Frequent hypermethylation and loss of heterozygosity of the testis derived transcript gene in ovarian cancer

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Testis derived transcript (TES) is a candidate tumor suppressor gene located at the human chromosome 7q31, and its function in ovarian cancer is still unknown. Using ovarian cancer cell lines and tissue samples, we demonstrated that both loss of heterozygosity and hypermethylation of the *TES* gene occurred in ovarian cancer at high frequencies, and there were significant correlations between TES expression and hypermethylation or loss of heterozygosity. We also detected methylation in ovarian cancer cell line A2780 after treatment with 5-aza-2-deoxycytidine. The expression level of TES was enormously up-regulated, then caused changes to the biological behaviors of A2780 cells: cell growth properties were greatly impaired, colony formatting abilities were suppressed to very low levels, and the apoptosis rate was highly raised compared to the control group. Our findings suggest that the *TES* gene functions as a tumor suppressor gene and is frequently silenced by hypermethylation and loss of heterozygosity in ovarian cancers. (*Cancer Sci* 2010; 101: 1255–1260)

Nowadays, we realize that tumors are a complex process made up of multiple steps. In past decades, a great number of genes was proved to be involved in the development and progression of tumors. In recent years, several genes were identified to be able to suppress tumor growth (as tumor suppressor genes), such as P53,[1] phosphatase and tensin homologue,[2] and p16,[3] highlighting the optimistic prospect of research into these genes. A great many studies showed that alterations of tumor suppressor genes like loss of heterozygosity[4,5] and promoter hypermethylation[6,7] were detected in almost every type of cancers.

Ovarian cancer is the third most frequent female cancer type and the leading cause of death by cancer among women in China. More than 70% of patients were already in the late stage on diagnosis, and lost the chance to have an operation, which then required a great deal of money and medical resources.

It has also been demonstrated that some tumor suppressor genes play a notable role in ovarian cancer; the *testis derived transcript (TES)* gene is one of them. The TES gene is located at 7q31, within the fragile chromosomal region FRA7G.[8,9] It spans about 48 kb encompassing seven exons. There are three isoforms of human TES, which differ from each other in the 3-untranslated region (3-UTR).[10] At its COOH terminal, the TES protein has three zinc-binding domain present in Lin-11, Isl-1 and Mec-3 domains which play a very important role in focal adhesion targeting.[11] TES was proved to be a putative tumor suppressor gene;[12] in the past few years, much evidence has indicated TES anticancer functions in the mechanisms of tumorigenesis, angiogenesis, and metastasis. Frequent loss of heterozygosity at 7q31 happened in a variety of malignances, implying that loss of gene expression within this region

had a relationship with cancer. Moreover, studies also reported a great rate of TES hypermethylation in glioblastomas and other malignances, leading to the TES expression decline or total loss.

Although there some research reported TES anticancer functions in other malignancies, we still didn't get information about its role in ovarian cancer. In this study, we investigated both loss of heterozygosity and hypermethylation in 41 cases of ovarian cancer. The results showed that TES expression had significant correlations with loss of heterozygosity and hypermethylation. Then we up-regulated TES in ovarian cancer cell line A2780 with 5-aza-2-deoxycytidine (5-aza-dC) and apparent changes of cell biological behaviors were detected, further implying that TES is a tumor suppressor gene and is often silenced in ovarian cancer because of either hypermethylation or loss of heterozygosity.

Materials and Methods

Cell lines and 5-aza-dC treatment. Epithelial ovarian cancer cell lines (SKOV3 and A2780) were routinely cultured in RPMI-1640 growth medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS at 37°C, 5% CO_2. 5-aza-dC was dissolved in double-distilled H_2O and filtered with a 0.22-μm filter membrane. 5×10^5 cells were plated into a 25-cm² flask with RPMI-1640. Twenty-four hours later, cells were treated with 5 μm 5-aza-dC, and the medium was changed every 3 days. Total RNA was prepared with Trizol reagent (Invitrogen) 10 days later in accordance with the manufacturer's instructions. Five-hundred ng RNA was used for quantitative RT-PCR on Stepone Plus (Applied Biosystems, Foster City, CA, USA). TES expression was evaluated with the following primers: forward, 5-CCTTCAAAGTGCCATGAGTTGTCTC-3; reverse, 5-TTC-ATACTCAGTTTGCAGCAATAGC-3. PCR conditions were as follows: 95°C for 30 s, 35 cycles of 95°C for 5 s, and 60°C for 32 s. GAPDH was used as the endogenous control.

