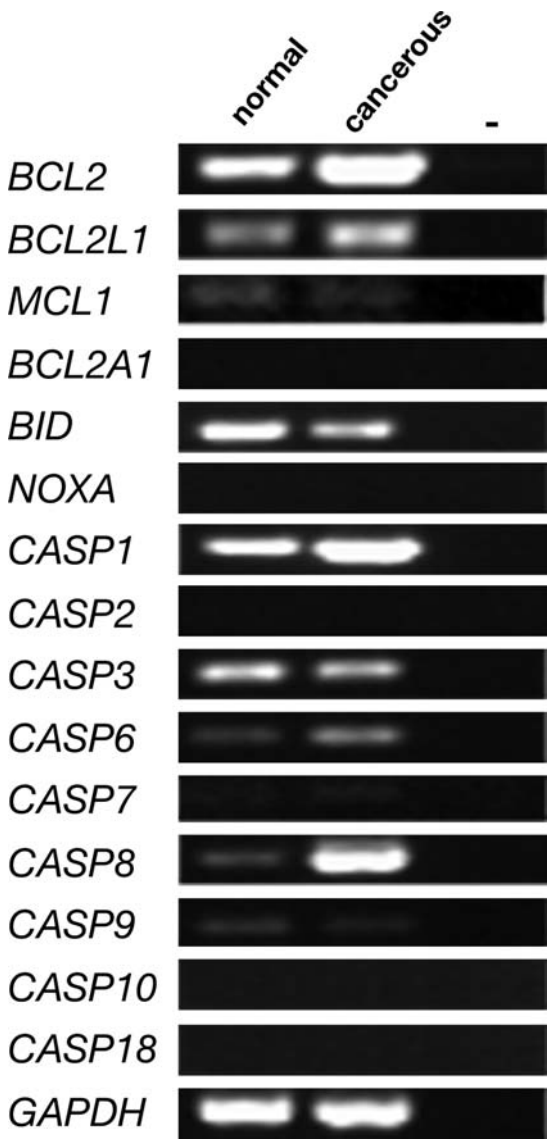

Figure 3. The expression pattern of apoptosis-related genes in normal and cancerous chicken ovaries using RT-PCR. Expressions of four genes in BCL2 family (*BCL2*, *BCL2L1*, *MCL1* and *BID*) and five genes in caspase family (*CASP1*, *CASP3*, *CASP6*, *CASP8* and *CASP9*) were detected by RT-PCR.

ovaries (Fig. 5A), but was localized in the neoplastic region of cancerous ovaries (Fig. 5B, C). In the latter, tumor cells with tubular structures weakly expressed *CASP1* gene mRNA (Fig. 5B, C).

Discussion

Apoptosis serves as a safety process by removing abnormally-controlled cells, such as DNA-damaged, abnormal-proliferated or dedifferentiated cells (13). Studying the apoptotic status dysregulation of cancer cells will provide meaningful findings for human cancer therapy. In this study, we investigated dysregulation of apoptosis in a chicken ovarian cancer model to enhance the value of the animal model for human epithelial ovarian cancer. Our results showed that few apoptotic cells were detected in chicken ovarian cancer in contrast to the apoptotic status in normal ovaries. To better understand apoptotic dysregulation in cancer, the expression pattern of antiapoptotic and proapoptotic genes were examined. Among the *BCL2* gene family, *BCL2*, *BCL2L1* and *BID* were expressed, and the expression of *MCL-1* was weakly detected in both normal and cancerous ovaries. Among the *CASP* gene family, *CASP1*, *CASP3*, *CASP6*, *CASP8* and *CASP9* were expressed, and the expression of *CASP7* was faintly observed in both normal and cancerous ovaries. Furthermore, the expression level of *BID* was decreased in chicken ovarian cancer compared with that in normal ovaries, and the expression level of *CASP1* was greatly increased. *CASP1* mRNA was also detected in chicken ovarian cancer cells with tubular structure.

