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ORIGINAL ARTICLE

Comparative study of BRAF gene mutations in ovarian serous tumors by immunohistochemistry and DNA sequencing

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Summary

Purpose: *Somatic mutations in the BRAF gene are common in several types of cancer, especially in ovarian serous cancer (OSC). Normally, the BRAF protein is switched on and off in response to signals that control cell growth and development.*

Methods: *To investigate the correlation between the mutation of BRAF gene and the expression of BRAF protein in OSC, pyrosequencing was performed to detect the mutation of the 600th codon in BRAF gene (written as Val600Glu or V600E) in 23 cases of high-grade serous ovarian cancer (HGSC), 28 cases of low-grade serous ovarian cancer (LGSC) and 72 cases of serous borderline ovarian tumors (SBT). Meanwhile, immunohistochemistry which stained with the specific antibody VE1 were used to clarified the expression level of BRAF V600E mutant protein.*

Results: *Finally, we found that V600E mutation in LGSC and SBT of occurred in 2 of 23 (7.1%) and 21of 72 (29.2%), respectively. The V600E mutation was not detected in 23 cases of HGSC. One case of HGSC (1, 4.3%), 3 cases of LGSC (3 of 28, 10.7%) and 25 cases of SBT (25 of 72, 34.7%) were positive expression detected by immunohistochemistry. Compared with BRAF gene mutation, the sensitivity, specificity and consistency of BRAF V600E protein were 91.3%, 92% and 91.9%, respectively.*

Conclusion: *Our findings indicate that BRAF mutations in LGSC and SBT, which are closely related to tumor staging. The specific antibody VE1 could be used as a preliminary screening for the mutation of BRAF gene.*

Key words: *BRAF gene, mutation, ovarian serous cancer, borderline ovarian tumors*

Introduction

Ovarian cancer is one of the reproductive system tumors that continue to pose a serious threat to women's lives. Although its morbidity is lower than cervical cancer and uterine cancer, its mortality is the highest among all gynecologic tumors. Ovarian serous cancer (OSC) is the most common ovarian malignancy [1]. According to the difference in molecular biology and histomorphology, it can be divided into high-grade serous ovarian cancer (HGSC), low-grade serous ovarian cancer (LGSC) and borderline ovarian tumors (SBT) [2]. The nuclei of LGSC cells show low-frequency mitosis, while those of HGSC cells, show strong mitosis. LGSCs are believed to originate from pre-existing cystadenomas or serous borderline ovarian tumors (SBT) [3] that eventually develop to an invasive cancer. While most SBT do not have a typical invasive carcinoma phenotype, microinvasion is not uncommon. Some authors reported that up to 60% of LGSCs were associated with SBT [4].

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The molecular analysis of carcinogenic mutations and the development of targeted biotherapy constitute a milestone in the diagnosis and treatment of ovarian malignant tumors. The BRAF gene provides information for making a protein that helps transmit chemical signals from outside the cell to the cell's nucleus [5]. This protein is part of a signaling pathway known as the RAS/MAPK pathway [6], which controls several important cell functions. Specifically, the RAS/MAPK pathway regulates the growth and proliferation of cells, the process by which cells mature to carry out specific functions (differentiation), cell movement (migration), and the self-destruction of cells (apoptosis) [8]. The BRAF gene belongs to a class of genes known as oncogenes [9]. When mutated, oncogenes have the potential to cause normal cells to become cancerous [10], including melanoma, non-Hodgkin lymphoma, colorectal cancer, and thyroid cancer. According to some authors, BRAF may be also an important oncogene for the progression of SBT to invasive cancer [11].

The mutation of the 600th codon in BRAF gene (V600E) is its most common mutation found in human cancers [6]. This mutation has been frequently found in cancers of the colon and rectum, ovary, and thyroid [10]. Several other somatic mutations in the BRAF gene have also been associated with cancer [12]. In clinical practice, evaluation of BRAF mutation status has routinely been performed by pyrosequencing [13,14]. However, molecular testing is not available at many hospitals since it is time-consuming, expensive, and requires expertise in molecular technique [15]. The first BRAF V600E-specific antibody was reported in 2011 (clone VE1) [16]. Therefore, we reviewed the correlation between immunohistochemistry and molecular results and deliberate how BRAF immunohistochemistry might be utilized in the evaluation of OSC.

