Clinical significance of 9P21 gene combined with BAP1 and MTAP protein expression in diagnosis and prognosis of mesothelioma serous effusion

GUAN-YING MA[1], SHUAI SHI[2], PING WANG[2], XING-GUANG WANG[2] and ZHI-GANG ZHANG[2]

[1]Department of Clinical Pathology, Chengde Medical University, Chengde, Hebei 067000;
[2]Department of Pathology, Cangzhou People's Hospital, Cangzhou, Hebei 061000, P.R. China

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Abstract. The diagnostic value of the 9P21 gene determined using fluorescence *in situ* hybridization (FISH) combined with BRCA1-associated protein 1 (BAP1) and methylthioadenosine phosphorylase (MTAP) expression detection by immunohistochemistry, was investigated in serous effusion samples of malignant mesothelioma. A total of 70 serous disease samples with serous effusion were collected from June 2017 to June 2020. Following biopsy specimen pathological diagnosis, samples were divided into malignant mesothelioma and benign mesothelioma. Differential expression of BAP1 and MTAP genes were identified in mesothelioma and mesenchymal hyperplasia. The 9P21 gene fragment was lost in mesothelioma. The positive rates of FISH, BAP1 and MTAP in biopsy specimens were 98.00, 94.00 and 90.00%. The specificity of the three were 96.00, 85.71 and 77.27%, the sensitivity were 90.00, 95.92 and 93.75%, and the positive rate of the combined detection of the three was 93.33%. The positive rate of serous fluid samples detected by the three methods (9P21 FISH probe combined with BAP1 and MTAP expression detected immunohistochemically) was 96.00, 92.00 and 88.00%, the specificity were 90.00, 77.27 and 71.43%, the sensitivity was 96.00, 93.75 and 89.80%, and the positive rate of the three combined detections was 91.33%. It was demonstrated that there was a high consistency between serous fluid samples and biopsy samples. According to clinicopathological analysis, sex, age, lesion site, Ki67 had little association with the occurrence and development of malignant mesothelioma, while asbestos exposure history was closely associated to the occurrence of mesothelioma. A high level of BAP1 gene was positively associated with the prognosis of mesothelioma, while a high level of MTAP gene was negatively associated with the prognosis of mesothelioma ($P<0.05$). Therefore, 9P21 FISH probe combined with BAP1 and MTAP can be used as a new method for the detection of malignant mesothelioma, and provide an important basis for the early diagnosis of mesothelioma.

Correspondence to: Dr Zhi-Gang Zhang, Department of Pathology, Cangzhou People's Hospital, 7 Qingchi Avenue, Cangzhou, Hebei 061000, P.R. China
E-mail: Zhzhg001518@126.com

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Introduction

Malignant mesothelioma (MM) is a highly aggressive tumor originating from serous mesothelioma cells (1), which is characterized by cryptic onset, difficult diagnosis, advanced stage upon discovery and short survival period. In recent years, the number of individuals exposed to asbestos chemical materials has increased, and the prevalence of malignant mesothelioma is increasing year by year. According to previous studies, the median survival of patients with malignant mesothelioma after diagnosis is only 12-15 months (2,3). Although the survival of patients can be prolonged by existing chemotherapy drugs, the 5-year survival rate of patients with intermediate and advanced mesothelioma remains <15% (4). Early screening and accurate diagnosis are important means to prolong the survival of patients with mesothelioma. Poorly differentiated epithelial mesothelioma is often difficult to distinguish from cancer. Currently, using biopsy specimens (obtained by puncture and endoscopic methods) to diagnose mesothelioma is relatively common, but it is extremely difficult to diagnose mesothelioma in specimens of serous effusion, especially mesothelial cells in the lungs. It is difficult to differentiate between adenocarcinoma cells and ovarian serous cancer cells, and invasive testing is occasionally required. Therefore, in the present study, the use of small traumatic serous fluid samples from patients with mesothelioma was considered to improve the cytological diagnosis accuracy of malignant mesothelioma through FISH probe detection combined with immunohistochemical staining.

Early studies have shown that numerous biochemical molecular markers are involved in the occurrence and development of tumors and can be used for early screening of tumors (5). Therefore, it is necessary to further explore new and highly specific diagnostic markers in the occurrence and development of malignant mesothelioma. According to the latest diagnosis and treatment and clinical practice guidelines for malignant

mesothelioma in 2022 (6), the deletion of BRCA1-associated protein 1 (BAP1) and multiple tumor suppressor 1 (MTS1), more often called CDKN2A gene, is regarded as the gold standard for molecular pathological diagnosis of malignant mesothelioma, but only biopsy specimens are mentioned for detection. At present, there are few cytological methods for the detection of specimens using serous cavity effusion.