Resistance to apoptosis is a hallmark of several human cancers, including ovarian cancer, and is closely related to chemotherapy resistance (12). Although CASP1 is well known as one of the cytokine-processing caspases for producing IL-1ß and IL-18, it also plays a major role in apoptosis and is linked to certain pathologic conditions (29,30). The down-regulation of *CASP1* is particularly associated with the occurrence of human ovarian cancer, with overexpression of *CASP1* triggering apoptosis (24). However, our results revealed overexpression of *CASP1* in cancerous ovaries of chickens compared with normal ovaries and detected *CASP1* mRNA in cancerous ovaries. This may be due to an association between CASP1 overexpression and endogenous stimuli for obstruction of tumor progression. INF-γ prohibits growth of human pancreatic carcinoma cells through the up-regulation and activation of CASP1 (31), and treatment of tumor necrosis factor-α (TNF-α) in A549 cells increases mRNA levels of *CASP1*, resulting in the stimulation of apoptosis (32). It is assumed that the overexpression of *CASP1* by external signals is an endogenous response to block cancer progression.

Another possibility is that the up-regulation of *CASP1* does not induce apoptosis in cancerous ovarian cells, with this status closely connected to modified *BID* expression levels. Our results indicated decreased expression of *BID* in cancerous chicken ovaries compared with normal ovaries. Generally, the activation of the RIP2/CASP1 pathway is converged to the cleavage of BID, an intrinsic apoptotic pathway, which subsequently triggers activation of *CASP9* and *CASP3* to induce apoptosis (29). Our results support the hypothesis that despite *CASP1* overexpression to induce apoptosis of transformed cells, a successive intrinsic apoptotic pathway cannot progress due to down-regulation of *BID*. Considered to be the origin of epithelial ovarian carcinomas, ovarian surface epithelial cells (OSE) greatly express *CASP1*, resulting in the production of proinflammatory cytokines such as IL-1 and IL-18 (33,34). In light of induction of apoptosis by CASP1, CASP1 may also contribute to the removal of damaged cells generated by continuous ovulation. Likewise, *CASP1* is highly expressed in CaOV3 human ovarian cancer cells, and CASP1-dependent apoptosis is dysregulated (24). It is hypothesized that the dysregulation of the intrinsic apoptosis pathway, which includes BID cleavage stimulated by CASP1, is subsequently able to survive damaged cells and could increase the risk of cancer incidence.

In conclusion, we have shown that *CASP1* is overexpressed and *CASP1* mRNA is detected in cancerous ovarian tissue of chickens *in vivo*. Also, *BID* acts as an intrinsic apoptosis

