p73 Gene Expression in Ovarian Cancer Tissues and Cell Lines

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ABSTRACT

The *p73* gene, a homology of *p53*, is a new candidate of imprinting and tumor suppressor gene. To investigate the role of *p73* in ovarian cancer, we studied the allelic expression in 56 cases of ovarian cancer using *Sty*I polymorphism analysis. We also examined *p73* expression by semi-quantitative reverse transcription-PCR as well as by Western blot analysis and DNA methylation study of the CpG island in exon 1 in ovarian cancer tissues and cell lines. Loss of heterozygosity was found in 8.3% (2 of 24) of the cases. Biallelic expression was demonstrated in 91.7% (22 of 24) of the tumor samples, in 70.8% (17 of 24) of the normal samples, and in 1 ovarian cancer cell line. Imbalanced expression and monoallelic expression were found in three and two pairs of matched samples, respectively. Overexpression of *p73* was found in advanced ovarian cancer rather than in early-stage disease or in borderline ovarian tumor. No significant difference was found in the *p53* expression. Three cell lines with absent *p73* protein expression and one tumor sample with monoallelic expression were methylated in the CpG island. Demethylation in SKOV3 cell line using 5-azacytidine can reactivate the expression of this gene in both the mRNA and the protein level. Our results indicated that *p73* was not imprinted in most of the ovarian cancer and normal tissues, but it could be involved in the advanced ovarian cancer through overexpression. DNA methylation may contribute to the lack of *p73* expression.

INTRODUCTION

Genetic alteration of *p53* plays important role in the development of human cancer (1). *p73*, a novel family member of *p53*, has recently been identified and found to activate *p21Waf1/Cip1* and to induce apoptosis. (2). The *p73* gene was also considered as a candidate of the imprinted tumor suppressor gene because: (*a*) *p73* was mapped on 1p36.33, a region frequently found to be deleted in the maternal chromosome of neuroblastoma and other tumors (3, 4); (*b*) monoallelic expression of this gene was found in numerous cancer cell lines and in

normal tissues (2); and (*c*) activation of the silent allele was found in renal cell carcinoma and lung cancer (5, 6) that is similar to LOI. However, biallelic expression of this gene was also observed in some normal tissues and lymphocytes as well as in both bladder cancer and normal bladder (2, 7). These data suggest that *p73* is not monoallelically expressed in all human tissues, and it could be tissue dependent (7).

In addition, overexpression of *p73* was found in several cancers such as lung, bladder, prostate, and colorectal (6–9). But no mutations were revealed in most of the cancer cells (7–12). The expression of *p73* was increased in lung cancer independent of *p53* gene mutation (13). Transcriptional silencing of *p73* in leukemia was associated with 5' CpG island methylation (14, 15). However, there was no evidence of methylation in any of the solid tumors analyzed, including breast, renal, and colon cancers (14).

In ovarian cancer, several genetic alterations have been identified, including mutations of *p53* tumor suppressor, *Ras*, *BRAC1*, *BRAC2*, *C-erb2*, and *Bcl-2* oncogenes. LOI of *IGF2* and *H19* genes was also involved in the genesis of ovarian cancer (16, 17). To date, the molecular mechanisms of ovarian cancer development are not well established. The new candidate tumor suppressor, *p73*, which is implicated in the pathogenesis of many types of cancer, may also be involved in the development of ovarian cancer. To elucidate the role of *p73* in the development of ovarian cancer and the allelic-specific expression as a potential imprinted tumor suppressor, we investigated the specific allelic expression of the *p73* gene in 56 cases of ovarian cancer, borderline ovarian tumor, and matched normal tissues. We also compared the expression level of *p73* by semi-quantitative RT-PCR[2] with *p53*. To further understand the expression of *p73*, we studied the *p73* expression at the mRNA level and at the protein level as well as elucidating the methylation status of *p73* gene in ovarian cancer tissues and cell lines.