Flow cytometry analysis of cell apoptosis. Cell apoptosis was analyzed by an Annexin V-FITC ⁄ propidium iodide (PI) kit according to the manufacturer's instructions. Briefly, harvested cells were resuspended in 100 μL Annexin V–FITC binding buffer and adjusted to about 1×10^6 ⁄ mL, then 5 μL Annexin V-FITC and 10 μL PI (20 μg ⁄ mL) were added, following 15-min incubation in the dark. Flow cytometry was conducted on a FACS caliber (BD Biosciences, Hercules, CA, USA).

Cell proliferation assay. 3×10^3 A2780 cells at approximately 80% confluence were plated per well into 96-well plates and incubated overnight, then cells were treated with different concentrations of 5-aza-dC separately. The cell growth curve was assessed using an 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium (MTT) assay according to the manufacturer's

instructions. The results were expressed as the absorbance at 490 nm at the indicated time points.

Colony formation assay. 0.5×10^3 A2780 cells were plated per well into six-well plates, and separated into two groups according to different 5-aza-dC concentrations. The culture medium was changed routinely and colony numbers were counted by inverted microscope 2 weeks later.

Western blotting. To investigate TES expression change after 5-aza-dC treatment, Western blotting was performed in a A2780 cell line. Briefly, 50 μg of protein samples were loaded onto SDS-PAGE gels and electroblotted to polyvinylidene difluoride membranes. The membranes were incubated with primary anti-TES (Abcam, Cambridge, MA, USA), followed by reaction with a secondary antibody. β-Actin was used as the endogenous control.

Patients and samples. Samples were collected from 41 patients diagnosed with ovarian cancer in Qilu Hospital (Shandong, China) between June 2008 and June 2009; all patients provided consent and approval was obtained from the ethics committee. One mL whole blood was extracted from each patient before the operation and tissues were obtained after surgical resection, then immediately stored at −80°C. All malignant cases were classified and graded according to the criteria of the International Federation of Obstetrics and Gynecology (FIGO). An additional 12 normal ovarian tissues were collected as controls.

DNA extraction. Genomic DNA was extracted according to the standard phenol/chloroform extraction. In brief, 25 mg tissue or 200 μL whole blood with Proteinase K (20 mg/mL) was incubated overnight at 50°C in a waterbath. DNA was extracted by phenol/chloroform twice, then precipitated with 100% ethanol and at the end dissolved in TE solution. For paraffin-embedded tissues used in loss of heterozygosity analysis, 8-μm tissue slides with 2-h dehydration by xylene were prestained with Toluidine blue, and the tumor cell zone was removed with a tiny needle on an inverted microscope.

Microsatellite analysis of loss of heterozygosity. Five microsatellite markers flanking chromosome 7q31 (D7S2502, D7S2543, D7S486, D7S2460, D7S522) were chosen from Genebank for the PCR-based analysis of loss of heterozygosity. One μg total DNA was used to perform PCR under the following conditions: denaturing at 95°C for 3 min, 30 cycles of 95°C for 15 s; and annealing of each primer for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. Ten μL of PCR products was separated by 8% denatured polyacrylamide gels (300 V for 2–3 h) and then the gels were silver-stained. Two experienced researchers separately analyzed the staining results.

Methylation-specific PCR (MS-PCR). One μg of each DNA sample was bisulfite modified using the CpGenome DNA modification kit (Chemicon, Temecula, CA, US) according to the manufacturer's instructions. The locations of CpG islands within the TES gene promoter have been described previously. Primers and PCR conditions for [MSP(13)] are available on request. DNA treated by M. Sss. I was used as a positive control.

Immunohistochemistry. Protocols for immunohistochemistry staining were described previously.[14] Sections stained without anti-TES were used as negative control. For evaluation of TES protein, classification standards were as follows: negative, totally no staining or <10% tumor cells showed positive staining; and positive, ≥10% tumors cells were positively stained.

