Introduction

Ovarian cancer is one of the most frequent female cancer types, it caused the leading mortality of malignancies among Chinese women. However, up to date, we could not yet discover a valid approach for the earlier diagnosis and therapeutic strategy of ovarian cancer. For that reason, more than seventy percent of ovarian cancer patients were already in very late stages (stage III/IV) when diagnosed, lost the best chance to perform an operation. More and more studies showed that ovarian cancer is a multiple-step process, involving many tumor suppressor genes (TSGs) inactivation, such as p53 (Farnebo et al., 2010), Rb (Simin et al., 2004), PTEN (Maehama et al., 2007) and APC (van Es et al., 2001), highlighting the optimistic prospect of research into these genes. A great many studies showed that epigenetic and genetic alterations of TSGs such as promoter hypermethylation, loss of heterozygosity, and mutation occurred very frequently in various human malignancies, which down-regulated the expression level of TSGs and impaired their anti-tumor functions (Fukuhara et al., 2002; Zhang et al., 2007; Hoebeeck et al., 2009; Qiu et al., 2010). These alterations were proved to be significantly correlated with the development, progression, invasion, metastasis and prognosis of various cancer types. Based on these findings, scientists proceeded huge numbers of researches to search and identify new and useful TSGs. Among all these achievements, studies focused on promoter hypermethylation of TSGs might shed new light on diagnosis and therapy for cancers.

SLIT2 is a newly candidate tumor suppressor gene. It belongs to the SLITs family which contains another two members-SLIT1 and SLIT3. The SLITs were found playing important roles in axon guidance and cell migration (Brose et al., 2000; Brose et al., 1999; Wang et al., 1999). The SLIT2 gene had been mapped to chromosome 4p15.2, it contains four tandem leucine-rich repeats (LRRs), seven epidermal growth factor (EGF)-like repeats, an Agrin-Laminin-Perlecanslitr(Alps) conserved space motif, and a cystine knot (Georgas et al., 1999). Its expression was detected in both neuronal and nonneuronal tissues, implicating its functions besides those in nervous system. Previous studies had reported frequent SLIT2 promoter hypermethylation in breast cancer (Dallol et al., 2002), colorectal cancer (Jin et al., 2009), colorectal cancer (Dallol et al., 2003a), gliomas (Dallol et al., 2003b), as well as lymphocytic leukemia (Dunwell et al., 2009). In these malignancies, promoter hypermethylation was revealed to be responsible for the downregulated SLIT2 expression. Then, after raised the SLIT2 expression in cancer cells, researchers found that SLIT2 could suppress the cancer cells proliferation, migration, invasion abilities, and induced more apoptosis. In addition, other researchers et al., 1999). DNA methylation 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 gene promoters. As the hypermethylation happened, it can downregulate the TSGs expression levels and as a result, the TSGs lost the ability to suppress tumor growth.

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analyzed by electrophoresis on 3.0% agarose gels. M.sssI was used as a positive control. MSP products were available on request to the first author. DNA treated by previously. Primers and PCR conditions for MSP are islands within the SLIT2 promoter have been described to the manufacturer’s instructions. The locations of CpG modification kit (Chemicon, Temecula, CA, US) according sample was bisulfite modified using the CpGenome DNA as described previously. Briefly, one μg of each DNA Bisulfite treatment of genomic DNA was performed at 50℃. DNA was extracted by phenol ⁄ chloroform with proteinase K (20 mg/ml) was incubated overnight. Genomic DNA was extracted according to the standard DNA extraction

Materials and Methods

Patients and samples

Samples were collected from sixty-six patients diagnosed with ovarian cancer in the fourth affiliated hospital of Suzhou University (Wuxi, PR. China) between June 2010 and January 2011. All cases were classified and graded according to the criteria of the International Federation of Obstetrics and Gynecology (FIGO). All patients provided consent and approval was obtained from the ethics committee. Tumor tissues were obtained immediately after surgical resection and stored at -80℃. According to our findings, specific therapy targeting SLIT2 might have bright prospects in the future.

DNA extraction

Genomic DNA was extracted according to the standard phenol/chloroform extraction. In brief, 25mg samples with proteinase K (20 mg/ml) was incubated overnight at 50℃. DNA was extracted by phenol / chloroform twice, precipitated with 100% ethanol and dissolved in TE solution.

Methylation specific PCR(MSP)

Bisulfite treatment of genomic DNA was performed as described previously. Briefly, 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 SLIT2 promoter have been described previously. Primers and PCR conditions for MSP are available on request to the first author. DNA treated by M.sssI was used as a positive control. MSP products were analyzed by electrophoresis on 3.0% agarose gels.

Cell lines and 5-aza-dC treatment.