MATERIALS AND METHODS

Characteristics of Patients. Ovarian tumors and adjacent normal tissues including ovary, cervix, endometrium, and peripheral blood were collected from 56 patients with ovarian cancer at Queen Mary Hospital, Hong Kong. The tissues were frozen and stored in liquid nitrogen until analysis. Histological diagnosis and clinical staging were performed according to the International Federation of Gynecologists and Obstetricians criteria. Among the 56 patients, 16 were in stage I (28.7%), 15 were in stage II (26.8%), 18 were in stage III (32.1%), and 7 were in stage IV (12.5%). The histology included 20 serous cystadenocarcinomas, 12 ovarian mucinous cystadenocarcinomas, 8 endometrioid adenocarcinomas, 9 clear-cell carcinomas, 5 borderline mucinous tumors, and 2 borderline serous cystadenomas. Five ovarian cancer cell lines, OVCA3, SKOV3, A2780s, A2780cp, and OV2008, were also studied.

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[2] The abbreviations used are: RT-PCR, reverse transcription-PCR; LOI, loss of imprinting; LOH, loss of heterozygosity; 5-aza, 5-azacytidine.

DNA, RNA, and Protein Extraction and Purification. Genomic DNA and RNA were isolated from frozen tissues and lymphocytes. The tissues were pulverized in liquid nitrogen and the powder was transferred to tubes with 75 mM NaCl and 25 mM EDTA. DNA was extracted by proteinase K-phenol/chloroform methods. RNA and protein were isolated using Tripure Isolation Reagent (Boehringer Mannheim) according to the manufacturer's protocol.

Allelic Expression of *p73*. To identify heterozygous samples, primers for exon 2 of the *p73* gene were used. PCR was performed using 100 ng of tumor and normal matched DNA under the following conditions: (*a*) 95°C for 5 min; (*b*) 35 cycles of 95°C for 20 s; (*c*) 62°C for 15 s; (*d*) 72°C for 30 s; and (*d*) a final extension for 4 min at 72°C. A specific 229-bp fragment was obtained. Five μl of PCR product was digested with 10 units of *Sty*I restriction enzyme at 37°C overnight. Ten μl of product was electrophoresed on 3% NuSieve (3:1) agarose gel and stained with ethidium bromide. The size of the undigested DNA band 229 bp and the *Sty*I digested band 157 bp were named as *a* and *b* allele, respectively. A known sample of *b* allele was used as a control to ensure complete digestion for each experiment. The primers used were as follows: primer 1, 5′-CAGGAGGACAGAGCACGAG-3′; and primer 2, 5′-CGAAGGTGGCTGAGGCTAG-3′. Heterozygous samples were chosen for RT-PCR using cDNA primers flanking the GC/AT polymorphism and *Sty*I RFLP. Two μg of total RNA was reverse-transcribed by Superscript II (Life Technologies, Inc.). One-twentieth of the cDNA volume was used for PCR amplification. The PCR reaction was performed using primer 3 and primer 5. A specific band of 285 bp was obtained. One-fiftieth of the first PCR product was used for the nested PCR with primer 3 and primer 4. The primers used were as follows: primer 3, 5′-GGGCTGCGACGGCTGCAGAGC-3; and primer 4, 5′-GAGAGCTCCAGAG GTGCTC-3′; and primer 5, 5′-ACCAGATGAGCAGCCGCG-3′. The final size of the band is 116 bp. Each of the amplifications was 25 cycles using the previous PCR conditions. Contamination by genomic DNA in total RNA was determined by the presence of DNA bands of different sizes and by the experiments without reverse transcriptase. No band was seen when reverse transcriptase was omitted. After RT-PCR, the *Sty*I digestion was performed as described previously, and a size of 84 bp was seen for complete digestion that represented A/T polymorphism.

Semi-quantitative RT-PCR. Because advanced ovarian cancer tends to spread bilaterally and both ovaries are affected, it's difficult to collect both tumor and normal ovarian tissue samples from the same patient. As a result, only three matched normal ovarian and tumor samples were obtained. The other normal tissues were either normal endometrium or normal cervix from the same patient. We first compared the expression level of *p73* in two patients with normal endometrium, cervix, ovary, or lymphocytes. The expression level of *p73* was nearly the same in the adjacent normal tissues in the two patients and a little bit lower in the lymphocytes. So, we investigated the expression level of *p73* in 14 advanced ovarian cancer specimens (stages III and IV), in 9 samples of early stages (stages I and II), and in 6 borderline ovarian tumors. All of them were compared with the expression level in normal tissues. PCR was performed according to conditions stated previously, except that 28 cycles were used for *p73* with primers 6 and 7 and 24 cycles