Statistical analysis. The software SPSS version 16.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. The appropriate χ^2-test was chosen to analyze the association between two categorical variables. The level of statistical difference was defined at 0.05.
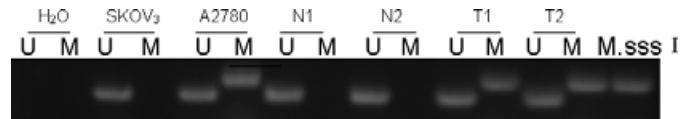

Fig. 2. Methylation analysis of testis derived transcript in ovarian cancers. C, cancer samples; M, methylated; M. Sss I, positive control; N, normal ovary tissues; U, unmethylated. (DNA modified by CpG methyltransferase M. Sss I.)

Table 1. Relationship between TES expression and methylation

	TES expression		P-value
	Negative	Positive	
Methylation			
Negative	7	19	0.001
Positive	12	3	

TES, testis derived transcript.

Results

TES protein expression in ovarian cancer. We immunohistochemically assayed TES protein levels in 41 ovarian cancer samples and 12 normal ovarian tissues. All the normal samples stained positively for TES antibody; however, loss of TES protein was identified in 56.1% (23/41) of ovarian cancer samples (Fig. 1).

Frequent TES hypermethylation in ovarian cancer. We observed hypermethylation in 36.5% of ovarian cancer tissues (15/41); in contrast, none of the 12 normal ovary tissues was hypermethylated (Fig. 2). We also detected hypermethylation in the A2780 cell line. There was a significant correlation between hypermethylation and TES protein level ($P = 0.001$; Table 1).

Allelic loss of TES in ovarian cancer. Twenty-six of all 41 cases were informative for loss of heterozygosity analysis. We detected TES locus loss in 34.6% (9/26) of ovarian cancers (at least one marker was lost) (Fig. 3). When we examined the relationship between TES expression and the frequency of loss of heterozygosity, eight of nine cases with loss of heterozygosity presented lack of TES protein expression (Table 2). Previous studies had reported a correlation between methylation and loss of heterozygosity; however, our results showed that only one case presented both hypermethylation and loss of heterozygosity
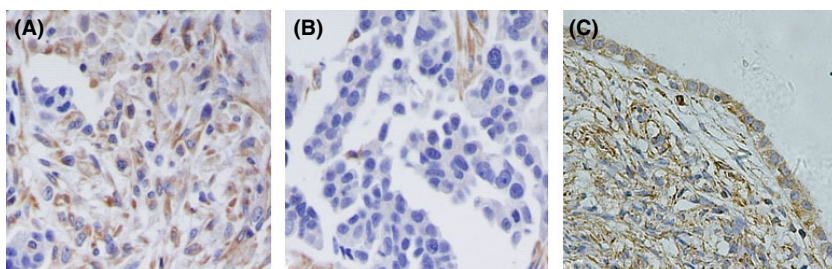

Fig. 1. Testis derived transcript (TES) expression in normal ovary and ovarian cancer tissues. (A) Positive staining of TES protein in ovarian cancer. (B) TES expression was lost in ovarian cancer. (C) Normal ovarian tissue was stained positively.
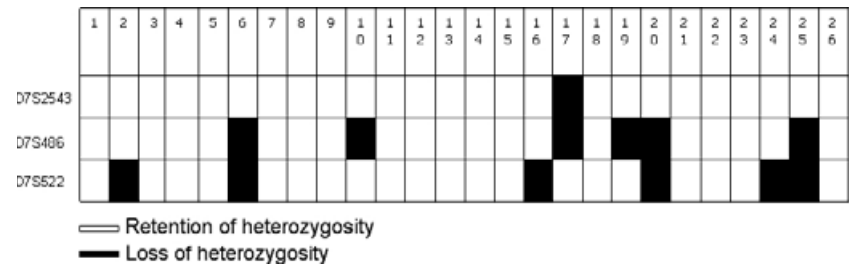
Fig. 3. Loss of heterozygosity in ovarian cancer.

☐ Retention of heterozygosity
■ Loss of heterozygosity

Table 2. Relationship between TES expression and locus loss

	TES locus loss		P-value
	ROH		
TES expression			
Negative	8	8	0.037
Positive	9	1	

ROH, retention of heterozygosity; TES, testis derived transcript.