Four ovarian cancer cell lines (SKOV3, A2780, 3AO, OVCAR3) were routinely cultured in RPMI-1640 growth medium (Invitrogen, Carlsbad, CA, USA) supplemented with10% FBS at 37℃,5%CO2. 5-aza-dC was dissolved in double-distilled H2O and filtered with a 0.22-µm filter membrane. 5×105 cells were plated into a 25-cm2 flask with RPMI-1640.Twenty-four hours later, cells were treated with 10 μm 5-aza-dC (Sigma-Aldrich, St. Louis, MO,USA), and the medium was changed every 3 days. Total RNA was prepared with the Trizol reagent (Invitrogen, Carlsbad, CA, USA) nine days later according to the manufacturer’s instructions. Five-hundred ng total RNA was used for quantitative RT-PCR on StepOnePlus (Applied Biosystems, Foster City, CA, USA). SLIT2 expression was evaluated with the following primers: 5'- GGTGTCTCTGTGATGAAGAG-3' (forward); and 5'-GTGTGAAGACACACCTCG-3' (reverse). PCR conditions were as follows: 95℃ for 30s, 40 cycles of 95℃ for 5s, and 60℃ for 30s. GAPDH was used as the endogenous control. PCR reactions were performed in triplicate.

Flow cytometry analysis of cell apoptosis

Cell apoptosis was analyzed by an Annexin V-FITC/ propidium iodide (PI) kit (Huajing Bioscience, Shanghai, China) according to the manufacturer’s instructions. Briefly, harvested cells were resuspended in 100 μl Annexin V–FITC binding buffer and adjusted to about 1×10⁶/ml, 5 μl Annexin V-FITC and 10 μl PI (20 μg/ml) were added, then a 15 minutes incubation in the dark. Flow cytometry was conducted on a FACS caliber (BD Biosciences, Hercules, CA, USA).

Cell proliferation assay

3×10⁴ SKOV3 cells at approximately 80% confluence were plated per well into 96-well plates and incubated over night, then cells were treated with 10 μm 5-aza-dC. The cell growth ability 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 490nm at the indicated time points.

Colony formation assay

0.5×10³ SKOV3 cells were plated per well into six-well plates, and separated into two groups with or without 5-aza-dC. The culture medium was changed routinely and colony numbers were counted by inverted microscope two weeks later.

Wound healing assay

SKOV3 cells were plated in six-well plates at a density of 6×10⁵ cells per well and allowed to reach suitable confluence. The cell monolayer was then gently scratched using a sterile 20 μl pipette tip. To remove cell debris, the wells were washed twice with PBS, and then incubated in RPMI-1640 with 5% FBS for 24 hours. Then, the cells were washed in PBS, fixed and stained with crystal violet. The distance that cells had migrated was calculated under microscope. The experiment was performed in triplicate.

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Statistical analysis
The software SPSS version 16.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. The appropriate \( \chi^2 \)-test was chosen. The statistical difference level was defined at 0.05.

Results

SLIT2 expression in ovarian cancer
We investigated SLIT2 mRNA levels in all the ovarian cancer samples and normal ovary tissues. Totally loss or very low level of SLIT2 mRNA were detected in 78.8\% (52/66) of ovarian cancer samples; by contrast, all cases from control group expressed SLIT2 normally (Figure 1).

Frequent SLIT2 promoter hypermethylation in ovarian cancer
We detected SLIT2 promoter hypermethylation in 84.8\% (56/66) of ovarian cancer tissues; but only one in the control group (Figure 2). By statistical analysis, there was a significant correlation between hypermethylation and SLIT2 expression level (p<0.01). In addition, SKOV3 cells also showed SLIT2 promoter hypermethylation.

Correlation between SLIT2 hypermethylation and clinicopathological factors
In our group of ovarian cancers, there was no statistical relationship between SLIT2 promoter hypermethylation and age, pathologic pattern, stage/grade, CA125 level (data not shown).

SLIT2 expression was restored by 5-aza-dC treatment
Quantitative RT-PCR was preformed to evaluate whether the SLIT2 mRNA level in ovarian cancer cells changed pre and after treatment with 5-aza-dC. As much as ten-fold upregulation of SLIT2 expression was detected in SKOV3 cells (p<0.05).

Cell proliferation assay
We treated SKOV3 cells with 10 \( \mu \)m 5-aza-dC. As shown in the growth curve, there was significant differences between the two groups. Compared to the control group, growth properties of SKOV3 cells treated by 10 \( \mu \)m 5-aza-dC were seriously impaired, indicated by the much lower absorbance at 490nm (p<0.05, Figure 3).

Colony formation assay
Colony formation assay showed enormous differentiation between the control group and 10 \( \mu \)m 5-aza-dC group. SKOV3 cells treated with 5-aza-dC only formed colonies much smaller (p<0.01).