for β-*actin* using primers 8 and 9. The conditions for *p53* were the same, except that the annealing temperature was 54°C and 28 cycles with primers 10 and 11. The primers used were as follows: primer 6, 5′-AACGCTGCCCCAACCACGAG-3′ and primer 7, 5′-GCCGGTTCATGCCCCCTACA-3′ (for *p73*); primer 8, 5′-ATCTGGCACCACACCTTC TACAATGAGC-TGCG-3′ and primer 9, 5′-CGTCATACTCCTGCTTGCT-GATCCACA TCTGC-3′ (for β-*actin*); and primer 10, 5′-CTGAGGTTGGCTCTGACTGTACCACCATCC-3′ and primer 11, 5′-CTCATTCAGCTCTCGGAACATCTCGAAGCG-3′ (for *p53*). The cycles were determined by the standard curve amplified from 16, 20, 24, 28, 32, 36, and 40. Conditions were chosen to give a linear relationship between the amount of amplified product and the input RNA (data not shown). A 231-bp band was seen for *p73*, 373 bp for *p53*, and 400 bp for β-*actin*. Ten μl of PCR product was electrophoresed on a 2.5% agarose gel and stained with ethidium bromide. The software UVP Gel Works 1.0 for Windows was used to analyze the expression levels of *p73* and *p53* in both tumor and normal specimens with β-*actin* as a control. Each PCR and electrophoresis procedure was repeated twice. We first calculated the average of *p73* and β-*actin* expression for each sample, then we calculated the ratio of *p73* and β-*actin* in both tumor and normal tissues, and finally we compared the *p73* expression ratio between tumor and normal tissues (T/Tβ/N/Nβ). Student's *t* test was used for statistical analysis. A $P < 0.05$ was considered a significant difference. The same procedure was performed for *p53* expression.

Western Blot and Methylation Analysis of the *p73* Gene in Ovarian Cancer. Ovarian cancer cell lines were cultured in DMEM medium supplemented with 10% FCS. Western blot was performed in ovarian cancer cell lines using 50 μg of protein and a goat antihuman polyclonal anti-p73 antibody (Santa Cruz Biotechnology, Inc.) with a dilution of 1:500 and visualized with the ECL chemiluminescent detection kit (Amersham). The filters were also reprobed with β-*actin* (Sigma). Seven advanced ovarian cancer samples, five early-stage, and three borderline ovarian tumor specimens and matched normal tissues were also studied by Western blot using 20 μg of protein. The methylation state of *p73* in ovarian cancer cell lines and tissues was detected as described previously (14). Two-tenths μg of genomic DNA was digested with 20 units of either methylcytosine-sensitive enzyme *Hpa*II (Promega) or its methylation-resistant isoschizomer, *Msp*I (Promega), for 3 h at 37°C, then phenol/chloroform-extracted, ethanol-precipitated, dried, and resuspended to 10 μl of Tris-EDTA buffer. One μl (20 ng) was amplified by PCR using primers 5′-GGGGACGCAGC-GAAACCG-3′ and 5′-CTGCAGCCGTCGCAGCC-3′, which amplified the CpG island in exon 1 with a 77-bp band. Normal placenta DNA was used as a negative control. The band existed after *Hpa*II digestion, but not if there were methylation alleles.

RESULTS

Frequent Biallelic Expression of the *p73* Gene in Ovarian Cancer Tissues, Normal Ovaries, and Adjacent Tissues or Lymphocytes. Of the 56 samples, 24 were heterozygous. LOH was found in 2 of 24 informative samples (8.3%). Among the 24 heterozygous samples, biallelic expression of *p73* was found in 22 tumor samples (91.7%) including 2 LOH speci-

Table 1   *p73* allelic expression in 24 informative ovarian cancers and normal tissues[a]