In summary, in our study, we analysed the BRAF V600E mutation in 123 cases of OST, and clarified the relationship between V600E mutation [17].

Methods

Patients and tissue samples

Samples from 123 patients diagnosed with OSC were obtained from Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine. For all cases, pathological and clinical follow-up data were collected after obtaining ethical approval from the China Ethical Review Committee. OSC was diagnosed based on endocrine evaluations, clinical symptoms, and imaging examinations.

DNA extraction from OSC paraffin tissue

Eight sections of 5 μm OST paraffin-embedded tissue were put into 1.5 ml Eppendorf tube.

Added were xylene 1.0 ml, and cells were incubated at 60°C for 10 min, centrifuged at room temperature at 12000 rpm/min for 3 min and discarded the supernatant.

Then 1.0 ml of absolute ethanol was added, mixed it upside down at room temperature for 10 s, centrifuged for 3 min at 12 000 rpm/min, discarded the supernatant, and these steps were repeated twice. Eppendorf tube was opened and the remaining anhydrous ethanol was dried at 55°C.

Subsequently 400 μl tissue digestion buffer was added and also 20 μL protease K (10mg/ml) to the treated tissues with vortex mixing. Digestion at 56°C for 90 min or overnight followed until the sample was completely digested. Incubation at 90°C for 30 min was performed and collection liquid on the tube wall was carried out by rapid centrifugation (12,000 rpm/min) transferring the supernatant to the matching Eppendorf tube. Centrifugation for 5 min followed and the clarified tissue mixture after centrifugation was placed into the matching sample tube. The above samples were placed in the fourth channel of T-frame and the program code 401 was carried out. After extraction, DNA was stored in the refrigerator at -20°C for reserve.

Pyrosequencing (Pyrosequencing of biotin-labeled PCR amplicons)

Direct sequencing based on primer extension is the gold standard for mutation detection, and the entire biotinylated PCR mixture was sequenced using the PyroMark ID and PyroMark Q96 reagents (GE company, Cat. No. 122-611) with the corresponding target-specific parameters (Table 1). Using PyroMark Identifire v1.05.0 software, the identity of the sequences was generated within seconds after comparison with our extensive reference library, which includes validated sequences from previously identified clinical bacterial isolates, ATCC reference strains, and those found in NCBI GenBank using the BLAST algorithm. One hundred percent identity was required to make a genus or species level identification. The data were analyzed by PSQ-96MA/Pyromark ID pyrophosphate sequencer. First, SNP analysis was carried out. For sites with heterozygotes, further frequency-based (AQ) analysis was needed.

Immunohistochemistry (IHC)

Immunohistochemical method using antiBRAF V600E (VE1) antibody was developed at Ventana Medical Systems, Inc. Sections (4μm) were cut from the paraffin blocks and the testing was performed on a Benchmark XT IHC staining instrument with Cell Conditioning 1 for 64 min, pre-peroxidase inhibition and primary antibody incubation for 32 min. The final concentration of the Anti-BRAF V600E (VE1) Mouse Monoclonal Primary Antibody was 12μg/ml. BRAF detection was performed by using OptiView HRP Multimer for 12 min at 37°C. OptiView DAB IHC Detection Kit was used to detect BRAF V600E protein expression for 8 min. To counterstain tissues the slides were incubated with Hematoxylin II and Bluing Reagent for 4 min. To measure the level of non-specific

background signal, each tissue was also stained with a mouse monoclonal antibody (MOPC-211) [Negative Control (Monoclonal), Ventana Medical Systems, Inc.].