The fragment of chromosome 9P21 is the 21.3 region of the short arm of chromosome 9, and tumor suppressor genes in this region mainly include CDKN2A and MTAP genotypes (7). CDKN2A is a cycle-dependent protease inhibitor, which is closely associated to negative regulation of the cell cycle (8). Previous studies have verified the effectiveness of CDKN2A gene deletion, and found that CDKN2A gene expression is variable and silenced in the tumor epigenetic mechanism of basal cell carcinoma, breast, non-small cell lung and colorectal cancer as well as other tumors (9,10). In the present study, CDKN2A gene FISH probe was used to detect serous cavity effusion in biopsy specimens. A previous study revealed that homozygous deletion (HD) occurs in 60% of malignant mesothelioma (11). Methylthioadenosine phosphorylase (MTAP), belonging to the PNP/MTAP phosphorylase family, is a gene with P16 telomeres of ~100 kb (12,13), and its antibody localization is mainly in the cytoplasm. This encoding enzyme plays a major role in saving adenine and methylene during polyamine metabolism. Previous research has shown that the expression of MTAP is closely related to the malignant transformation of cells, and there are varying degrees of deletion in various types of tumors (14). A previous study has shown that MTAP and CDKN2A are both deleted in malignant mesothelioma, and ddPCR detection of both genes can distinguish mesothelioma from benign mesothelioma (15). Cigognetti *et al* (16) determined that in pancreatic cancer, the combined deletion of MTAP and CDKN2A protein expression is considered as a substitute marker for CDKN2A homologous deletion. BAP1 is a histone deubiquitination enzyme encoded by genes. BAP1 is an important tumor suppressor gene encoded in the 21.1 region of the short arm of chromosome 3 (17) and can significantly increase the stability of the KLF5 protein. Righi *et al* (18) established that the most commonly mutated genes in malignant mesothelioma genome research results are BAP1, NF2 and CDKN2A/B. BAP1 deletion promotes cell proliferation by upregulating enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2). EZH2, a histone lysine-methyltransferase, is overexpressed in numerous cancers. A previous study has shown that BAP1 accelerates the G(1)-S checkpoint process by influencing the cell cycle and induces cell death through processes characterized by apoptosis and necrosis (19).

In the present study, FISH was used to detect serous fluid samples with evident advantages for the diagnosis of mesothelioma. In addition, MTAP and BAP1 protein expression combined with FISH 9P21 gene expression were used to distinguish malignant mesothelioma from reactive mesothelioma, laying the foundation for a diagnostic method with low trauma and high sensitivity for malignant mesothelioma.

Materials and methods

Clinicopathological data. The pathological specimens of all enrolled patients were collected, and questionnaires were issued to obtain information concerning the general situation, nature of work, educational level, and personal hygiene habits of the patients. The present study was approved (ethical approval document no. AF/SC-08/02.0.) by the Ethics Committee of Cangzhou People's Hospital (Cangzhou, China). Following screening, 70 patients with thoracic and peritoneal diseases treated at Cangzhou People's Hospital from June 2017 to June 2020 were selected. The pathological diagnosis met the diagnostic criteria of clinical guidelines for diagnosis and treatment of malignant mesothelioma. The inclusion criteria were as follows: i) Age range, 18-80 years old; ii) pathological type, patients with a clear pathological diagnosis of thoracic and peritoneal mesothelioma (epithelial); iii) informed consent obtained from patients and their families as well as follow-up with patients; and iv) following detection of D2-40, WT1 as well as other markers, the specimen was confirmed as mesothelioma in the official pathological report. The exclusion criteria were as follows: i) Patients with incomplete pathological data and lost to follow-up; ii) patients without hereditary diseases in their family history medical records; and iii) patients with contaminated specimens caused by improper specimen handling (insufficient specimen fixation, antibody cross-binding, etc.) in the experiment.

Reagents and experimental equipment. BAP1, a mouse monoclonal antibody (cat. no. sc-28383) directed against amino acids was obtained from Santa Cruz Biotechnology, Inc. The dilution performed for immunohistochemistry (paraffin-embedded sections) was 1:200. MTAP, a rabbit monoclonal antibody (product code ab126770; dilution, 1:200) was obtained from Abcam. CDKN2A(P16) gene deletion FISH probe reagent (*in situ* hybridization) (cat. no. FP-032) was obtained from Wuhan HealthCare Biotechnology Co., Ltd. Red fluorescein was used to label the P16 probe, and green fluorescein was used to label the CEP9 probe. P16 and CEP9 probes can be combined to the target detection site through *in situ* hybridization.

Research methods. The expression of BAP1 and MTAP proteins in serous effusion were detected by FISH probe of 9P21 gene and immunohistochemistry. The enrolled patients were retrospectively analyzed by diagnostic test evaluation method. The patients meeting the inclusion and exclusion criteria were assessed by diagnostic testing, and the enrolled patients were assessed by FISH *in situ* hybridization. The test results were recorded. The cases that did not meet inclusion criteria were removed from the study, and the cases were re-numbered randomly by double-blind method. A new round of diagnostic tests were conducted, in which four pathologists with senior professional titles diagnosed the pathophysiology of each case, and then all the cases were assessed by immunohistochemical staining of BAP1 and MTAP, and the sensitivity and specificity of the new combined diagnosis method were recorded to evaluate the efficacy of the diagnostic tests. The improved paraffin sectioning method of pleural effusion was as follows: No less than 60 ml of serous cavity fluid with natural settlement over 0.5 h was used. The serous cavity fluid samples were placed in 6 centrifuge tubes, and centrifuged 3 times (depending on the size of the centrifuged precipitated cells) at room temperature (15-25˚C). The first round of centrifugation was performed at 694 x g for 5 min, followed by smearing and