Table 3. Relationship between TES locus loss and methylation

	TES locus loss		P-value
	ROH		
Methylation			
Negative	13	8	0.833
Positive	4	1	

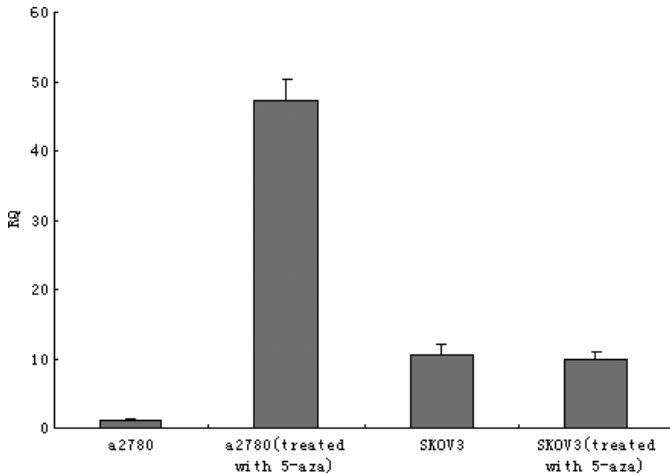ROH, retention of heterozygosity; TES, testis derived transcript.

Fig. 4. RT-PCR analysis of testis derived transcript with or without 5-aza-2-deoxycytidine treatment in A2780 and SKOV3 cell lines.
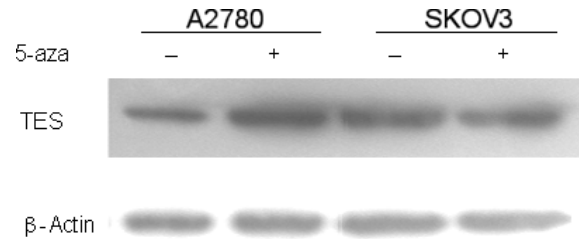

Fig. 5. Western blot of testis derived transcript (TES) in SKOV3 and A2780 cell lines before and after 5-aza-2-deoxycytidine treatment. β-Actin was used as an endogenous control.
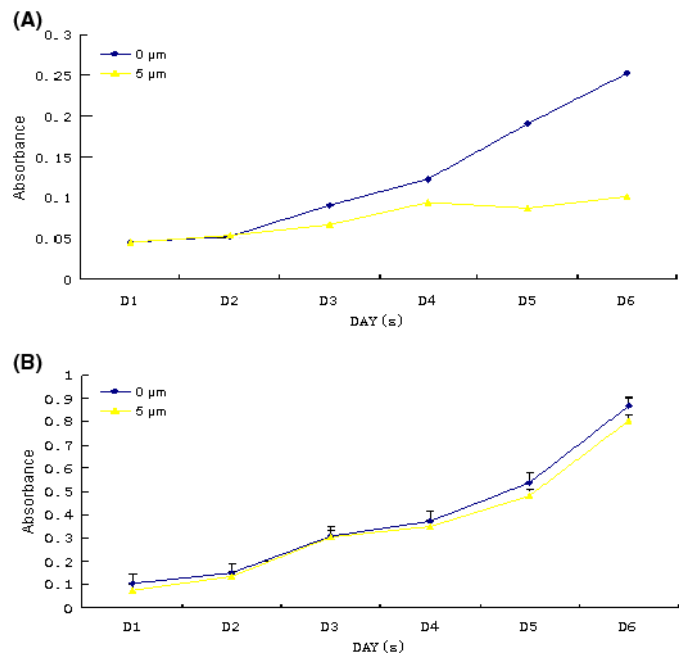

Fig. 6. Cell proliferation curve with or without 5-aza-2-deoxycytidine. (A) A2780 cell line; (B) SKOV3 cell line.

(Table 3). There wasn't an obvious correlation between methylation and loss of heterozygosity ($P = 0.833$; Table 3).

TES expression in ovarian cancer cell lines. Quantitative RT-PCR and Western blotting were preformed to evaluate the expression changes after treatment with 5-aza-dC. The results indicated an enormous up-regulation in both transcriptional (more than 45-fold) and translational levels in the A2780 cell line after treatment with 5 μm 5-aza-dC, but no obvious change was detected in SKOV3 cells (Figs 4,5). In addition, by quantitative RT-PCR, we found no changes in the 1-μm group compared to the 0-μm group for both cell lines (data not shown).

Cell growth analysis. We used 5 μm 5-aza-dC for treating the two cell lines. In A2780 cells, significant difference was observed in cell growth curve of the two groups. Compared to the 0-μm group, the growth properties of A2780 cells in the 5-μm group were obviously impaired, indicated by the much lower absorbance at 490 nm ($P = 0.013$; Fig. 6A). However, as for the SKOV3 cell line, no apparent changes were detected between the two groups ($P = 0.796$; Fig. 6B).