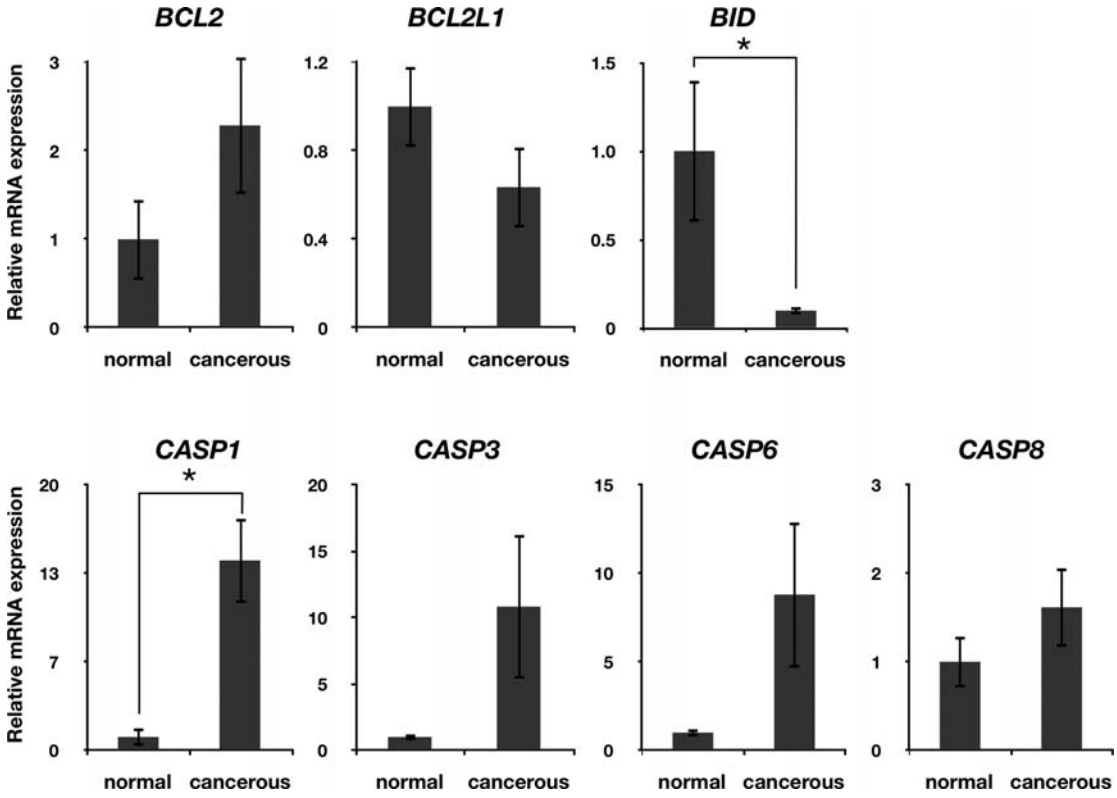

Figure 4. The expression level of apoptosis-related genes in normal (n=3) and cancerous (n=5) ovaries using quantitative RT-PCR. The *GAPDH* gene was simultaneously run as a control and used for normalization. Each bar represents the mean ± SEM of independent experiments. Statistical significance was determined using a Student's t-test. *p<0.05.

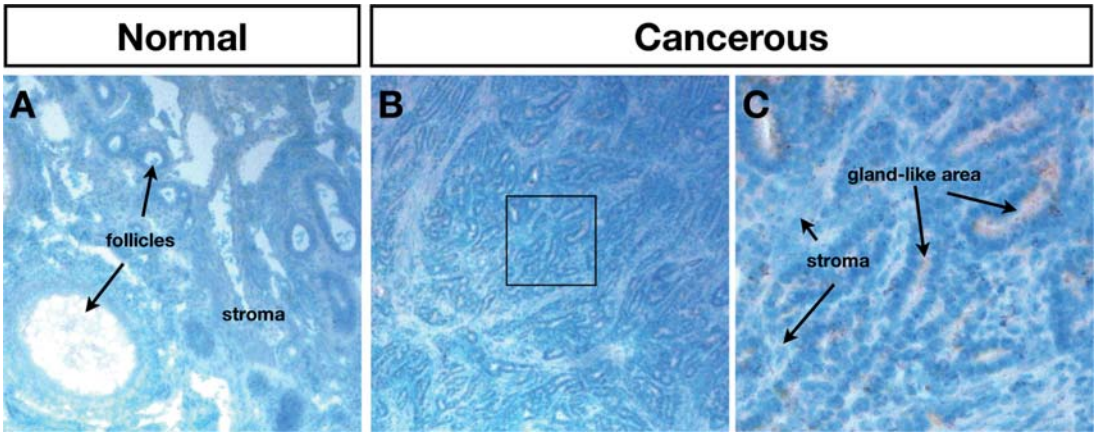

Figure 5. mRNA localization of *CASP1* in normal and cancerous chicken ovaries. The dark brown color is the signal for probe staining of *CASP1* mRNA. Methyl green was used for counterstaining of the nucleus region. *CASP1* was weakly expressed in chicken ovarian cancer cells, and morphology of *CASP1*-expressed cells is like typical glandular structures. The expression of *CASP1* was not detected in normal ovaries. (A) Normal ovary (x40); (B) cancerous ovary (x40); (C) cancerous ovary (x200).

regulator that is down-regulated in chicken ovarian cancer. Through this study, we have provided a new application of the apoptotic status of chicken ovarian cancer as an excellent model of human epithelial ovarian cancer. Through the combination of our transgenic techniques in chickens (35), our findings will be useful in addressing the incidence of cancer due to dysregulated apoptosis. In conclusion, our study provides new insight into the development of therapeutic agents based on the apoptotic pathway in human ovarian cancer.

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