Each immunostained slide was examined and scored independently by two pathologists (Qizhi He and Liu Fangfang) without prior knowledge of any clinicopathological or molecular data. All sections for which the two observers disagreed were reevaluated with a third pathologist, and after discussion, final agreement was achieved. For B-raf, cytoplasmic immunostaining in tumour cells was considered to be positive. A 4-tiered semiquantitative system used in the previous study by He et al [1] was followed: Staining intensity was categorized into 4 groups by comparing the staining intensity of tumour cells. The staining intensity was scored as 0 (no staining), 1 (faint yellow), 2 (brown-yellow), or 3 (dark yellow). The extent of staining was scored as 0 (less than 5% positive cells), 1 (5-25% positive cells), 2 (26-75% positive cells), or 3 (more than 76% positive cells). The final score of 0-6 was obtained by summing the above 2 scores. The expression categories of nestin were defined as follows: - (0), + (1-2), ++ (3-4), and +++ (5-6).

Statistics

We used SPSS 19.0 software to analyse the data obtained in this study. Statistical comparisons were performed using GraphPad Prism, version 5.0. The results were expressed as mean±standard deviation (SD) and were compared with Student's t-test and x^2 test for categorical data. Paired *Kappa* analysis was used to detect the consistency between IHC and pyrophosphate sequencing results (Table 1).

Results

DNA sequencing

Of the 123 ovarian serous tumors included in the study, in initial DNA sequencing, SBT occurred in 2 of 28 (7.1%) and 21 of 72 (29.2%), respectively. The V600E mutation was not detected in 23 cases of HGSC. Based on pyrosequencing, the mutation of codon 600 of BRAF gene was detected and the resulting glutamic acid was replaced by valine (V600E) (Figure 1).

Table 1. The specific parameters

Entries	*Sequence to analysis*	*Nucleotide dispensation order*
BRAF-15	TACAGT / AGAAATCTCGAT	GTACAGTACGATC

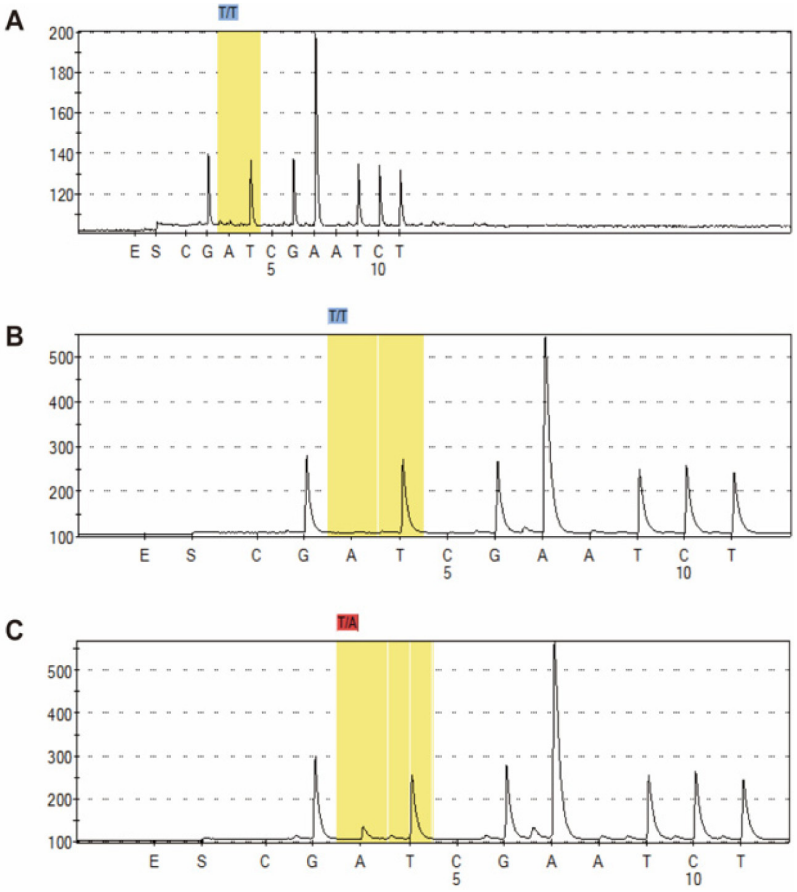

Figure 1. DNA Sequencing. **A:** standard wild type diagram. **B:** testing diagram. **C:** Testing diagram.

