

Original Article

A fine decision tree consisted of CK5/6, IMP3 and TTF1 for cytological diagnosis among reactive mesothelial cells, metastatic adenocarcinoma of lung and non-lung origin in pleural effusion

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Abstract: The utility of combination with CK5/6, IMP3 and TTF1 to differentiate among reactive mesothelial cells (RMs), metastatic adenocarcinoma of lung (LAC) and non-lung (NLAC) origin was investigated by using immunocytochemistry (ICC) and conventional PCR (C-PCR) in pleural effusion. A total of 108 cell blocks (32 RMs, 51 LAC and 25 NLAC) were evaluated by ICC for CK5/6, IMP3 and TTF1 protein expression. In addition, we further performed C-PCR for amplification of CK5/6, IMP3 and TTF1 DNA from 28 specimens (9 MAC and 7 RMs, 6 LAC and 6 NLAC) for molecular diagnosis. CK5/6 staining was observed in the majority of reactive specimens (78.1%) and was rare in adenocarcinoma cells (14.5%), whereas the opposite was true for IMP3 and TTF1. We found a high frequency of TTF1 positivity (76.5%) in LAC, but not in NLAC (4.0%); while there was no significant difference of IMP3 expression in LAC (88.2%) and NLAC (88.0%). The 487 bp DNA fragments of IMP3 was expected to be amplified in 6/9 of adenocarcinoma cases showed negative in ICC; and the 394 bp DNA fragments of CK5/6 was also expected to be amplified in 4/7 of RMs cases showed negative in ICC. Consistent with ICC results, there was significant difference of TTF1 expression in the LAC and NLAC compared with IMP3 expression. The combination with CK5/6, IMP3 and TTF1 immunostaining appears to be useful to improve the accuracy of cytological diagnoses between RMs, metastatic adenocarcinoma of lung and non-lung origin in pleural effusion. In addition, C-PCR may act as a useful supplemental approach for ICC, especially negative cases in ICC for differential cytological diagnosis.

Keywords: CK5/6, IMP3, TTF1, pleural effusion, adenocarcinoma, PCR

Introduction

Metastatic lung adenocarcinoma (LAC) is the most common pathological type of metastatic adenocarcinoma (MAC) in malignant pleural effusion (MPE) [1, 2]. The type of MAC cells in pleural MPE is an important factor to determine the prognosis and treatment of the patients. Thus, the differential cytological diagnosis of metastatic adenocarcinoma of lung (LAC) and non-lung (NLAC) origin is critical. However, the cytomorphologic distinction among reactive mesothelial cells (RMs), LAC and NLAC is not always straightforward and is sometimes extremely challenging, such as the often deceptively bland appearance of tumor cells and the presence of MAC cells; the primary sites of

tumor cells are unclear and cytological specimens also add to the difficulty of proper diagnosis.

In recent years, many immunocytochemical (ICC) stains have been investigated for both RMs and MAC [3-6]. Insulin-like growth factor-II mRNA-binding protein 3 (IMP3) is a newly identified oncofetal protein; It is expressed in the developing epithelium, muscle and placenta, but it is expressed at low or undetectable concentrations in adult tissues [7]. However, the usefulness of IMP3 as an ICC biomarker used to differentiate MAC from RMs in cell blocks prepared from effusion specimens are limited [8-10]. In addition, whether it has significant utility in the differential diagnosis between LAC

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Table 1. Antibodies used for immunocytochemical analysis in the study

Antibody	Clone	Dilution	Source	Pretreatment
CK5/6	D5/16B4	1:50	DAKO ^a	Heat
IMP3	L523S	1:100	DAKO ^a	Heat
TTF1	8G7G3/1	1:50	DAKO ^a	Heat

^aDAKO (Carpinteria, CA).

and NLAC remains unknown. Also, investigators have recommended some panels of ICC stains distinguishing between LAC and NLAC in serous effusions [11, 12]. Antibodies against thyroid transcription factor-1 (TTF1) antigen were well established for the differentiation among primary LAC from other sites and malignant mesothelioma [13-16], and they had also been applied to effusion cytology. In addition, Conventional PCR (C-PCR) based techniques coupled with new developments in the extraction of DNA from paraffin-embedded tissue now enable pathologists to use such archival material for a variety of purposes [17, 18]. The C-PCR targeting 372-bp region of the 56-kDa TSA has been reported as a good tool for molecular diagnosis and has also led to the identification of new genotypes from India [19]. However, there have been few studies done using C-PCR for cytological diagnosis in MPE, which is much simpler and economical.