Case no.	Cancer stage	Histology	Normal tissues	Expressed alleles (normal tissues)	Expressed alleles (cancer tissues)
1	IV	Clear[b]	Cervix	GC/AT	GC/AT
5	Ic	Serous	Lymphocyte	GC/AT	GC/AT
8	IIc	Serous	Lymphoctye	GC/AT	GC/AT
13	IIIc	Muci	Lymphocyte	GC	GC/AT
14	I	Serous	Cervix	GC/AT	GC/AT
17	IV	Serous	Endo[c]	GC/AT	GC/AT
20	IIIc	Muci	Endo	GC/AT	GC/AT
21	IIIb	Muci	Lymphocyte	GC	GC
30	IIc	Serous	Lymphocyte	GC/AT	GC/AT
33	IIIc	Serous	Lymphocyte	GC/AT	GC/AT
36	IIc	Serous	Endo	GC	GC
38	Border[d]	Muci	Endo	GC/AT	GC/AT
41	IIIc	Serous	Endo	GC/AT	GC/AT
42	IIIc	Muci	Endo	GC/AT	GC/AT
45	IIb	Endoca	Endo	GC/AT	GC/AT
46	IIb	Endoca	Endo	AT	GC/AT
47	Ic	Endoca	Cervix	GC/AT	GC/AT
48	Ia1	Muci	Ovary	GC/AT	GC/AT
51	IIIc	Serous	Lymphocyte	GC	GC/AT
54	IIa	Clear	Cervix	GC/AT	GC/AT
58	III	Endoca	Lymphocyte	GC/AT	GC/AT
60	II	Clear	Lymphocyte	GC/AT	GC/AT
24 LOH	IIIc	Serous	Lymphocyte	GC/AT	GC/AT
61 LOH	II	Muci	Lymphocyte	GC/AT	GC/AT

[a] Allelic expression in the *GC/AT* polymorphism site. Samples 13, 46, and 51 showed allelic imbalanced expression with biallelic expression in tumor and monoallelic expression in normal tissues; samples 21 and 36 showed monoallelic expression in both tumor and normal tissues. T24 and T61 showed LOH and biallelic expressed in both tumor and normal tissues.

[b] Clear, clear cell carcinoma; serous, serous adenocarcinoma; muci, mucinous adenocarcinoma; endoca, endometroid carcinoma.

[c] Endo, endometrium.

[d] Border, borderline tumor.

Table 2   Allelic expression of the *p73* gene in ovarian cancer and cell lines

Expression type[a]	Ovarian cancer	Normal tissues	Cell lines
Biallelic	22/24 (91.7%)	19/24 (79.2%)	1/1 (100%)
Monoallelic	2/24 (8.3%)	5/24 (20.8%)	0
Imbalance	3/24 (12.5%)		

[a] The allelic expression was analyzed using *GC/AT* polymorphism as shown in Table 1 and Fig. 1.

Fig. 1   Analysis of allelic expression of *p73* in tumor (*T*) and normal (*N*) samples. *p73* allelic expression was analyzed in eight matched samples and one ovarian cancer cell line (OVCA3) using a C/T polymorphism in exon 2. The size of the band (116 bp) was seen after nested RT-PCR. Biallelic expression was identified for both 116 bp and 84 bp after *Sty*I digestion. Biallelic expression of the *p73* gene in both tumor (*T*) and normal (*N*) samples was found in samples 1, 14, 17, 20, 30, and 33. Imbalanced expression was shown in sample 13, with biallelic expression in tumor and monoallelic expression in normal lymphocytes. Both *T21* and *N21* remained monoallelic in expression. *OVCA3* also showed biallelic expression. *Ctr*, control of complete digestion.

mens. Nineteen matched normal samples were also proved to be biallelically expressed (79.2%). Eight of them were lymphocytes. Only three samples showed imbalanced expression of *p73*, with biallelic expression in tumors and monoallelic expression in normal specimens. Among these three samples, two were lymphocytes and one was endometrium. The remaining two samples showed monoallelic expression in both tumor and normal specimens. Interestingly, two samples with LOH also showed biallelic expression for both tumor and normal tissues. One informative cell line, OVCA3, was also expressed biallelically. The results are summarized in Tables 1 and 2 and in Fig. 1.