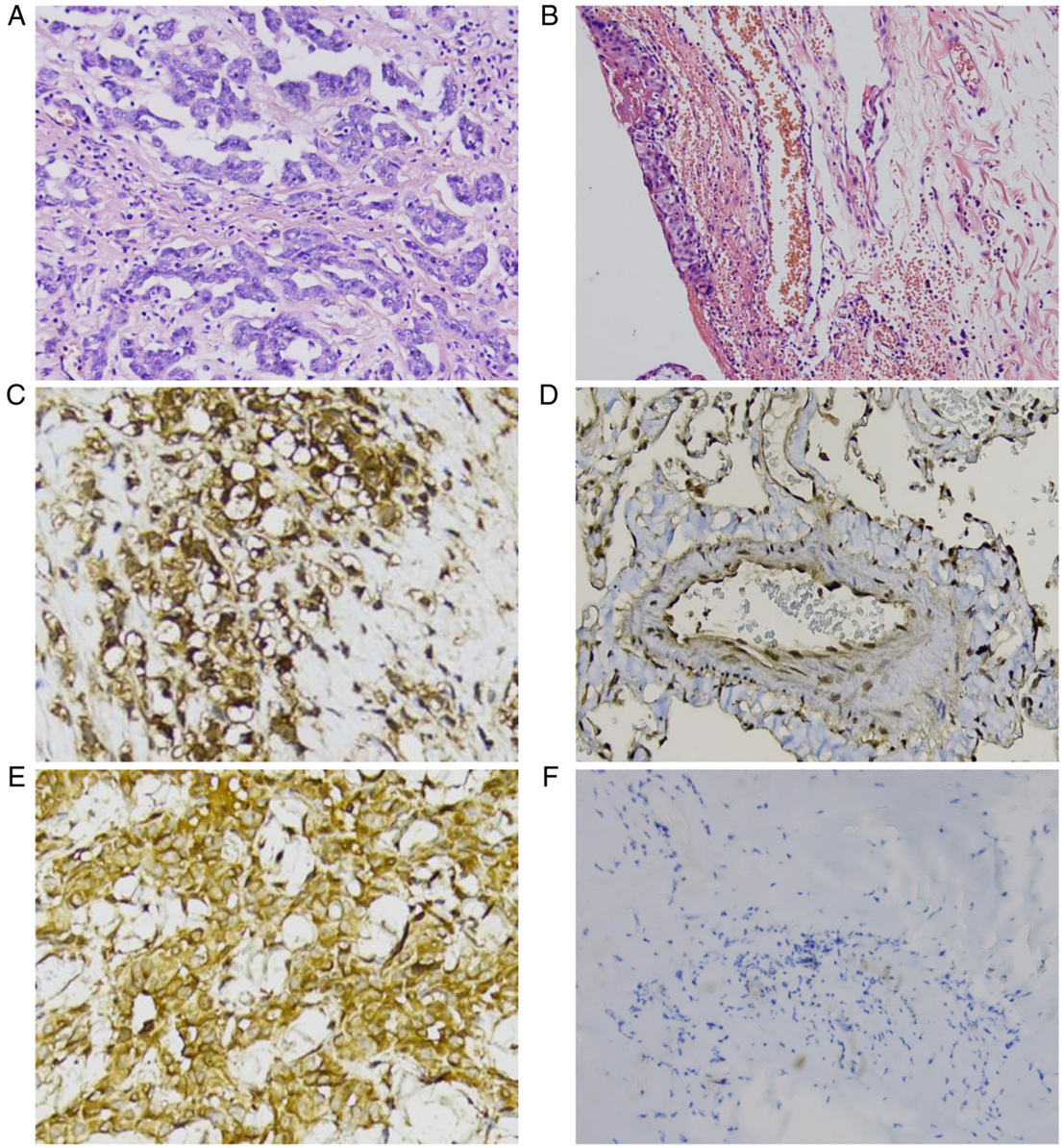

Figure 1. H&E staining results from (A) biopsy of malignant mesothelioma and from (B) biopsy of reactive mesothelioma (x200). (C) BAP1 expression in a mesothelioma biopsy specimen and (D) BAP1 expression in a reactive mesothelioma biopsy specimen (x200). The results of (E) the high expression of MTAP in mesothelioma biopsy specimens and (F) low expression in reactive mesothelioma tissues (x200).

the supernatant was discarded. At the second centrifugation, several drops of protein, glycerin and 90% ethanol were added to resuscitate the precipitate. The precipitate was centrifuged again at 694 x g for 5 min. The third centrifugation was the same as the second centrifugation. Following centrifugation, the supernatant was discarded, cell masses were removed, fixed, dehydrated, embedded and sectioned. A FISH probe was used to detect 9P21 gene expression, and the processed paraffin sections were placed in the hybridization apparatus for denaturation, washing and drying. 4'6-Diamidine-2-phenylindole (DAPI) was added, observed under a fluorescence microscope, and the results were recorded. FISH fluorescent sections were stored at -20˚C, and protected from light. Paraffin sections of mesothelioma tissues and benign mesothelioma tissues were dewaxed, hydrated with alcohol of different concentrations, then incubated with 3% H_2O_2 at room temperature (15-25˚C) for 10 min, immersed and rinsed with PBS solution, and then incubated with antibody reagent drops at 37˚C for 60 min. Reagents on tissues were rinsed 3 times with PBS solution. DAB chromogenic solution was added for incubation, and hematoxylin was finally applied. For immunohistochemistry, tissue sections with a thickness of 4 μm were baked in a 70˚C toaster for 2 h, and then removed and cooled to room temperature. The paraffin sections were then placed into two xylene sample bottles successively and each bottle was soaked for 30 min. The samples were then placed into two absolute ethanol sample bottles successively, and each bottle was soaked for 10 min. The sections were then placed in sample bottles containing 95, 85 and 75% ethanol successively, and each bottle was soaked for 5 min. The sections were then rinsed with tap water for 2 min. Citric acid buffer was added to the pressure cooker for antigen repair, the slices were added and soaked, heated for 90-180 sec, removed from the pressure cooker, and washed 3 times for 5 min each time with PBS

Table I. Biopsy specimen pathological morphology combined with immunohistochemical staining of BAP1 and MTAP, and FISH diagnosis results.