In this study, our goal was to evaluate the diagnostic usefulness of the combination with cytokeratin 5/6 (CK5/6), IMP3 and TTF1 and propose a decision tree in differentiating among RMs, LAC and NLAC in pleural effusion by using cytomorphology, ICC and C-PCR as alternate methods.

Materials and methods

Case selection

A total of 108 cases were diagnosed based on histopathology and clinical data, including 32 RMs, 51 LAC and 25 NLAC (14 breast, 9 stomach, 2 unconfirmed primary sites of origin), were obtained from the formalin-fixed, paraffin-embedded block archived from The Pathology Department of Nanfang Hospital and the Third Hospital affiliated to Southern Medical University. Cell blocks were prepared for each case. All the cases were collected between 2009 and 2013.

Immunocytochemistry

Immunocytochemistry (ICC) of paraffin sections from clinical samples were carried out, using the ChemMate™ EnVisio™ Detection kit (Dako, Carpinteria, CA, USA). Multiple 4 μm thick sections from the paraffin-embedded cell blocks were used for ICC studies. Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) for 5 min at 100°C. The sections were first blocked for endogenous peroxidase activity with 3% H₂O₂ for 15 min at room temperature. Then the sections were incubated overnight at 4°C with antibodies; a summary of the antibodies used is provided in **Table 1**. After washing three times, sections were then incubated with biotin-labeled goat anti-rabbit antibody or biotin-labeled goat anti-rat antibody (Zhongshan Inc., Zhongshan, China) accordingly for 10 min at room temperature, and subsequently were incubated with streptavidin-conjugated horse-radish peroxidase (HRP) (Maixin Inc., Shenzhen, China). Sections were treated with DAB and counterstained with hematoxylin, cover slipped with neutral gum. Appropriate positive and negative controls were used for each antibody.

The ICC staining reactions were recorded for each antibody by at least two pathologists separately, and discrepant staining reactions between the two pathologists were jointly reviewed and agreeable interpretations were reached. For CK5/6 and IMP3 staining, diffuse, easily visible, cytoplasmic staining in 5% or more of target cells was scored as positive. For TTF1 staining, dark brown nuclear staining in 5% or more of target cells was scored as positive.

DNA extraction and PCR assay

Ninety-one paraffin-embedded cell blocks (9 MAC and 7 RMs; 6 LAC and 6 NLAC) were selected for DNA extraction. DNA was extracted using phenol-chloroform-isoamyl alcohol as previously described [20]. PCR primers were designed and synthesized by Biotech (shanghai, china). The sequences obtained were identified and compared using BLAST software (<http://blast.ncbi.nlm.nih.gov>) and the sizes of the PCR products were showed in **Table 2**. Reactions was 50 ul, including 2× MightyAmp Buffer Ver. 2 (Takara, Otsu, Japan), 10 μM of each primer, respectively, 5 ul DNA templates,

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Table 2. Sequences for primers

Gene name	Primer sequence	Product size
CK5/6	Forward 5'-TAAATCATCAAACAGAATCCCCAC-3'	394 bp
	Reverse 5'-CAAATCATCTCACCTTGCATAGG-3'	
IMP3	Forward 5'-GATGTCCTATTCTTGCCTTCTCTA-3'	487 bp
	Reverse 5'-GTTTCTCCTTCTACTCTCCTGTTC-3'	
TTF1	Forward 5'-TCCAGCCATAAACAAGATAACCCAT-3'	447 bp
	Reverse 5'-AACACATTCCAATCCCAGAAAGTA-3'	

Table 3. CK5/6, IMP3 and TTF1 expression in reactive mesothelial cells (RMs) and metastatic adenocarcinomas (MAC) in pleural effusion

Diagnoses	Total	CK5/6		IMP3		TTF1	
		+	-	+	-	+	-
^a RMs	32	25	7	3	29	2	30
^b MAC							
Lung origin	51	7	44	45	6	39	12
No-Lung origin	25	4	21	22	3	1	24

^aReactive Mesothelial Cells; ^bMetastatic Adenocarcinomas; +, positive staining; -, negative staining.