Cell apoptosis assay
SKOV3 cells treated with 10 \( \mu \)m 5-aza-dC were assayed for cell apoptosis. In the controls, the total rate of apoptosis was very slight (1.01\%). After the treatment of 10 \( \mu \)m 5-aza-dC, more apoptosis was detected (3.28\%, p<0.05, Figure 4).

Wound healing assay
SKOV3 cells treated with 10 \( \mu \)m 5-aza-dC migrated
more slowly than those cultured without 5-aza-dC (p<0.01).

Discussion

In ovarian cancer, it had been demonstrated that several TSGs such as BRCA1/2 (Chiang et al., 2006; Chan et al., 2002), PTEN (Schondorf et al., 2004), RASSF1 (Bol et al., 2010) and TES (Qiu et al., 2010) were hypermethylated in their promoter regions, leading to the expression levels downregulated and the anti-tumor functions impaired. In the last ten years, promoter hypermethylation of SLIT2 had been detected in colorectal cancer (72%) (Dallol et al., 2003a), lung cancer (36% in SCLC, 53% in NSCLC) (Dallol et al., 2002), breast cancer (43%-58%) (Dallol et al., 2002; Sharma et al., 2007), glioma (59%) (Dallol et al., 2003b), cervical cancer (52%) (Narayan et al., 2006), hepatocellular carcinoma (83.3%) (Jin et al., 2009), neuroblastoma (29%), Wilms' tumor (38%), renal cell carcinoma (25%) (Astuti et al., 2003). According to our findings, SLIT2 promoter was methylated in 84.8% ovarian cancer samples, compared with that just 1 of 30 showed hypermethylation in the control group. Promoter hypermethylation of SLIT2 was well correlated with a reduction of expression in ovarian cancer samples, indicating that hypermethylation is critical for expression of SLIT2 and may play an important role in the biological behaviors of ovarian cancer.

In our further experiments, we found that hypermethylation was also detected in ovarian cancer cell line-SKOV3, in which SLIT2 expressed at a very low level. By treating SKOV3 cells with the demethylating agent (5-aza-dC), SLIT2 expression was obviously restored (as much as ten-fold).

Regulation of cell proliferation and apoptosis had been suggested as the mainly mechanism of SLIT2-mediated suppression of tumor growth (Jin et al., 2009; Dallol et al., 2003a; Kim et al., 2008; Marlow et al., 2008). By MTT assay, a significant difference was observed between the groups with or without treatment of 5-aza-dC. SKOV3 cells treated by 5-aza-dC showed much less absorbance at 490nm, implying very poor growth properties (p<0.05). In addition, we found apparent induction of apoptosis in 5-aza-dC treated SKOV3 cells by flow cytometry analysis. After the restoration of SLIT2 expression, the total apoptotic rate of SKO3 cells raised up enormously(from 1.01% to 3.28%, p<0.05). We also performed the colony formation assay. 5-aza-dC treatment impaired the abilities of SKOV3 cells to grow into colonies as long as they did in the non-5-aza-dC group (p<0.05). SLIT2 expression inhibits cells migration of hepatocellular cell carcinoma (Jin et al., 2009) and lung cancer (Tseng et al., 2010). In this study, we performed wound-healing assay to investigate how SLIT2 impact on the migration of SKOV3 cells. According to our data, cells treated with 5-aza-dC migrated much slower than those without any drug. This result was also supported by previous studies in vascular smooth muscle cell and esophageal squamous cell carcinoma (Kim et al., 2008; Liu et al., 2006).

Up to now, there are only a few reports about the mechanisms of SLIT2. In one research, SLIT2 attenuated the migration of vascular smooth muscle cells by suppressing the activation of a small GTPase Rac1 (Liu et al., 2006); in another one, SLIT2 had been demonstrated to suppress tumor growth by coordinating regulation of the beta-catenin and phosphoinositide 3-kinase (PI3K)/AKT pathways in cell and animal models of breast cancer.

In the most recent study, SLIT2 was proved to inhibit glioma cell invasion in the brain by suppressing the cdc42 activity. In the future, we need further studies to delineate pathways that SLIT2 functions through and mechanisms of its anti-tumor activities in ovarian cancer.

In summary, we are the first to report highly frequent promoter hypermethylation of the SLIT2 gene in ovarian cancer. We restored the SLIT2 expression in SKOV3 cells using the demethylating agents, the proliferation, migration and colony formation ability of SKOV3 cells were significantly suppressed compared to the control group; besides that, more apoptosis was induced after the SLIT2 expression restoration. According to our results, we concluded that loss of SLIT2 expression plays an important role in tumorigenesis and progression of ovarian cancer. Therefore, reactivating the SLIT2 function by reversing epigenetic inactivation may represent a novel therapeutic opportunity to attenuate human ovarian cancer.

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References