Overexpression of p73 with Normal Expression of p53 in Advanced Ovarian Cancer.   The expression levels of *p73* and *p53* in tumors were compared with those of the normal matched tissues. The results are shown in Table 3 and in Fig. 2. The expression level of *p73* in the advanced ovarian cancer was 2–10 times higher than that in the normal tissue, with an average

ratio of 4.4:1. The *P* is <0.05 when compared with early-stage and borderline tumors exhibiting significant statistical difference. However, the *p73* level was only 1.5 and 1.1 times as high as in paired normal tissues in the early-stage disease and the borderline tumor, respectively. There was no statistically significant difference between the early-stage ovarian cancer and the borderline ovarian tumor. The *p53* expression was uniform among different types of tissues. There is no relationship between *p53* and *p73* expression in mRNA level.

Overexpression of p73 Protein in Advanced Ovarian Cancer in Agreement with mRNA Level.   To further understand whether *p73* mRNA expression correlates with protein

Table 3 Expression level of *p73* and *p53*[a]

Tumor type	No.	*p73* mRNA level	*p53* mRNA level	*p73* protein level	
		T/Tβ/N/Nβ[b]	T/Tβ/N/Nβ	No.	T/Tβ/N/Nβ
Advanced stage	12	4.4 ± 2.4	1.3 ± 0.9	7	5.2 ± 2.9
Early stage	9	1.5 ± 0.5	1.3 ± 1.0	5	1.5 ± 0.6
Borderline tumor	6	1.1 ± 0.2	0.9 ± 0.2	3	1.2 ± 0.3

[a] $P = 0.016$ and 0.008, respectively, for *p73* mRNA between advanced and early stage cancer or borderline tumor. $P = 0.019$ and 0.029, respectively, for p73 protein between advanced stage and early stage or borderline tumor. $P > 0.05$ between early stage and borderline in both *p73* mRNA and protein level. *P* of *p53* is > 0.05.

[b] T, tumor; N, normal; Tβ, β-*actin* expression level in tumor; Nβ, β actin expression level in normal tissues.

Fig. 3 p73 *methylation in the CpG island of exon 1 in ovarian cancer cell lines. DNA from the cell lines was digested with 20 units of either methylcytosine-sensitive enzyme HpaII (Promega) or its methylation-resistant isoschizomer, MspI (Promega), for 3 h at 37°C, then phenol/chloroform-extracted, ethanol-precipitated, dried, and resuspended to 10 μl of Tris-EDTA buffer. One μl (20 ng) was amplified by PCR using primers 5'-GGGGACGCAGCGAAACCG-3' and 5'-CTGCAGC-CGTCGCAGCC-3', which amplified the CpG island in exon 1 (77bp). Normal ovary DNA was used as a negative control. U, uncut; H, HpaII; M, MspI. The PCR band was shown in the uncut. The HpaII digestion band can be seen in SKOV3, OVCA3, and A2780s, which is shown as methylation. No band was found in OV2008.*
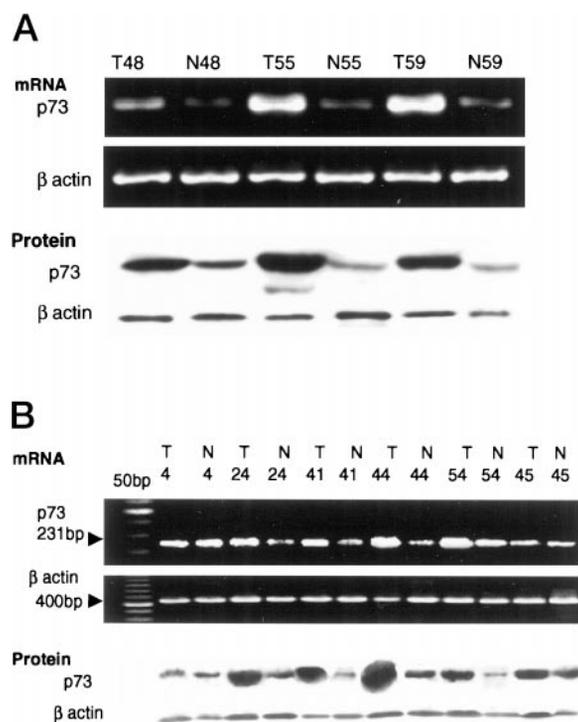
Fig. 2 p73 *expression in tumor and normal ovaries. A,* three matched tumor (*T*) and normal (*N*) ovary samples were examined for *p73* expression by semi-quantitative RT-PCR and Western blot. β-*actin* was used as a control. For RT-PCR, the cycles were 28 for *p73* (231 bp) and 24 for β-*actin* (400 bp), respectively, according to the standard curve. Western blot was performed using 20 μg of protein and a goat antihuman polyclonal anti-p73 antibody (Santa Cruz Biotechnology, Inc.) with a dilution of 1:500 and visualized with the ECL chemiluminescent detection kit (Amersham). The filters were also reprobed with β-actin (Sigma). The expression ratio between tumor (*T*) and normal (*N*) ovary was 1.8:1, 4.8:1, and 3.7:1 for mRNA and 1.6:1, 4.7:1, and 4.5:1 for protein in *T48, T55,* and *T59,* respectively. T55 and T59 were advanced stage, T48 was stage Ia1. *B, p73* mRNA and protein expression in different stages of ovarian cancer and in normal tissues. *T24, T41,* and *T44* were advanced-stage cancer, *T54* and *T45* were early stage, and *T4* was a borderline ovarian tumor.