1.25 U MightyAmp DNA Polymerase (Takara, Otsu, Japan) and 16 ul dH₂O. Reactions were hot-started at 98°C for 2 minutes. Amplification of the three steps PCR was carried out for 40 cycles (98°C/10 sec, 60°C/15 sec, 68°C/29 sec). The PCR products were analyzed with electrophoresis in a 2% agarose gel and visualized with ethidium bromide staining. Negative controls (PCR mix without DNA template) were included in each amplification run.

Statistical analysis

The χ^2 test was used to assess the association between categorical variables. *P* values of less than 0.05 were considered statistically significant with two-sided tests.

Results

A total of 108 cases were diagnosed based on histopathology and clinical data, including RMs, 76 MAC (51 LAC and 25 NLAC). Among these patients with MAC, the mean age was 60 years (range, 27-84 years); 39 were men, and 37 were women. Among these patients with RMs, the mean age was 56 years (range, 17-80 years); 28 were men, and 4 were women.

The ICC results of CK5/6 IMP3 and TTF1 expression were summarized in **Table 3**. To dis-

tinguish MAC from RMs, representative staining patterns for CK5/6 and IMP3 were showed in **Figure 1**. Immunostaining for detection of CK5/6 was generally concentrated cytoplasmic staining in RMs (**Figure 1B**) and negative staining in MAC (**Figure 1C**); In contrast, cytoplasmic staining of IMP3 was often observed in MAC (**Figure 1F**) but neg-

ative staining in RMs (**Figure 1E**). CK5/6 was positive in 25/32 (78.1%) of RMs and in 11/76 (14.5%) of MAC (*P*=0.000); IMP3 was positive in 3/32 (9.4%) of RMS and 67/76 (88.2%) of MAC (*P*=0.000) (**Table 3**). We further evaluated the expression of IMP3 and TTF1 in LAC and NLAC. Representative staining patterns for IMP3 and TTF1 were showed in **Figure 2**. Immunostaining for detection of IMP3 was generally concentrated cytoplasmic staining both in LAC (**Figure 2B**) and NLAC (**Figure 2E**); Nevertheless, nuclear staining of TTF1 was often observed in LAC (**Figure 2C**), but negative staining in NLAC (**Figure 2F**). IMP3 was positive in 45/51 (88.2%) of LAC and 22/25 (88.0%) of NLAC (*P*=1.000); Unlike IMP3, TTF1 was positive in 39/51 (76.5%) of LAC, but 1/25 (6.7%) of NLAC (*P*=0.000).

The 487 bp DNA fragments of IMP3 were expected to be amplified 6/9 in MAC specimens showed negative in ICC (**Figure 3A**); and the 394 bp DNA fragments of CK5/6 was expected to be amplified 4/7 in RMs specimens showed negative in ICC (**Figure 3B**); As with ICC staining, the 487 bp DNA fragments of IMP3 in 6/6 of LAC and in 6/6 of NLAC specimens were detected by C-PCR (**Figure 3C**). In contrast, almost all 447 bp DNA fragments of TTF1 (6/6) were showed positive expression in LAC specimens but scarcely expression (0/6) in NLAC specimens (**Figure 3D**).