Detection method	Malignant mesothelioma		Benign mesenchymal tissue		Positive rate (%)	Specificity (%)	Sensitivity (%)
	Negative	Positive	Negative	Positive			
CDKN2A	2	48	18	2	98.00	96.00	90.00
BAP1	47	3	2	18	94.00	85.71	95.92
MTAP	5	45	17	3	90.00	77.27	93.75

The positive rate was calculated as follows: Positive rate (%) = number of malignant mesothelioma cases/total number of cases. BAP1, BRCA1-associated protein 1; MTAP, methylthioadenosine phosphorylase; FISH, fluorescence *in situ* hybridization.

buffer. Subsequently, 50 μl endogenous peroxidase blocker was added to the sections, followed by incubation at room temperature for 30 min, and washing 3 times for 5 min each time with PBS. A total of drops of goat serum blocking solution were added to each section and then incubated in an incubator at 37˚C for 30 min. After removal of the blocking solution at room temperature, BAP1 and MTAP antibody reagents (cat. no. sc-28383 and product code ab126770, respectively; as aforementioned) were added (50 drops of antibody reagents per section). The slices were then placed in a refrigerator at 4˚C overnight (12-18 h), removed from the wet box, returned to room temperature, and washed three times with PBS buffer for 5 min each time. After removal of the PBS buffer, 50 drops of enzyme-labeled goat anti-mouse/rabbit HRP IgG polymer (product codes ab97040 and ab7090, respectively; Abcam) were added to each section and incubated for 30 min in a 37˚C incubator. Subsequently, the sections were washed 3 times with PBS buffer for 5 min each time. A total of 50 drops of newly prepared DAB color developer was added to each microliter slice. and color was developed at room temperature for 3-7 min. Following color development and rinsing with tap water, hematoxylin was used for staining at room temperature for 5-8 min, and then the sections were washed again with tap water for 2 min. After differentiation with hydrochloric acid and alcohol, the sections were placed in flowing tap water for 30 min. Subsequently, the sections were dehydrated in 75, 85 and 95% gradient alcohol sample bottles for 3 min, and dehydrated in anhydrous alcohol sample bottles, 2 times for 5 min each time. The sections were then rendered transparent using newly configured xylene for 30 min, and finally sealed with neutral gum.

Interpretation criteria. 9P21 gene FISH probe detection results showed that the CDKN2A locus signal was red, and the CEP9 locus signal was green; therefore, 2 red:2 green were considered negative cells, 1 red:2 green or 2 green were considered positive cells. When the positive cells were >15% it indicated that CDKN2A expression was missing, and malignant mesothelioma in the experimental results could be diagnosed. BAP1 antibody is positive in the nucleus, and the positive expression is brown under the microscope. The expression of BAP1 in malignant mesothelioma was mostly negative. The positive control for the MTAP antibody is lung adenocarcinoma, localized in the cytoplasm. Positive staining is brown, and malignant mesothelioma is mostly positive. The results were evaluated according to the depth of staining. Any discrepancies were verified by both observers until a consensus was reached. The expression positivity was graded and counted as follows: 0, negative; 1, 1-50%; 2, 51-74%; 3, ≥75%. The staining intensity score was graded as follows: 1, weak; 2, intermediate; 3, strong. The scores for BAP1 and MTAP expression positivity and staining intensity were multiplied to obtain a final score categorized as: (-), 0; (+), 1-2; (++), 3-5; and (+++), 6-9. A score of 0-3 was considered negative and 4-9 as positive.

Statistical analysis. Statistical software SPSS 21.0 (IBM Corp.) was used for statistical analysis of data. Association analysis was used to detect the association between BAP1 and MTAP protein expression levels and clinicopathological features of mesothelioma. The χ^2 test was used to assess the association between BAP1 and MTAP protein expression and clinicopathological characteristics of mesothelioma. $P<0.05$ was considered to indicate a statistically significant difference. Kaplan-Meier plotter with log-rank testing was used to analyze the association between the expression levels of MTAP and BAP1 in mesothelioma tissues and the overall survival (OS) of patients.

Results

Protein expression and diagnostic results. The experimental results demonstrated that the CDKN2A gene on the 9P21 gene fragment was lost in malignant mesothelioma, BAP1 protein expression level in malignant mesothelioma was lower than that in normal serous tissue, and MTAP gene protein expression level in malignant mesothelioma was higher than that in normal serous tissue. In biopsy specimens, the positive rates of CDKN2A FISH, BAP1 and MTAP were 98.00, 94.00 and 90.00%, respectively (Fig. 1A-F and Table I), with specificity of 96.00, 85.71 and 77.27%, and sensitivity of 90.00, 95.92 and 93.75%. The positive rate of the combined test of biopsy specimens was 93.33% (Table I). The positive rates of CDKN2A FISH, BAP1 and MTAP in serous fluid samples were 96.00, 90.00 and 88.00% respectively (Fig. 2A and B and Table II), with specificity of 90.00, 77.27 and 71.43% (Fig. 2C-E and Table II). The sensitivity was 96.00, 93.75 and

Table II. Results from the diagnosis of serous effusion samples detected by FISH and immunohistochemical staining of BAP1 and MTAP.