Colony formation assay. Colony formation assay showed strong differentiation between the 0-μm and 5-μm groups for A2780 cells, but not SKOV3 cells. The A2780 cells treated with 5 μm 5-aza-dC lost the capacity to grow into large colonies,
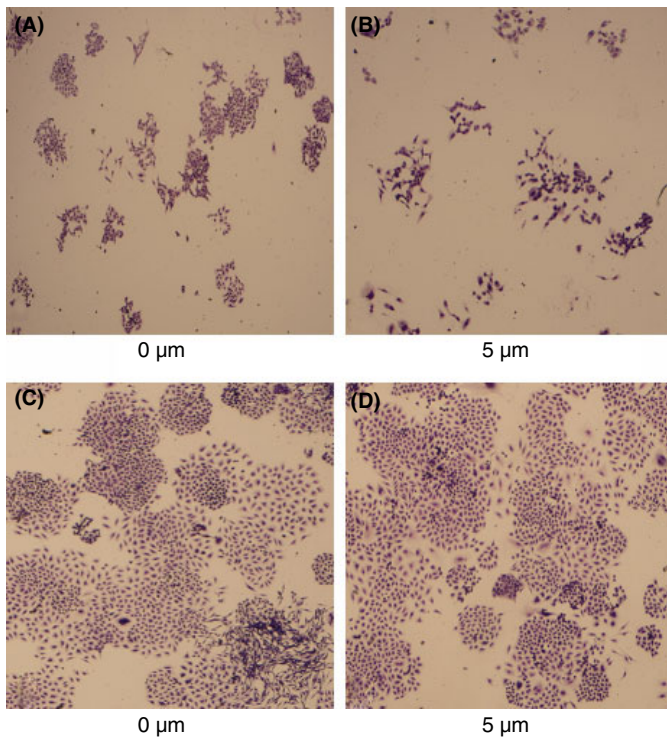
Fig. 7. Colony formation assay with or without 5-aza-2-deoxycytidine. (A,B) A2780 cell line; (C,D), SKOV3 cell line.

compared with the cells in 0-μm group; moreover, the colonies in the 5-μm group were more dispersive. However, we didn't detect obvious changes in SKOV3 cells after treatment with 5-aza-dC (Fig. 7).

Cell apoptosis assay. The two cell lines treated with 5 μm 5-aza-dC were assayed for cell apoptosis. In the A2780 control group (no 5-aza-dC was given), the total rate of apoptosis was very slight, just 0.99 (early apoptotic rate, 0.3; late apoptotic rate, 0.69). However, many more apoptotic cells were detected in A2780 cells treated with 5-aza-dC: the rate raised to 3.31 (early apoptotic rate, 1.38; late apoptotic rate, 1.93). As for the SKOV3 cell line, the apoptotic conditions didn't vary much before and after treatment with 5-aza-dC (1.59 *vs* 1.43; Fig. 8).

Discussion

Because we have not yet found a valid approach for the early detection of ovarian cancer, much research focuses on early diagnosis and cure. In this field, studies of tumor suppressor genes might show us a bright future. Epigenetic and genetic alterations of tumor suppressor genes like promoter hypermethylation, loss of heterozygosity, and mutation occur frequently in various malignances, down-regulating the expressions of these genes, and giving tumors a chance.

To date, nearly 100 articles have reported loss of heterozygosity in ovarian cancer at chromosome arms 4p, 5q, 7q, 8p, 9p, 10q, 12q, 13q, 14q, 16q, 17p, 17q, and so on.[15,16] Tumor suppressor genes located in these sites were proven to be low expressed or even non-expressed, such as phosphatase and tensin homologue,[17] breast cancer 1,[18] cyclin-dependent kinase inhibitor 2A,[19] and adenomatous polyposis coli[20] providing evidence for the antitumor character of these genes. Loss of heterozygosity at 7q31 was common in breast carcinoma (40–83%),[21,22] prostate cancer (more than 80%),[23] and renal cell carcinoma (30%).[24] In addition, loss of heterozygosity was also found in pancreatic carcinoma,[25] colonic carcinoma,[26]

and many other malignancies.[27–30] Recently, more and more studies have indicated that TES may act as a tumor suppressor gene in tumorigenesis,[31] metastasis,[11] and prognosis.[32,33] In this study, when we tested the frequency of loss of heterozygosity by microsatellite analysis, 34.6% cases presented at least one marker lost. As to our results, eight of nine cases indicated loss of heterozygosity with a decline of TES expression. In contrast, TES protein level was normal in nine of 17 retention of heterozygosity cases; there is a statistical difference ($P =$ 0.037).