The staining pattern using a combination of CK5/6 and IMP3 using ICC staining and C-PCR was divided into six categories for detecting RMs and MAC detailed in **Table 4**. The staining pattern of CK5/6⁺/IMP3⁻ using ICC had a higher specificity than CK5/6⁺ ICC alone (98.3% vs. 85.5%), but a lower sensitivity for detecting RMs (71.5% vs. 78.1%). Whereas, a higher sensitivity was observed in the staining pattern of CK5/6⁺/IMP3⁻ using combination with ICC and C-PCR than ICC alone for detecting RMs (83.0%

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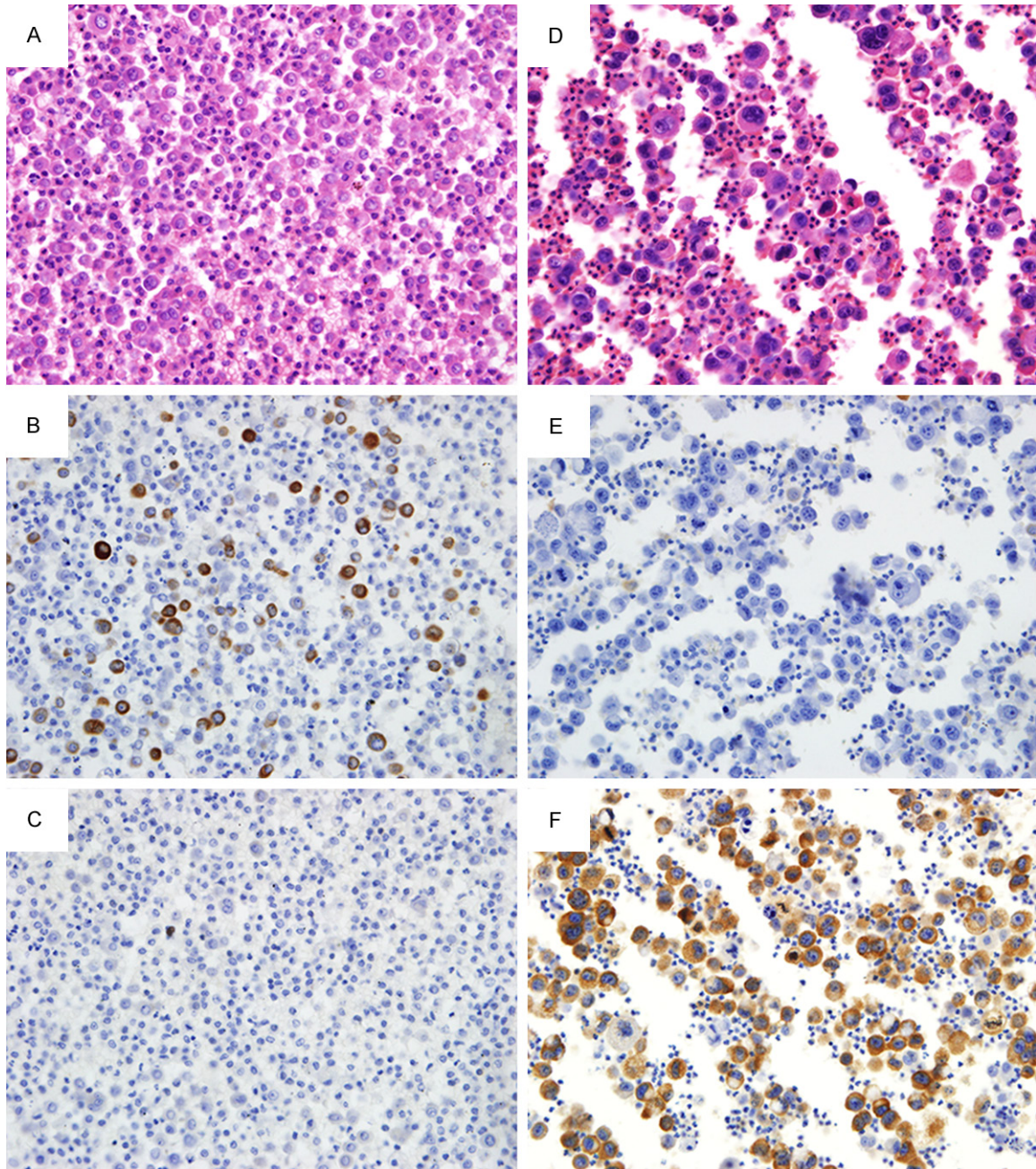