Detection method	Malignant mesothelioma		Benign mesenchymal tissue		Positive rate (%)	Specificity (%)	Sensitivity (%)
	Negative	Positive	Negative	Positive			
CDKN2A	2	48	18	2	96.00	90.00	96.00
BAP1	45	5	3	17	90.00	77.27	93.75
MTAP	6	44	15	5	88.00	71.43	89.80

The positive rate was calculated as follows: Positive rate (%) = number of malignant mesothelioma cases/total number of cases. BAP1, BRCA1-associated protein 1; MTAP, methylthioadenosine phosphorylase; FISH, fluorescence *in situ* hybridization.

Table III. Results of CDKN2A (FISH) fluorescence signals.

Specimen type	Signal position point	Malignant mesothelioma	Benign mesenchymal tissue	Total
Biopsy specimens	1 red:2 green (+)	31	2	33
	2 green (+)	17	0	17
	2 red:2 green (-)	2	18	20
Samples of serous cavity effusion	1 red:2 green (+)	26	1	27
	2 green (+)	22	1	23
	2 red:2 green (-)	2	18	20

1 Red represents 1 red signal as a dot, the red signal as a dot is the CDKN2A gene; 2 green represents 2 green signals as a dot, and the green is the CEP9 gene. FISH results were interpreted according to the ratio of red signal sites to green signal sites. FISH, fluorescence *in situ* hybridization.

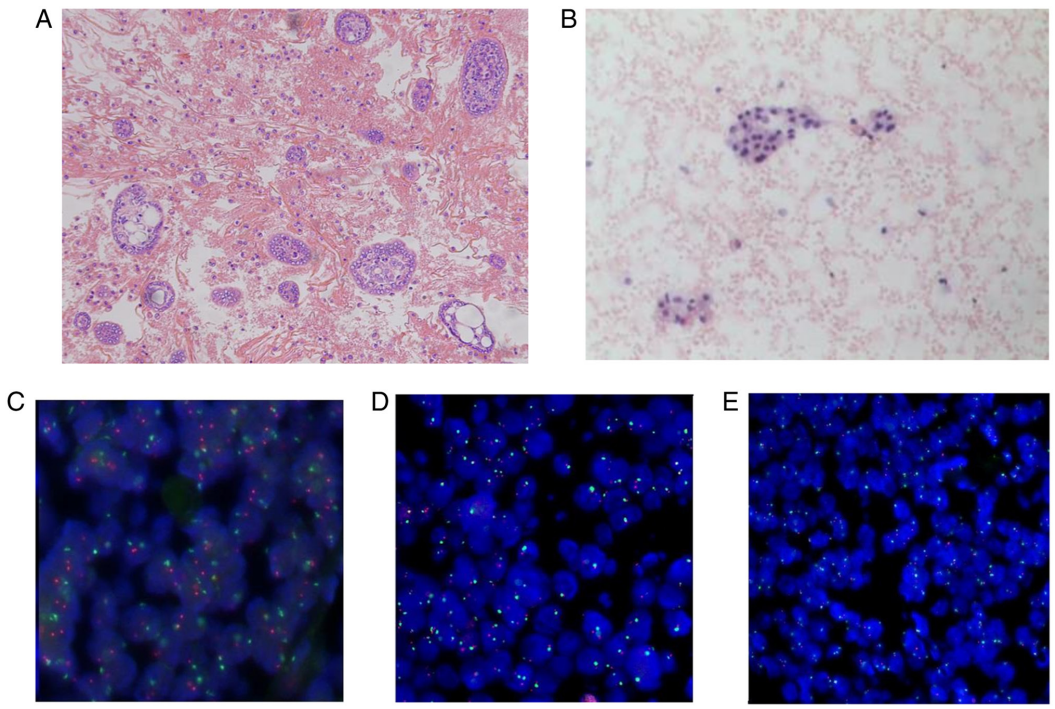

Figure 2. (A) H&E staining results of mesothelioma serous effusion specimens, and (B) H&E results of reactive mesothelioma serous effusion specimens (x200). The results of FISH on chromosome 9P21 in (C) lung adenocarcinoma, (D) mesothelioma and (E) benign mesothelioma (x1,000). The CDKN2A site signal is red, the CEP9 site signal is green; 2 red:2 green were negative cells, 1 red:2 green or 2 green were positive cells. Mesothelioma was identified, when positive cells were >15% in the microscopic field. 1 Red represents 1 red signal as a dot, the red signal as a dot is the CDKN2A gene; 2 green represents 2 green signals as a dot, and the green is the CEP9 gene.

Table IV. BAP1 expression and clinicopathological features of patients with malignant mesothelioma.

Clinicopathological features	No. of patients	Expression of BAP1			
		-	+	PR (%)	P-value
Sex					
Male	22	2	20	90.91	0.639
Female	28	3	25	89.29	
Age (years)					
<60	12	4	8	66.67	0.816
≥60	38	1	37	97.37	
Asbestos exposure history					
Yes	26	1	25	96.15	**<0.01**
No	24	4	20	83.33	
Pathological changes					
Pleural	17	2	15	88.24	0.980
Peritoneal	25	2	23	92.00	
Other (greater omentum, ovary, appendix, etc.)	8	1	7	87.50	
Ki67					
Negative	11	3	8	72.73	0.356
Positive	39	2	37	94.87	

Bold indicates a statistically significant difference. BAP1, BRCA1-associated protein 1; PR, positive rate.

Table V. MTAP expression and clinicopathological features of patients with malignant mesothelioma.