expression, Western blot was also performed in seven advanced ovarian cancers and in five early-stage and three borderline ovarian tumors. The protein level of p73 was in accordance with mRNA level in all of the samples, as shown in Table 3 and in Fig. 2. The p73 protein level was 2–10 times higher in advanced
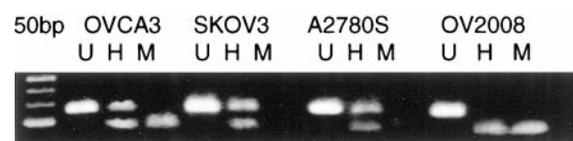
ovarian cancer, with a mean value about 5.2 ± 2.9, but not in early-stage (1.5 ± 0.6) and borderline (1.2 ± 0.3) ovarian tumors.

Loss of p73 Protein Expression Was Related to CpG Island Methylation in Ovarian Cancer Cell Lines. Among the five ovarian cancer cell lines studied, p73 protein was detected in two, OV2008 and A2780cp. We hypothesized that the absence of p73 protein might be caused by methylation of the CpG islands. The results showed that the protein-negative cell lines OVCA3, A2780s, and SKOV3 were methylated in exon 1. In OV2008 and A2780cp with *p73* expression, exon 1 was not methylated (Fig. 3). SKOV3, exhibiting no *p73* expression in mRNA and protein level, was treated with 10 μM of 5-aza for 8 days. RT-PCR and Western blot showed reexpression of this gene at both the mRNA and protein levels, as shown in Fig. 4. No evidence of methylation was found in ovarian cancer tissues and normal tissues, except that one cancer sample (T21) with monoallelic expression was methylated.

DISCUSSION

Kaghad *et al.* first discovered *p73* gene on chromosome 1p36, which showed lack of mutations in many cancer cell lines (2). However, LOH has been found in 5.3–19% of cancers (8–11). In this study, 2 of 24 heterozygous (8.3%) samples showed LOH, both of them stage III. Our data suggest that the loss of one of the parental alleles of *p73* gene is uncommon in ovarian cancer and LOH does not play an essential role in the ovarian carcinogenesis.
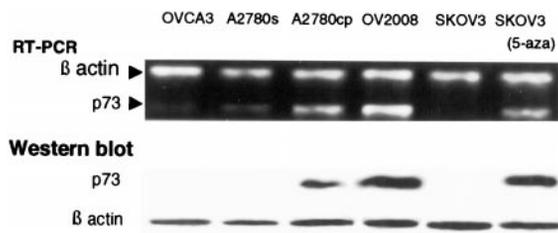
Fig. 4 *p73* expression in ovarian cancer cell lines by Western blot and RT-PCR. Western blot using 50 μg of protein and goat antihuman polyclonal anti-p73 antibody (Santa Cruz) was shown in ovarian cancer cell lines OVCA3, A2780s, A2780cp, OV2008, SKOV3, and SKOV3 after 5-aza treatment for 8 days. p73 protein was identified in OV2008 and A2780cp. After 5-aza treatment, p73 protein was detected in the SKOV3 cell line. A similar result was shown using RT-PCR in the same ovarian cancer cell lines. RT-PCR was performed using 28 cycles for *p73* and β-*actin*; the annealing temperature was 62°C.