Figure 1. Reactive mesothelial cells (RMs) are shown in Pleural Effusion using by (A) HE, (B) CK5/6 ICC stain, (C) IMP3 ICC stain; Metastatic adenocarcinoma (MAC) cells are shown in pleural effusion using by (D) HE, (E) CK5/6 ICC stain, (F) IMP3 ICC stain. HE, Hematoxylin & Eosin staining; ICC, Immunocytochemistry. ($\times 400$).

vs. 71.5%). The staining pattern of IMP3⁺/CK5/6⁻ using ICC had a higher specificity than IMP3⁺ alone (98.2% vs. 91.6%), but a lower sensitivity for detecting MAC (75.4% vs. 88.2%). Whereas, a higher sensitivity was observed in the staining pattern of IMP3⁺/CK5/6⁻ using combination with ICC staining and C-PCR than ICC alone for detecting MAC (82.2% vs. 75.4%).

Discussion

Cytomorphologic differentiation among RMs, LAC and Other NLAC in pleural effusion (PE) can be a diagnostic challenge alone. The difficulty is compounded when the primary sites of tumor cells are unclear or neoplastic cells exhibit only slight atypia. Currently, ICC staining

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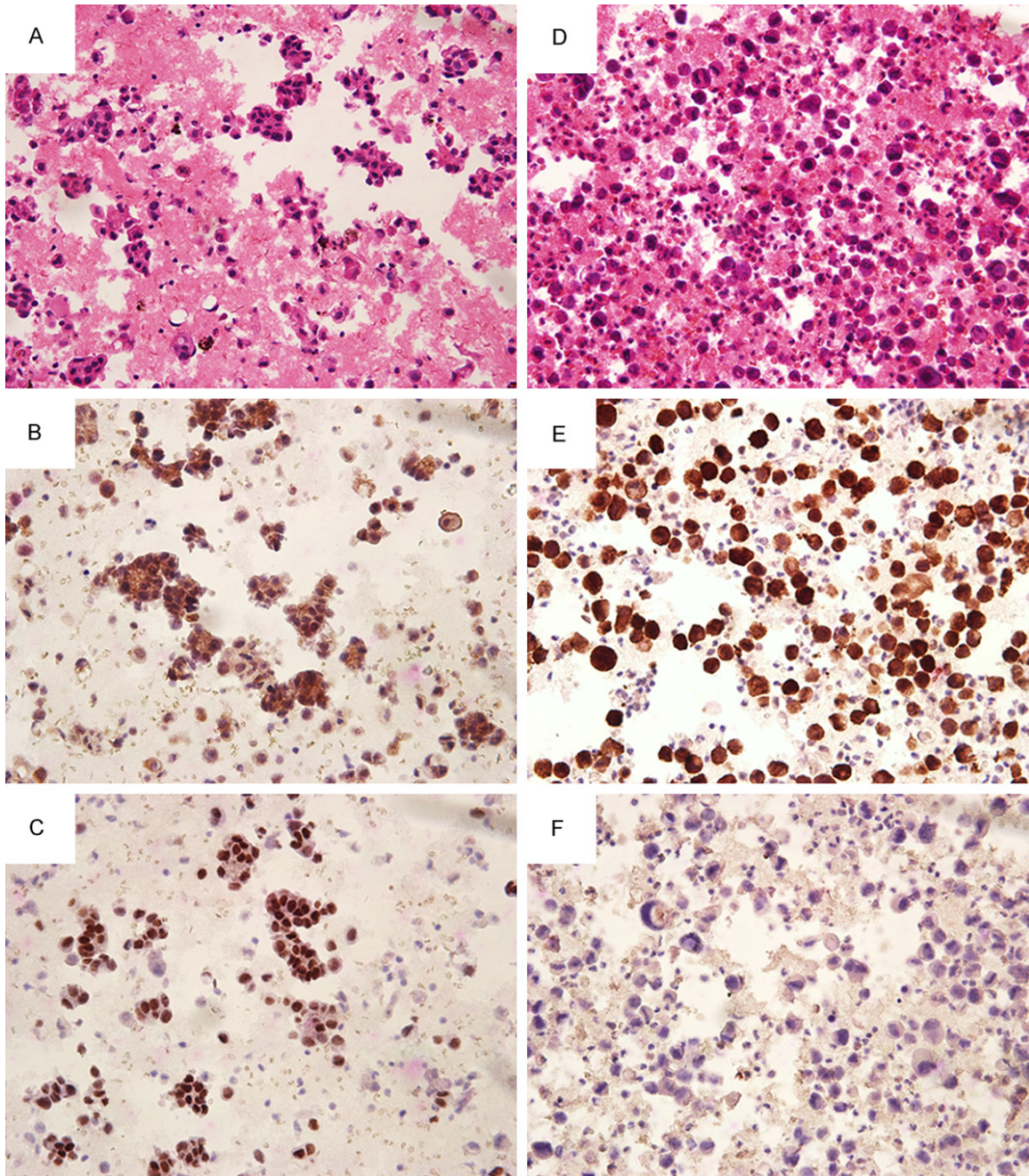