Clinicopathological features	No. of patients	Expression of BAP1			
		-	+	PR (%)	P-value
Sex					
Male	22	2	20	90.91	0.10
Female	28	4	24	85.71	
Age (years)					
<60	12	4	8	66.67	0.927
≥60	38	2	36	94.74	
Asbestos exposure history					
Yes	26	1	25	96.15	**<0.01**
No	24	5	19	79.17	
Pathological changes					
Pleural	17	2	15	88.24	0.831
Peritoneal	25	3	22	88.00	
Other (greater omentum, ovary, appendix, etc.)	8	1	7	87.50	
Ki67					
Negative	11	4	7	63.64	0.461
Positive	39	2	37	94.87	

Bold indicates a statistically significant difference. MTAP, methylthioadenosine phosphorylase; PR, positive rate.

89.80%, respectively (Fig. 3A-D and Table II). The positive rate of serous cavity effusion specimens diagnosed using the combination of the three methods (CDKN2A by FISH probe, and BAP1 and MTAP by immunohistochemistry) was 91.33%

Table VI. Association of the expression of CDKN2A, BAP1 and MTAP genes in pathological biopsies and effusion specimens of patients with malignant mesothelioma.

Measurements		Sample of serous cavity effusion		No. of cases	χ^2/Fisher	P-value
		Positive	Negative			
Biopsy specimen (FISH)	Positive	46	2	48	35.280	0.001
	Negative	2	0	2		
Biopsy specimen (BAP1)	Positive	44	3	47	106.000	0.001
	Negative	1	2	3		
Biopsy specimen (MTAP)	Positive	43	4	47	92.462	0.001
	Negative	3	2	5		

FISH, fluorescence *in situ* hybridization; BAP1, BRCA1-associated protein 1; MTAP, methylthioadenosine phosphorylase.

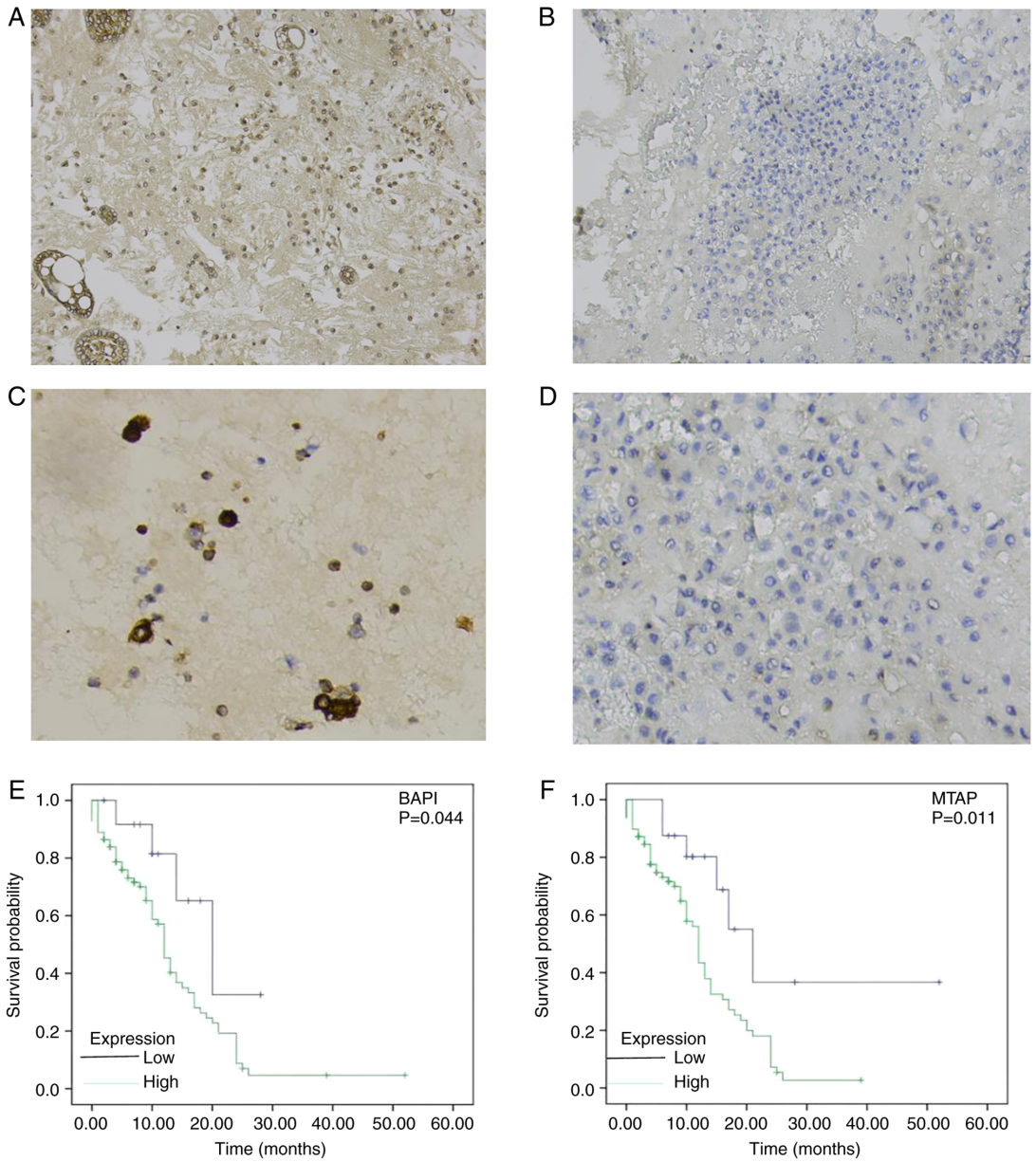

Figure 3. Expression of BAP1 antibody in (A) serous effusion of mesothelioma and (B) reactive mesenchymal hyperplasia (x200). MTAP expression antibody in serous effusion specimens of (C) mesothelioma and (D) reactive mesenchymal hyperplasia (x200). (E) Survival analysis of patients with malignant mesothelioma and BAP1 gene expression level (P=0.044). (F) Survival analysis of patients with malignant mesothelioma and MTAP gene expression level (P=0.011).

