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Original Research

Sequencing of SMAD4 somatic variation in patients with serous ovarian cancer

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Summary

Objective: A previous study has indicated SMAD4 mutations identified in patients with serous ovarian cancer. The aim of study is to analyze the SMAD4 mutation in Chinese people with primary serous ovarian cancer and attempt to build the correlation between the genotype and clinical phenotype or parameters of clinical pathological; thus to explore the precise general theory for individual treatment of serous ovarian cancer. *Materials and Methods:* The authors collected 90 serous ovarian cancer cases with primary samples that were identified by pathologist. DNA was extracted from paraffin-embedded tumor tissues. The exon 2, 8, 9 and 11 of SMAD4 mutation hotspots were screened by Sanger sequencing. *Results:* The authors detected neither heterozygous mutations nor homozygous mutations in exon 2, 8, 9, and 11 of SMAD4 in 90 cases of serous ovarian cancer. However, they identified a single nucleotide polymorphism (SNP) (rs77389132) in the intron 2 regions and searched the ExAC website (http://exac.broadinstitute.org/) for the SNP at Chr18: 48573689 and allele is A/G. *Conclusions:* The mutational rate of exons 2, 8, 9, and 11 of *SMAD4* in serous ovarian cancer may be rare in Chinese people with primary serous ovarian cancer. Therefore, Seeking SMAD4 mutation for ovarian cancer susceptible population and individual treatment still need further pursuing.

Key words: Serous ovarian cancer; SMAD4; Somatic variation; Clinico-pathological parameters.

Introduction

Serous ovarian cancer is a subtype of epithelial ovarian cancer and is one of the most common and aggressive type, especially high-grade serous ovarian cancer (HGSOC). According to the degree of histological differentiation, serous ovarian cancer is divided into borderline serous ovarian cancer, low-grade serous ovarian cancer, and high-grade serous ovarian cancer, among which the latter is the most common type, approximately accounting for 75% of epithelial ovarian cancer [1]. Furthermore, HGSOC is characterized chiefly as a high degree of tumor heterogeneity and genomic instability, rapid development, and strong invasiveness. The spatial and temporal tumor heterogeneity have potential influences on platinum-based chemotherapy in ovarian cancer; more accurate study that assessed intratumour heterogeneity on HGSOC, reported that it may be a predictive indicator for survival after chemotherapy treatment [2]. In recent years, genetic sequencing has been used to detect somatic mutations and provide theoretical basis for tumor resistance and individualized therapy, which become a research hotspot in solid tumors [3, 4].

As a main tumor suppressor pathway TGF-β/SMADs formed by TGF-β1 ligand and its receptor, SMADs proteins, intracellular signaling molecular, and target gene of transcription regulation transfers information directly from the membrane to the nucleus, which is a vital signaling molecule in the TGF-β signaling pathway [5]. SMADs super family consist of eight proteins in mammals according to the DNA-binding domain, receptor-regulated SMADs (SMAD1, 2, 3, 5, 9), a common SMAD (SMAD4), and inhibitory SMADs (SMAD6, 7), respectively. SMAD4 protein, located in the central position of TGF-β/SMADs pathway, is a transcriptional coactivator and promotes that binding SMADs protein to homologous DNA and improves the adsorption force both of them [6]. SMAD4 is located in chromosome 18q21.2 [7], and contains 12 exons and encode 552 amino acid proteins, involving the tumorigenesis and metastasis [8, 9].

There is evidence that the majority of SMAD4 mutational hotspots are located in exons 8 and 11, and the mutations in MH2 domain were associated with poor prognosis in pancreatic cancer patients [10]. Tone *et al.* [11] identified SMAD4 mutation at the position of the exon 9 in 37 cases primary low-grade serous ovarian cancers. Therefore, the present study concentrating on exons 2, 4, 8, 9, and 11 of *SMAD4* whose exons are prone to deletion and mutation in malignant tumors [12]. The present authors aimed to screen SMAD4 mutations in 90 cases of serous ovarian cancer in order to build a correlation between the genotype and clinical phenotype or parameters of clinical pathologicy, and thus explore the general theory for individual targeted accurate treatment for serous ovarian cancer.

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Table 1. — *Participants' specific information.*

General characteristic	Property	Data
Median age (years)	—	52 (19~78)
Family history of malignant tumors	—	17(18.90%)
	Digestive tumors (gastric cancer, liver cancer and esophageal cancer)	8
	Lung cancer	3
	Ovarian cancer	2
	Breast cancer	2
	Both cervical and lung cancer	1
	Both ovarian and lung cancer	1
Marital status	Married	89 (98.9%)
Age of menarche (years)	13~16	59 (65.6%)
	<13	1 (1.11%)
Menopausal status	47 cases	Median age 49 yrs
Gravidity (times)	>4	13 (14.4%)
	2~4	60(66.7%)
Age of first gestation (years)	—	Median age 24
Clinicopathological features	—	—
Tumor site	Bilateral	53 (58.9%)
	Left ovary	19 (21.1%)
	Right ovary	18 (20%)
Pathology diagnosis	Borderline	6 (6.7%)
	HGSOC	76 (84.4%)
	LGSOC	8 (8.9%)
FIGO Stage	I	5 (5.6%)
	II	15 (16.7%)
	III	60 (66.7%)
	IV	10 (11.1%)
Differentiation	Well	12 (13.3%)
	Moderate	4 (4.4%)
	Poor	74 (82.2%)
Diameter of the tumor (cm)	≥ 8	45 (50%)
	< 8	45 (50%)
Ascites (ml)	≥ 500	37 (41.1%)
	< 500	53 (58.9%)
Lymph node metastasis	—	44 (48.9%)