Figure 2. Metastatic lung adenocarcinoma (LAC) cells are shown in pleural effusion using by (A) HE, (B) IMP3 ICC stain, (C) TTF1 ICC stain; Metastatic adenocarcinoma of non-lung (NLAC) origin are shown in pleural effusion using by (D) HE, (E) IMP3 ICC stain, (F) TTF1 ICC stain. HE, Hematoxylin & Eosin staining; ICC, Immunocytochemistry. ($\times 400$).

is widely used as a tool to enhance correct cytological diagnoses of body fluids. Numerous antibodies have been applied to distinguish RMs from MAC [3-6]. However, to pursue a biomarker of high sensitivity and specificity remains to be the hot spot of clinical research.

In MAC, IMP3 expression was reported to be correlated with more poorly differentiated histological grade, advanced stage of disease and lymph node metastases [21]. IMP3/L523S was also reported to be sensitive and specific markers for differentiating RMs from malignant

CK5/6, IMP3 and TTF1 in pleural effusion

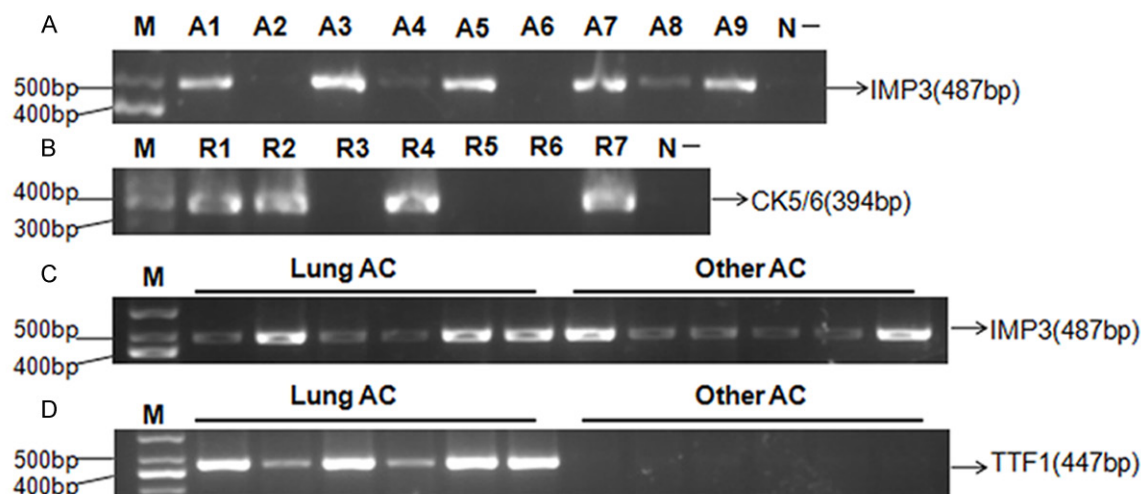


Figure 3. Conventional PCR amplified products of CK5/6, IMP3, TTF1 DNA in paraffin-embedded cell blocks of patients with reactive mesothelial cells (RMs), metastatic adenocarcinoma of lung (LAC) and non-lung (NLAC) origin in 2% agarose gel. A. IMP3 expression in 6/9 of MAC showed negative in ICC; B. CK5/6 expression in 4/7 of RMs showed negative in ICC; C. IMP3 expression in 6/6 of LAC and 6/6 of NLAC; D. TTF1 expression in 6/6 of LAC and 0/6 of NLAC. M, DNA Maker (DL1000); A1-A6, Paraffin-embedded MAC cell blocks; R1-R6, Paraffin-embedded RMs cell blocks; N, Negative control.

Table 4. Sensitivity and specificity of cytological diagnoses from CK5/6 and IMP3 by using immunocytochemistry and conventional PCR

Diagnosis	Staining pattern	Sensitivity (%)	Specificity (%)
RMs	CK5/6 ⁺ (ICC ^a)	78.1%	85.5%
	CK5/6 ⁺ (ICC)/IMP3 ⁻ (ICC)	71.5%	98.3%
	CK5/6 ⁺ (ICC+C-PCR ^b)/IMP3 ⁻ (ICC)	83.0%	98.3%
MAC	IMP3 ⁺ (ICC)	88.2%	91.6%
	IMP3 ⁺ (ICC)/CK5/6 ⁻ (ICC)	75.4%	98.2%
	IMP3 ⁺ (ICC+C-PCR)/CK5/6 ⁻ (ICC)	82.2%	98.2%