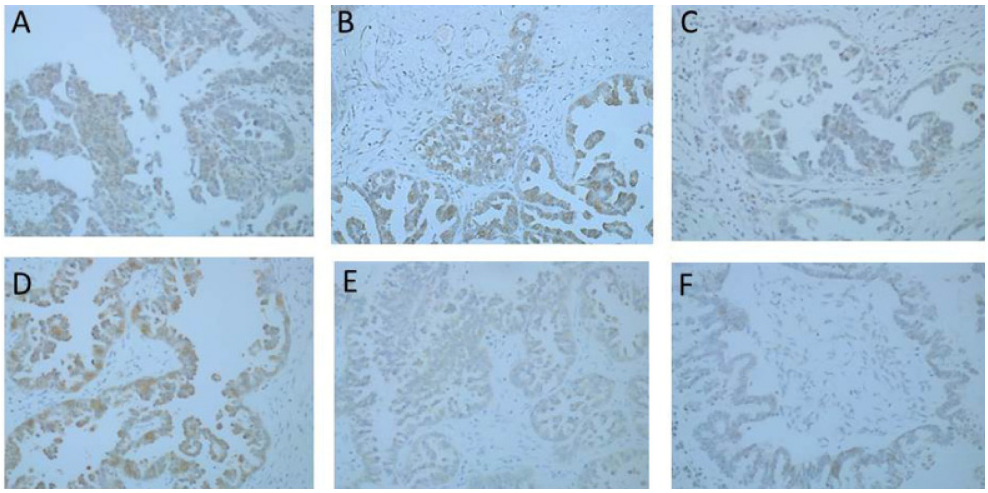

Figure 2. Images of serous ovarian tumors tissues stained with anti-BRAF V600E (VE1) mouse monoclonal antibody (×200). **A:** high-grade SOC-wild-type BRAF with weak, nonspecific staining. **B:** BRAF V600E-mutated low-grade SOC with strong cytoplasmic staining. **C:** BRAF V600E-wild type low-grade SOC with weak cytoplasmic staining. **D:** BRAF V600E-mutated borderline tumor with strong cytoplasmic staining. **E:** BRAF V600E-wild type borderline tumor with moderate cytoplasmic staining. **F:** BRAF V600E-wild type borderline tumor with moderate cytoplasmic staining.

Table 2. Estimation of *Kappa* value and strength of agreement.

Kappa value	*Strength of agreement*
< 0.20	Poor
0.21 - 0.40	Fair
0.41 - 0.60	Moderate
0.61 - 0.80	Good
0.81 - 1.00	Very good

Table 3. Contingency table for determination of BRAF mutation status using immunostaining

BRAF Mutation status		
Mutated		*Total*
BRAF immunohistochemistry status		
Wt	*Mutant*	*Total*
94	0	94
6	23	29
100	23	120

Sensitivity= 91.3%; Specificity= 92%; Consistency= 91.9%; Kappa= 0.854

VE1 immunohistochemistry

BRAF protein expression was located in the cytoplasm, with finely granular cytoplasmic staining (Figure 2). Any nuclear staining was ignored and not scored. Of 23 patients with high-grade SOC, 22 showed complete absence of staining with the VE1 antibody, whereas in 1 case, very weak, diffuse background staining of both nuclei and cytoplasm (Figure 2A) was observed, interpreted as nonspecific staining. In contrast, 3 (10.7%) of 28 patients with low-grade SOC was positive. One showed strong cytoplasmic positivity (Figure 2B), two cases were weak (Figure 2C), whereas the remaining 25 cases remained negative. Twenty-five (34.7%) of 72 patients with SBT showed positive immunostaining, including 9 cases with strong (Figure 2D) and 12 cases with moderate immunoreactivity (Figure 2E), 4 cases were weak (Figure 2F). Of the remaining 47 negative cases, 29 showed a very weak background staining, whereas 18 were completely negative.

The sensitivity of immunostaining in detecting a BRAF mutation was calculated at 91.3%, whereas the specificity of this method was calculated at 92%, and consistency was 91.9%. The *Kappa* was calculated at 0.854, representing a very good strength of agreement (Tables 2 and 3).

Discussion

The research on signal transduction has become the frontier and hotspot of life science research in the 21st century. In 1980, Martin Rodbell first used "signal transduction" to describe the role of GTP and G protein in biochemical regulation. This review was published in Nature, and the term signal transduction was widely used in biology [18].