Materials and Methods

A total of 90 patients, who were diagnosed with serous ovarian cancer according to the WHO (2014) ovarian tumor histological classification and confirmed by molecular pathology expert, and visited to the Department of Gynecology, Affiliated Hospital of Qingdao University between January 2012 and March 2017, were included. Clinical stages and medical records were clearly and completely assessed. The specific information including median age, family history of malignant tumors, menstrual obstetrical history, and clinico-pathological parameters are shown in Table 1. All recruited participants were Chinese Han women.

Paraffin-embedded tissue in 90 cases were taken as the study samples after obtaining written informed consent. Twenty-three patients were treated with platinum-based chemotherapy before surgery; the remaining were neither treated with any chemotherapy or hormone therapy. The research project was approved by the Ethics Committee of Affiliated Hospital of Qingdao University.

The obtained wax particles were successively dewaxed, xylene eluted, and enzymolyzed in order that the sample was fully digested and cracked. Subsequently, a DNA extraction kit from paraffin embedded tissue (version DP150804) was utilized. The concentration and purity of DNA were measured by a spectrophotometer. DNA was stored at -20°C for reserve. Primers were designed for exon 2, 8, 9, and 11 according to the human SMAD4 sequence published by PubMed, and primers were synthesized

PCR reaction system: using 20 μl reaction system containing 2 μl DNA template, 10 μl Mix, 7 μl double distilled water, both the sense and the antisense primers were 0. 5 μl. Cycle parameters: pre-denatured 94°C, 5 minutes, 94°C, 30 seconds, suitable annealing temperature, 30 seconds, 72°C, 30 seconds, 35 cycles, and then extended to 72 for 7 minutes. The four exons amplification products were successfully identified by 1.5% agarose gel electrophoresis, followed by subsequent sequence detection. PCR am-plification products of four exons of SMAD4 were sequenced by generation directly. The authors performed validation via reverse sequencing with specimens that were selected randomly and yet not found.

Results

No mutations were found in the four exons of SMAD4 studied in the present research. Analysis of the sequence

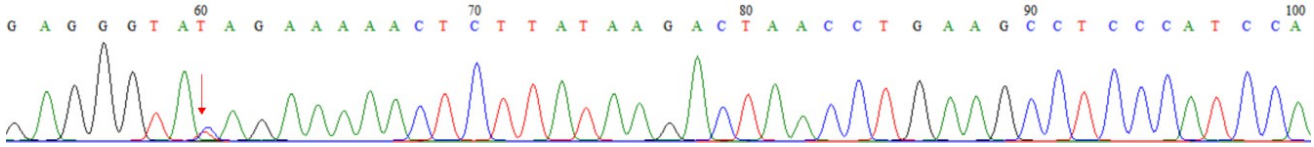

Figure 1. — One single nucleotide polymorphism (SNP) in *SMAD4* located at the intron domain of the exon 2 and exon 3 junctions, the variation ID and alleles is rs77389132 and A/G.

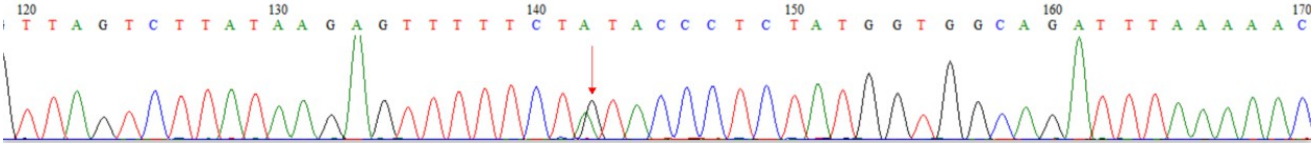

Figure 2. — This is the forward primer to verify the doublet.

diagram by BioEdit (version 7.1.3.0) software revealed no doublet, and no difference was found between the actual sequence and the target sequence by DNAMAN (version 6.0.3.99). That is, no heterozygous or homozygous mutations were found in this study. However, the authors observed one patient in which the samples showed single nucleotide polymorphism (SNP) in SMAD4 and then searched through NCBI and found that this SNP locus is at the intron domain of the exon 2 and 3 junctions, the variation ID and alleles is rs77389132 and A/G, respectively (Figure 1 and Figure 2). The authors also searched the ExAC website (http://exac. broadinstitute.org/) for the SNP at Chr18: 48573689 and A/G alleles. Interestingly, the total allele frequency is 0.006475, among them East Asian is 0.03977, European (Finnish) is 0.0006098, and European (Non-Finnish) is 0.0002733, suggesting this locus SNP is more inclined to occur for East Asian population.