^aImmunocytochemistry; ^bconventional PCR.

mesothelioma (MM) or metastatic carcinoma in recent years [22-26]. Ikeda et al. [9] used immunohistochemistry (IHC) studied IMP3 expression in large effusion samples and the utility of IMP3 as an IHC stain for distinguishing malignant cells from RMs. They observed IMP3 positivity in only 5.1% of cases of RMs, while IMP3 positivity was observed in 75.7% of MAC effusions. Consistent with previous literatures, our study had shown that IMP3 immunoreactivity was a significant difference between the staining of MAC (88.2%) and RMs (9.4%), and it was a perfect biomarker of high sensitivity and specificity for MAC. These results raised the question whether had a useful marker for detecting RMs. Ordonez et al. [27] showed that CK5/6 was positive in 60/60 cases of epitheli-

al mesothelioma while it was reactive in only 1/50 cases of MAC in tissue specimens. Few subsequent studies were conducted on effusion samples. In one study, CK5/6 was found reactive in 20/20 RMs cases, whereas it was positive in one of nine cases of MAC [28]. In our study, CK5/6 showed good sensitivity (78.1%) and specificity (85.5%) for detecting RMs. The use of this antibody was based on relatively

select expression (14.5%) in MAC compared with RMs (78.1%). Viewed in total, these results indicated that IMP3 was a useful biomarker for detection of MAC cells in PE, and it had significant utility in the differential diagnosis of MAC and RMs in combination with CK5/6 ICC staining; however, it was not appropriate for differentiation between LAC and NLAC.

The distinction between LAC and NLAC in PE remains very difficult or sometimes impossible when the primary sites of tumor cells is unclear [29-31]. TTF1 was mainly expressed in thyroid and lung during embryogenesis and it played a physiologic role in their development and morphogenesis [32, 33]. Antibodies against TTF-1 antigen were well established for the differen-