(Table II). The number of gene copies [CDKN2A (FISH) fluorescence signals] is presented in Table III. By comparing the diagnosis of malignant mesothelioma with the combination of serous fluid specimens and biopsy specimens, there was a high consistency. The positive rate of the combination of serous fluid specimens and biopsy specimens was 91.33 and 93.33%, respectively. The positive rate of FISH detection of serous fluid in specimens was also highly consistent with that of biopsy specimens. Comprehensive analysis revealed that this new combined diagnosis has high clinical application value, as well as high sensitivity and specificity.

Analysis of clinicopathological features and survival prognosis. According to the statistical analysis of BAP1 and MTAP protein expression and clinicopathological data, the development and prognosis of malignant mesothelioma were positively associated with asbestos chemical exposure history ($P<0.05$), and had no association with sex, age, lesion site and Ki67 expression (Tables IV and V). The results of these three detection methods (CDKN2A by FISH probe, and BAP1 and MTAP by immunohistochemistry) in biopsy specimens and effusion specimens were all associated ($P<0.01$; Table VI), which confirmed that the detection results of the biopsy specimens and effusion specimens had high consistency, and provided guidance and basis for clinical diagnosis. The survival of patients with malignant mesothelioma was observed. Kaplan-Meier plotter was used to analyze the association between the expression levels of MTAP and BAP1 in mesothelioma tissues and the OS of patients, and it was determined that BAP1 and MTAP genes were closely associated to the prognosis of patients with mesothelioma. The higher the expression level of MTAP gene, the worse the survival prognosis of mesothelioma patients ($P<0.05$). Similarly, when BAP1 gene was highly expressed, the survival prognosis of mesothelioma patients was poor ($P<0.05$; Fig. 3E and F).

Discussion

Malignant mesothelioma is one of the most common fatal primary pleural tumors, and the degree of disease is closely related to asbestos exposure (20,21). Mesothelioma is difficult to distinguish from reactive mesothelioma (RMH), especially in cytology, with pleural mesothelioma having the highest incidence (81%) and the worst prognosis. The mesothelioma mediators can also occur in other membranous structures, including the peritoneum (9%), pericardium and testicular sheath (22). WHO divides malignant mesothelioma into epithelioid (the most common), sarcomatoid and biphasic. The sensitivity of the cytological diagnosis commonly used in clinic is only 30-75% (23). Since mesothelioma cells are difficult to distinguish from the degenerative or proliferative mesothelioma mesenchymal cells, most patients with serous effusion of malignant mesothelioma are already in an advanced stage at the time of diagnosis. FISH probe combined with an immunohistochemical technique can easily identify these cells. Moreover, cell slices also have disadvantages such as overlapping cell blocks, a high false positive rate, and easy removal of slices. The present improved approach addresses some of these deficiencies. The serous cavity effusion specimens were centrifuged and then paraffin-embedded into sections. The obtained cells were smooth and clearly stained, which provided more accuracy and the diagnostic results were easily identified. This combined method provides an important basis for the accurate cytological diagnosis of malignant mesothelioma. At present, the clear diagnosis of mesothelioma is mostly obtained by endoscopic surgery or puncture specimens, which are difficult to obtain and harmful to patients. The sample of serous cavity effusion is easy to obtain through FISH, it does little harm to the patient, and it can reduce the clinical symptoms of the patient after extracting the effusion, which has evident advantages. Therefore, detection of serous fluid by FISH is a novel method, which has higher sensitivity, specificity and accuracy than clinical methods.

The CDKN2A gene is 8.5 kb in length and encodes 148 amino acids. It is a cell cycle-dependent protease inhibitor, which is closely related to negative regulation of the cell cycle. If the CDKN2A gene is mutated or lost, the inhibition of the cyclin D-CDK4 complex will be relieved, CDKN2A will function as a tumor suppressor gene, and an abnormal cell cycle will occur. Cells will acquire infinite proliferation (24). CDKN2A has been confirmed to be negatively correlated with BAP1 gene expression in colorectal cancer and non-small cell lung cancer (25). FISH probes detected homozygous deletion of CDKN2A gene in up to 80% of mesotheliomas, but not in reactive mesotheliomas (26). The results of the present study revealed that 9P21 gene was lost in malignant mesothelioma, with a positive rate and specificity >80% in biopsy specimens and serous effusion specimens. Chapel *et al* (27) determined that MTAP expression is deficient in non-small cell lung cancer and has a role in transcriptional expression change. MTAP is highly expressed in breast cancer, liver cancer, multiple myeloma as well as other cancer cells. A previous study revealed that the expression of MTAP gene in hepatocellular carcinoma cells was decreased after treatment with pathway inhibitors (28). In contrast, in a primary hepatocellular carcinoma model, the expression levels of VEGF and microvessel density in rats decreased after treatment with growth inhibitors, while tumor growth was also inhibited (29). However, there is no clear study on malignant mesothelioma. The present study also found that the expression level of MTAP in malignant mesothelioma was higher than that in normal serous tissue, and the high expression of MTAP was closely associated with the poor prognosis of mesothelioma ($P<0.05$).