A 50-year-old woman carrying the rs77389132 SNP and no family history of malignant tumors, menstrual, and obstetric history: menarche and menopause in 15 and 47 singly, regular periods, gestation 2 and production 2, the first pregnancy age at 21-years-old. Due to abdominal pain and abdominal distension, the woman visited the hospital in March 2016 and underwent transvaginal ultrasound, and a large solid mass was found in the right accessory region, with a mass sized 8.3×5.8×5.1 cm accompanied with mixed echo, and the resistance index (RI) value 0.58~0.80. Then the patient underwent surgical treatment, and it was observed that there were some foci adhesions with sigmoid colon densely and metastatic lesions on the omentum surface. The woman was diagnosed with high-grade serous ovarian cancer of which clinical FIGO Stage and histological grade was IIC and G2, respectively. Postoperative administration with paclitaxel combined platinum-based chemotherapy included six courses, and the patient is currently alive and well.

Discussion

SMAD4 is an antioncogene found in pancreatic cancer initially [13], and plays an important tumor suppressor in digestive tract adenocarcinoma. SMAD4 is related with the promotion of invasion and metastasis, while SMAD4 protein expression is correlated with the development of these tumors negatively. Specifically, it is illustrated that the deletion of SMAD4 not only plays an important initial action in the skin and upper gastrointestinal squamous cell carcinoma and gastrointestinal adenocarcinoma, but also accelerate or coordinate other carcinogenic pathways to promote cancer development in cholangiocarcinoma and pancreatic cancers [5, 8]. TGF-β receptor and SMADs protein related mutations may lead to the TGF-β signal transduction disorder, hence the function of antitumor becomes weakened [9]. In addition, the loss of SMAD4 expression involved in the occurrence and metastasis of breast and lung cancers [14, 15]. Mutations in SMAD4 may also be associated with tamoxifen treatment in breast cancer patients [16], and be potential time heterogeneity.

The germinal epithelium of the ovary, derived from the cavity of the mesoderm, is transformed into pelvic mesothelial and provided with characteristic of mesenchymal cells and epithelial cells [17]. This can modulate the ovarian self repair process following ovulation via epithelial mesenchymal transition (EMT). David *et al.* [18] believe that TGF-β induced EMT is usually the pathway that promotes tumorigenesis, which also provides a basis for ovulation tumorigenesis.

Although the lack of sensitivity of SMAD4 to TGF-β signaling can occur in human ovarian cancer cells, there is less research on this information in human ovarian cancer [19]. It is well-established that there are two mechanisms of tumor regulation and development: first, the genetic mutation of DNA sequence revealed in a genetic regulation and the second is that the DNA sequence does not change, but the gene expression alters. Using chromatin immunoprecipitation and massive parallel sequencing (ChIP-seq), Brian *et al.* [20] found that SMAD4-mediated TGF-β pathway shows a significant difference between abnormal and epithelial ovarian cancer cell lines. Interestingly, they also found that the TGF-β/SMAD4 target gene identified in epithelial ovarian cancer cell lines was associated with the survival time of the patient. Antony *et al.* [19] performed immunoprecipitation with anti-SMAD4 antibody and found that SMAD4 protein expression negative despite of

no specific changes in SMAD4 mutations or gene expression. Although loss or low expression of SMAD4 was found in 28% of serous carcinomas [21], SMAD4 may be involved in the development of serous ovarian cancer by collaboration with potential tumor suppressor genes.

While the loss of SMAD4 protein were found in ovarian cancer, it may be related to post-translational modification, especially ubiquitination [5]. Compared with other studies that the loss of SMAD4 protein expression in human ovarian cancer, it was associated with a weakened DNA binding capacity in the downstream pathway [19]. However, there is also research in which there was no significant difference between the SMAD4 expression level and the clinical stage analysis parameters, which may be related to the small differences in the study population [22]. Tone *et al.* [11] identified R361G mutation of SMAD4 in 37 cases of primary low-grade serous ovarian carcinoma. Regretfully, in the present research, the authors investigated 90 patients with serous ovarian cancer in the SMAD4 exons 2, 8, 9, and 11 and no genetic mutation was detected. Racial differences or histological type still require further research with larger samples or sequencing of the entire genome of SMAD4. Furthermore, deletion of the SMAD4 is common in adenocarcinomas of the colon, stomach, and pancreas, but is relatively rare in ovarian cancer, which provides a partial basis for undetected mutations in the present findings [23].

Genome wide sequencing and analysis showed that gene disruption in high-grade serous ovarian cancer usually inactivated tumor suppressor genes such as RB1, NF1, RAD51B, and PTEN and helped produce resistance to acquired chemotherapy [24]. In light of antecedent research, whether SMAD4 alterations are associated with chemoresistance in epithelial ovarian cancer, notably the genetic characteristics of serous ovarian cancer are urgently needed to be discovered.

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