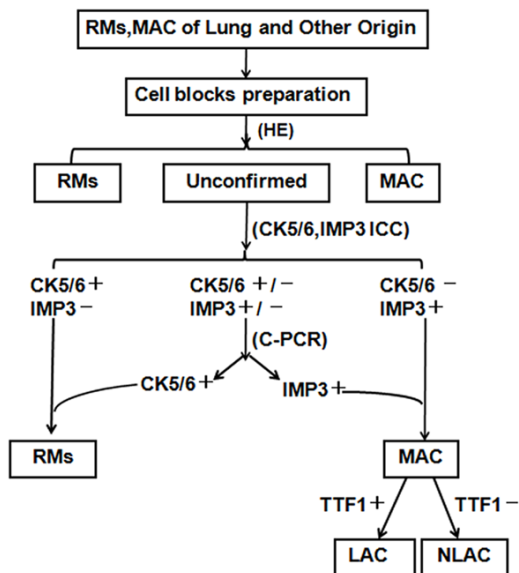


Figure 4. Decision tree for the cytologic diagnosis of reactive mesothelial cells (RMs), metastatic adenocarcinoma of lung (LAC) and non-lung (NLAC) origin by using CK5/6, IMP3 and TTF1 immunocytochemistry (ICC) and conventional PCR (C-PCR) in pleural effusion. HE, Hematoxylin & Eosin staining.

tiation among primary LAC, AC from other sites and MM [13-16], and they had also been applied to effusion cytology. However, with sensitivities varying from 54 to 88% [15, 34-37], we compared the reactivity of IMP3 and TTF-1 to biomarkers for LAC and NLAC in PE. It was noteworthy that TTF1 immunoreactivity in LAC (39/51) and NLAC (1/25) was significantly statistical difference; In contrast, IMP3 immunoreactivity in LAC (45/51) and NLAC (22/25) was no statistical difference. It seemed that TTF1 could be a complementary marker of IMP3 for differential diagnosis of MAC of lung and non-lung origins.

Although ICC staining can greatly aid the accuracy of cytological diagnosis, available markers have varying sensitivities and specificities for mesothelial cell or cells of epithelial differentiation [38, 39]. Real-time PCR also can be used to assess gene expression levels and evaluate the relationship between genes and disease [40]. But it is impossible that the total RNA could be extracted with high purity from paraffin-embedded cell blocks. C-PCR has the advantages of higher sensitivity and specificity, and the quality requirements of DNA templates are not demanding [41, 42]. Thus, we extracted

DNA from paraffin-embedded cell blocks and detected DNA fragments of CK5/6, IMP3 and TTF1 by using C-PCR assay. In this study, the 487 bp DNA fragments of IMP3 DNA was expected to be amplified 6/9 in MAC specimens showed negative cases in ICC staining; and the 394 bp DNA fragments of CK5/6 DNA was also expected to be amplified 4/7 in RMs specimens showed negative cases in ICC staining. Consistent with ICC results, there was significant difference of TTF1 expression between the LAC and NLAC group.

When we analyzed the sensitivity and specificity for using CK5/6/IMP3 to distinguish between RMs and MAC, we found that a higher sensitivity was observed in the combination of CK5/6/IMP3 using ICC staining and C-PCR for detecting RMs compared with using ICC staining alone; and a higher Specificity than using CK5/6 ICC staining. Also, for MAC, we observed a higher sensitivity in the combination of IMP3/CK5/6 using ICC staining and C-PCR compared with using ICC staining alone; and a higher specificity than using IMP3 ICC staining. Based on these data, we propose the use of a fine decision tree consisted of CK5/6, IMP3 and TTF1 for differentiating among RMs, LAC and NLAC by using ICC staining and C-PCR assay as alternate approaches in PE (Figure 4). To our knowledge, this is the first report to describe the detection of DNA fragments of CK5/6, IMP3 and TTF1 DNA from paraffin-embedded cell blocks via C-PCR as a supplementary approach of ICC staining for differential cytological diagnosis in PE.

In summary, the combination with CK5/6 and IMP3 immunostaining appears to be useful to improve the accuracy of cytological diagnoses between reactive mesothelial Cells and metastatic adenocarcinoma in pleural effusion. In addition, TTF1 could be a complementary biomarker of IMP3 for differential diagnosis of metastatic adenocarcinoma of lung and non-lung origin. C-PCR may act as a useful supplementary approach of ICC staining, especially negative cases in ICC for differential cytological diagnosis in pleural effusion.

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Disclosure of conflict of interest

None.

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