BAP1 also mediates deubiquitination and nuclear localization. BAP1 mutations are localized on chromosome 17q, a protein that contains a ring domain and has been shown to encode inhibitors in breast and ovarian tumors. A previous study revealed that BAP1 lost nuclear expression in malignant mesothelioma. The occurrence of mesothelioma is related to the failure of the inhibition of oncogene activity when the expression of the BAP1 gene cluster is insufficient or missing (30). Seastedt *et al* (31) proposed that the increased risk of skin melanoma, meningioma and renal cell carcinoma is associated with germline mutations of BAP1, which is a marker of tumor susceptibility syndrome. Henderson *et al* (32) reported that BAP1 mutation is associated with HPV-associated squamous cell disease, which is of great significance for the diagnosis of pathological grade, depth of invasion and lymph node metastasis, and is an important molecular marker and potential therapeutic target. Previous studies have shown

that BAP1 has a high specificity (81-99%) in detecting mesothelioma, but a general sensitivity (30-67%), and thus is generally not used for diagnosis alone (33,34). The present study revealed that the expression of BAP1 gene was different in mesothelioma and normal serous tissue, and the expression of BAP1 was closely related to the survival time of patients with mesothelioma ($P<0.05$). In recent years, tumor suppressor genes have been considered as important biomarkers for the progression and prognosis of various malignant tumors and precancerous lesions (35). In the present study, on the basis of traditional paraffin sections, the expression of 9P21 gene was detected immunohistochemically by MTAP and BAP1 protein expression combined with FISH in serous cavity fluid samples, and quantitative analysis was performed at different levels, providing a theoretical basis for accurate diagnosis of mesothelioma pathological sections.

Due to the characteristic industrial and economic development of the Cangzhou region (China), which has a large number of factories with asbestos chemical raw materials, the number of cases of malignant mesothelioma is larger in this population base than that of other regions. Therefore the present study is of great clinical significance. In the present study, it was determined that FISH test combined with BAP1 and MTAP immunohistochemical detection had high specificity for needle biopsy in the diagnosis of mesothelioma, with a positive rate, specificity and sensitivity, all >91%, which are standard detection values. The detection rate, sensitivity and specificity of serous cavity effusion specimens in FISH specimens were highly consistent with the results of the biopsy specimens. However, due to the small number of cells contained in the plasma cavity exudate specimen, the atypical nature of the exudate cells in general, the fact that the cells in the exudate were not extracted simultaneously, and the different positions (sitting, supine, lateral, standing) in which the exudate specimen was extracted from the patient. Both will affect the diagnostic accuracy of serous cavity effusion specimens. Therefore, the diagnostic accuracy is slightly lower, thus the combination of BAP1 and MTAP protein expression method can render the detection results more reliable, and the serous fluid sample is superior to the puncture biopsy. It is non-invasive, easy to perform and obtain clinical samples, and the patient experiences less pain. The present findings revealed that FISH combined with BAP1 and MTAP has a positive rate of >90% in the diagnosis of mesothelioma in the thorax, ascites or biopsies. Differences in immunohistochemical conditions, such as antibody cloning or fixation and/or staining procedures used, may contribute to the observed differences. Another possibility is that the CDKN2A protein may not be continuously expressed. It plays an important role in cell proliferation and its expression is strictly regulated. Hypermethylation of the promoter region is also the mechanism of reduced CDKN2A gene expression in some tumors (36). It was observed from comparison Tables I and II, that the new combined diagnosis method has high sensitivity and specificity and a high diagnostic value as well, which is expected to be applied in future clinicopathological diagnosis.

The diagnosis and differential diagnosis of mesothelioma is a difficult issue in pathology, thus biomarkers which have high specificity, good sensitivity and short half-life, can provide the basis for diagnosis and prognosis. Mesothelioma is a malignant tumor with strong heterogeneity, and it is difficult to achieve good diagnostic sensitivity with single biomarkers. In conclusion, in serous cavity effusion specimens, FISH probe (CDKN2A gene) detection combined with immunohistochemistry (BAP1 and MTAP genes) can be used for the diagnosis of malignant mesothelioma. Compared with needle biopsy, serous effusion specimen is easier to obtain and to perform, with less trauma to patients. It is expected to be applied in pathological diagnosis in future clinical work and provide an effective screening method for early detection and diagnosis of malignant mesothelioma. It also lays a foundation for future research on the prognosis of patients with malignant mesothelioma with BAP1 and CDKN2A genes.

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Availability of data and materials

The data sets generated and/or analyzed in the present study can be obtained from the Data storage repository of Cangzhou People's Hospital (Cangzhou, China) upon reasonable request.

Author's contributions

GYM and ZGZ conceived and designed the study and evaluated the results. Fluorescence *in situ* hybridization and immunohistochemical staining were performed by GYM and XGW. SS and PW collected tissue samples and evaluated the data of patients. XGW assisted in the evaluation of the results. GYM and SS participated in the theoretical organization, study design, manuscript modification and editing of the study. ZGZ and PW confirm the authenticity of all original raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. AF/SC-08/02.0) by the Institutional Review Committee of Cangzhou People's Hospital (Cangzhou, China). All tissue samples were provided from the biobank maintained by the Department of Pathology of Cangzhou People's Hospital, which is in accordance with the guiding principles of the Declaration of Helsinki. The study obtained written informed consent for the comprehensive study of clinical samples from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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