Monoallelic expression of *p73* has been reported previously, suggesting that *p73* may be a candidate of the imprinting gene (2, 5). In contrast to previous findings, we observed 91.7% (22 of 24) of the ovarian cancer and 70.8% (17 of 24) of the normal endometrium and normal cervix expressed biallelically. Even lymphocytes were biallelic expressed in eight of the twelve samples. Our results are in line with several other studies that biallelic expression of this gene was found in 25 of 26 normal lung specimens (18), in both bladder cancer and normal bladder (7), in 3 peripheral lymphocyte cell lines, 1 colon cancer cell line, and melanoma cell lines (12). These results supported the conclusion that biallelic expression was involved in most of the ovarian cancer and normal tissues. On the other hand, in our study 2 of 24 informative samples maintained monoallelic expression in both tumor and normal specimens. Imbalanced allelic expression was identified in 3 of 24 (12.5%) samples with biallelic expression in tumor and monoallelic expression in normal tissues. This phenomenon looks like LOI. The same phenomenon was found in (5 of 5) lung cancer (6), (1 of 26) lung cancer (19) and (7 of 11) renal carcinoma (5). Genomic imprinting has been suggested to play a role in many human cancers (20, 21). Activation of the silent allele with biallelic expression in cancer cells and monoallelic in normal tissues was considered as LOI. LOI has been reported in many types of tumors (21, 22). As shown by others (7, 12, 18), our results showed that *p73* was evidently biallelically expressed in both normal tissues and ovarian cancer. Therefore, the *p73* gene was not imprinted in ovarian cancer and LOI of this gene was not an important step in the tumorigenesis when compared with renal cell carcinoma and lung cancer (5, 6). It seems that allelic expression of *p73* may be tissue specific or individual specific.

In this study, we showed that expression level of *p73* is higher in advanced ovarian cancer than in normal tissues. However, our present study failed to show any correlation in the mRNA level between *p73* and *p53*. Overexpression of *p73* gene has also been reported in lung cancer, bladder cancer, and colorectal cancer without mutation (6, 7, 9). It is possible that wild type *p73*, not mutant *p73*, is overexpresssed in tumors. The wild type *p73* functions as a security guard when overexpressed

and works to arrest the cell cycle as a tumor suppressor gene. However, another possibility is that *p73* functions as an oncogene in the up-regulation of cell growth (19) and silences p*53* function by binding to its functional binding site (23).

To elucidate the imprinting and expression of *p73* gene, we studied the methylation of promoter CpG island in ovarian cancer tissues and normal tissues. Finding no evidence of methylation in these samples further suggests that it is not an imprinting gene. However, in ovarian cancer cell lines the lack of p73 protein expression with evidence of methylation supports that methylation of *p73* may play a role. Reactivating the expression by 5-aza also confirms the role of methylation in the control of expression of this gene. Our results in the ovarian cell lines support that epigenetic silencing of tumor suppressor genes via methylation of the promoter CpG island is involved in the ovarian malignancy. Although it is difficult to explain the high level of both mRNA and protein in advanced ovarian cancer and the absence of protein expression in ovarian cancer cell lines, this is the first report that loss of *p73* expression is attributable to methylation in ovarian cancer.

Our study revealed that LOH of the *p73* gene occurred in a low frequency in ovarian cancer. Biallelic expression of *p73* was found in the majority of ovarian cancer and normal tissues. Overexpression of *p73* was associated with advanced ovarian cancer when compared with the early-stage and borderline ovarian tumors, and we showed that *p73* overexpression was independent of *p53*. Lack of expression of *p73* was associated with CpG island methylation. Demethylation using 5-aza in SKOV3 can reactivate the expression of this gene in both the mRNA and protein levels. We conclude that the *p73* gene is not an imprinting gene in ovarian cancer. Overexpression of *p73* in both the transcriptional and translational levels is associated with ovarian cancer in advanced stages. DNA methylation is involved in the *p73* inactivation in ovarian cancer cell lines.

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