Cell signal transduction usually means that cells receive external signals through cell surface receptors (or endonuclear receptors) [19] and transfer extracellular signals into intracellular signals through cascade transmission mechanism, which

eventually induces physiological response or specific gene expression, and induces cellular response [20]. Ras-MAPK pathway is the joint action hub of many receptor-mediated signal transduction pathways [21]. Changes in the signal components of RAS itself or its upstream and downstream, which is closely related to human tumors. Mitogen-activated protein kinases (MAPKs) [22], which are highly conserved in human cells, are important transmitters of signals from the cell surface to the inside of the nucleus. They are mainly involved in the process of embryonic development, cell proliferation, cell differentiation and cell death. At present, at least 14 MAPKKs, 7 MAPKs and 13 MAPKs have been found in mammalian cells, which constitute a complex system. Classical MAPK cascade reactions consist of three sequential steps: activation of MAPK kinase (MAPKK), which belongs to serine/threonine kinase and can phosphorylate and activate MAPKK; subsequently, MAPKK activates MAPK by double phosphorylation of adjacent threonine and tyrosine [23].

Raf family belongs to MAPKK and is a highly conserved serine-threonine kinase, which is activated by interaction with Ras protein. Raf family members include A-Raf, B-Raf and Raf-1 [24]. Raf-1 is ubiquitous. A-Raf often plays a role in the urogenital tract, B-Raf is most common in nerve tissue and testis.

BRAF oncogene is a well-known proto-oncogene. Oncogenes [25], originally defined as cancerous genes, are actually normal genes that regulate cell growth and differentiation [26]. They encode key regulatory proteins and are the normal components of the cell genome. It includes viral oncogenes and cellular oncogenes. Proto-oncogenes exist in normal cells and are activated into oncogenes by various zfactors. The quality or quantity of the oncoprotein encoded by it changes, which causes the metabolic abnormality of its target cells and promotes the gradual transformation of cells into cancer cells. The activation of proto-oncogene is mainly through the following ways: point mutation, DNA rearrangement, insertion of promoter or enhancer, etc. Point mutation is the main way. About 95% of BRAF gene mutations are T1799A point mutations, which lead to abnormal activation of the protein encoded by BRAF [27]. It can activate downstream signal transduction and promote cell proliferation and differentiation. BRAFV600E mutation is reported to be a common molecular genetic change in ovarian serous carcinoma.

Protein is the final product of structural gene expression. It is protein that really plays a biological role in human body [28]. Therefore, studying the changes of protein quality and quantity can also reflect the expression activity of genes. At present, Western blot or IHC staining are mostly used to analyze proteins. The basic principle of IHC technique is to use the characteristic of specific binding of antigen and antibody to inject some substance in tissues or cells into another animal body as antigen or hapten to produce specific antibodies corresponding to the antigen. When a marker is added to the serum, it becomes a labeled antibody [29]. The labeled antibody was incubated with tissue sections, and the antibody was specifically bound to the corresponding antigen in the cells. The binding site was displayed by the labeled antibody. The distribution and content of the substance could be observed by microscopy. This method has become one of the intuitive and specific methods for gene analysis at the protein level. In this study, BRAF (VE1) Mouse anti-Human Monoclonal antibody was first used. The BRAF protein was detected by IHC Envision two-step method, and the mutation of BRAF V600E was analyzed at the protein level.

Nowadays, a number of gene sequencing techniques including pyrosequencing have been used to detect the BRAF mutation [30]. Although the first generation gene sequencing reading length was up to 1000 base pairs, and its accuracy was reaching 99.9%, it also had its own shortcomings, such as high cost and low throughput, which seriously affects its real large-scale commercial application in hospitals [31]. Compared with pyrophosphate sequencing, immunohistochemistry is widely used in hospitals, and the appliance of VE1 antibodies allows to quickly screen for BRAF mutations in OSC patients with the consistent detection rate. In summary, we suggest that immunohistochemistry with VE1 antibody is equivalent to gene sequencing technology in screening BRAF V600E mutation of OSC patients.

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Conflict of interests

The authors declare no conflict of interests.

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