DNA methylation is considered to be another mechanism for the second hit for Knudson's hypothesis.[34] It is an enzymatic process to add the methyl group at the fifth carbon of cytosines of the dinucleotide 5′-CpG-3′ sequence which is plentiful within nearly all promoters. Cytosine methylation can function on regulating the expression of endogenous genes, silencing transposons, and controlling the stability of the genome.[35] In recent years, studies on promoter hypermethylation of the tumor suppressor genes have reported great achievement. Hypermethylation of DAPK,[36] OPCML,[37] CDH13,[38] and GATA4[39] were reported to be frequent in ovarian cancer. Up to now, only two previous studies have detected methylation of the *TES* gene. In the first study, hypermethylation of the *TES* gene was detected in all 30 tumor-derived cell lines,[12] including the A2780 cell line, which was also proved to be methylated in our study. In the second study of glioblastomas, TES promoter methylation existed in 58% cases and this alteration was more common in primary than secondary glioblastomas.[40] Both the studies showed high frequency of hypermethylation in the *TES* gene, implying its potential role in suppressing tumor activities. Consistent with these studies, in our study, hypermethylation was another cause for TES silence in ovarian cancer: more than 35% of cases were observed hypermethylated, and in contrast, methylation happened in none of the 12 normal tissues. There was a significant difference between the ovarian malignant and nonmalignant groups. We also investigated the relationship between TES expression and hypermethylation. In the normal TES expression group, three in 22 cases presented hypermethylation, whereas 12 of 19 cases in the low TES expression group were methylated, indicating the important influence of hypermethylation on TES protein level regulation.

By Knudson's hypothesis, inactivation of tumor suppressor genes was caused by two hits: the first is usually a mutation at one of the alleles, and the second may occur by mutations, deletions, or some other mechanisms. The analysis for loss of heterozygosity and hypermethylation is a useful approach to identify tumor suppressor genes involved in tumorigenesis. But recent studies had suggested that some genes may be just one-hit genes, meaning that just one alteration can impair the expression of these genes. A previous study presented evidence that the *TES* gene is also a one-hit gene. In TES-knockout mice, semi(+/−) or total loss(−/−) of TES caused many more high risks for tumorigenesis (ORs were 38.9 and 27.6, respectively).[31] Interestingly, in our study we also observed the phenomenon that there was just one case in which both loss of heterozygosity and hypermethylation occurred. Further research needs to be undertaken to determine whether the one-hit mechanism really works on the *TES* gene.

In a previous study, the impact of TES on cells was studied; after transfecting the full-length TES coding cDNA into cell lines, TES expression was highly up-regulated, which resulted in cell growth deduction and less colony formation.[12] Another study proved that re-expression of TES in T47D cells can induce more apoptosis and impair the capability to form a mass in nude mice.[41] We raised the TES protein level in A2780 cells by treatment with 5-aza-dC; high TES expression greatly suppressed the biological behaviors of A2780 cells. As the cell proliferation curve indicated, the growth properties of A2780
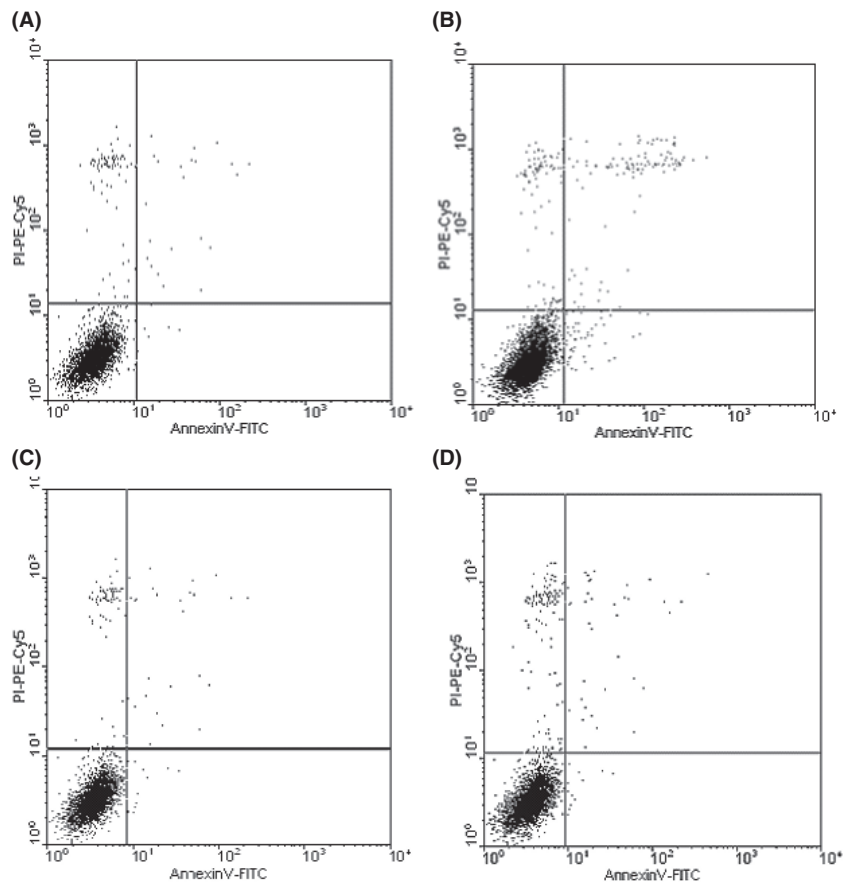
Fig. 8. Cell apoptosis induced by treatment with 5 μm 5-aza-2-deoxycytidine. (A) Apoptosis of A2780 cells in the group without 5-aza-dC treatment (early apoptotic rate, 0.3; late apoptotic rate, 0.69); (B) apoptosis of A2780 cells in the 5 μm 5-aza-2-deoxycytidine group (early apoptotic rate, 1.38; late apoptotic rate, 1.93); (C) apoptosis of SKOV3 cells in the group without 5-aza-dC treatment (early apoptotic rate, 0.44; late apoptotic rate, 0.99); (D) apoptosis of SKOV3 cells in the 5 μm 5-aza-dC group (early apoptotic rate, 0.57; late apoptotic rate, 1.02).

cells treated with 5 μm 5-aza-dC were much less than cells in the control group, suggesting that by treatment with the appropriate concentration of 5-aza-dC, we can prevent ovarian cancer from rapidly growing. In addition, colony formation assay results further indicated that the colonies in the 5-μm 5-aza-dC group were much fewer in number and smaller than those in the control group, and the cells appeared very scattered. Though we were unsure how re-expressed TES achieved this, the results indicated that TES has the potential to stop the ovarian cancer cells shaping into huge solid tumors. That is also further evidence for the negative functions of TES in malignant tumors. We also tested the apoptotic conditions in cells treated with 5-aza-dC and compared to the no-treatment group, more cells were induced to apoptosis by 5-aza-dC. This result implies that TES may cause cell apoptosis after the reversion of its hypermethylation. But 5-aza-dC didn't show obvious influences on SKOV3 cells (in which TES wasn't methylated) in any of the three assays. Above all, we proved that TES has the potential to slow the proliferation of cancer cells, weaken their capacity to format cell colonies, and can also induce more cells into apoptosis, implying the possibility of using TES as a therapy for ovarian cancer. Future research into *in vivo* tumorigenesis is required to reveal the functions of TES in ovarian cancer and other types of malignances.

In summary, we firstly investigated highly frequent promoter hypermethylation and loss of heterozygosity of the *TES* gene in ovarian cancer. There were significant correlations between TES expression and hypermethylation or loss of heterozygosity, which proved that the *TES* gene was a tumor suppressor gene in ovarian cancer. By restoring the TES expression, both the proliferation and invasive abilities of A2780 cells were enormously suppressed. At the same time, more cells were induced to apoptosis, showing that TES can suppress tumor growth by regulating various biological behaviors of ovarian cancer; this provided more and detailed evidence for the role of the tumor suppressor gene *TES* in ovarian cancer. According to our results, specific therapy targeting TES might have broad prospects in the future.

Acknowledgments

Disclosure Statement

The authors have no conflict of interest.

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