The diagnostic significance of the detection of cytokeratin 19 mRNA by quantitative RT-PCR in benign and malignant pleural effusions

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Abstract: Objective: To evaluate the diagnostic significance of detecting cytokeratin 19 (CK19) mRNA by quantitative reverse transcription polymerase chain reaction (RT-PCR) in benign and malignant pleural effusions. Methods: CK19 mRNA was examined by quantitative RT-PCR and CK19 was detected by Enzyme-linked immunoadsorbent assay (ELISA) in 32 patients with malignant pleural effusions and 35 patients with benign pleural effusions. Results: On the threshold of 200 copies/ μ l, the positive rate of CK19 mRNA in patients with malignant pleural effusions was 62.5%. The positive rates of CK19 mRNA and CK19 in the malignant pleural effusions were significantly higher than those in the benign group ($P < 0.01$). Furthermore, the positive rate of CK19 mRNA was higher than that of CK19 in the malignant group ($P < 0.05$). Conclusion: Detection of CK19 mRNA can be a promising diagnostic marker in differential diagnosis of benign and malignant pleural effusions.

Key words: Cytokeratin 19 mRNA, Quantitative reverse transcription polymerase chain reaction, Pleural effusions

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INTRODUCTION

Malignant pleural effusions often result from malignant tumors transferring into pleural cavity. Detecting tumor cells by RT-PCR is thought to be one of the most effective methods to judge micrometastases of malignant tumors. It is well known that cytokeratin 19 (CK19) is one of the most useful markers for diagnosis and management of tumor. CK19 mRNA is expressed in nearly all the epithelial malignancies and may be widely employed as a molecular marker for assessing the circulating tumor burden and diagnosing micrometastasis. Sequential quantification of CK19 mRNA levels in blood may potentially indicate

colorectal cancer patients response to treatment and help identify patients at high risk for metastasis (Wong *et al.*, 2001). Yuan *et al.* (2002) revealed the presence of circulating CK19-expressing cancer cells in the blood of patients with untreated early-stage cervical carcinomas. Lockett *et al.* (1998) had developed keratin-19, c-myc, prolactin inducible protein (PIP) RT-PCR based method to identify axillary lymph node metastases in patients with breast cancer and thought it appeared to be a readily available and highly sensitive method for detecting breast cancer micrometastases.

In our study, CK19 mRNA was detected by quantitative RT-PCR and CK19 examined by ELISA in benign and malignant pleural effusions.

Our aim was to evaluate the value of quantitative CK19 mRNA RT-PCR method in the diagnosis of malignant pleural effusions.

METHODS

Patient population

The study population consisted of 67 in-patients with pleural effusions at the Second Affiliated Hospital of College of Medicine, Zhejiang University from May 2001 to April 2003. Of 32 patients with malignant pleural effusions, 18 were male and 14 were female. Their ages were between 45 and 77. Their primary diseases were: lung cancer (23 cases), breast cancer (4 cases), gastric cancer (2 cases), liver cancer (1 case), colorectal cancer (1 case), and lymphoma (1 case). Ten malignant cases were confirmed by cytology and others were subjected to pathological examination (pleural biopsy or operation). Of 35 patients with benign pleural effusions, 24 were male and 11 were female. Their ages were between 26 and 82. Their primary diseases were: tuberculosis (21 cases), pneumonia (7 cases), uremia (5 cases), congestive heart disease (2 cases). All benign cases were confirmed by pathological examination or clinical manifestation and outcome.

Detection of CK19 mRNA

Pleural fluid samplings were performed before patients were treated by any chemical drug. To reduce the false positive risk by contamination of epidermal cells in the skin, 10 ml of the second tube of pleural fluid was collected after 5 ml of pleural fluid was aspirated. Cells from pleural fluid sampling were prepared by centrifugation at 1000 g for 10 minutes for two times. Total mRNA was isolated by Trizol reagent according to the procedure of the supplier (Gibco Inc., America). Approximately 2 µg mRNA from each sample was subjected to reverse transcription using RT Kit (Promega Inc., America) for 2–4 h at 42 °C, then PCR amplification was performed in a quantitative thermal cycler (7700 Sequence Detector, Perkin-Elmer, America) and the copies of CK19 mRNA were detected.

Forward and reverse primes of CK19 were bought from Jiu Sheng Inc. of Shanghai. PCR condition: 30 s at 4 °C, 15 s at 62 °C, 15 s at 72 °C for 30 cycles amplification, then extend at 72 °C for 10 min. Above 200 copies of CK19 mRNA was thought of as positive. β-actin as an internal control showed that all samples displayed detectable circulating cell RNA.

Detection of CK19

CK19 in the pleural fluid was detected by ELISA Kit (Mega Diagnostics, Inc., America). Above 3.7 ng/ml of CK19 was thought of as positive.

Statistical analysis

The data were analyzed by Fisher's exact test and Mann-Whitney U test. A value of $P < 0.05$ was regarded as statistically significant.

RESULTS

Levels of CK19 mRNA in benign and malignant pleural effusions

Detected by quantitative RT-PCR, the levels of CK19 mRNA in malignant pleural effusions ranged from 15 copies/µl to 1.73×10^8 copies/µl. Its P_{25} (Percentile 25) and P_{75} (Percentile 75) were 73 copies/µl and 38725 copies/µl respectively. The levels of CK19 mRNA in benign pleural effusions ranged from 0 copies/µl to 19520 copies/µl. Its P_{25} and P_{75} were 0 copies/µl and 160 copies/µl respectively. By Mann-Whitney U test, the levels of CK19 mRNA were much higher in the malignant pleural effusions than in the benign group ($P < 0.01$).

Comparison of positive rate of CK19 mRNA and CK19 in pleural effusions

Following our previous study, 200 copies/µl was selected as cut-off of CK19 mRNA by ROC curve analysis (Xu et al., 2003). The positive rates of CK19 mRNA in the group of malignant and benign pleural effusions were 62.5% (20/32) and 17.1% (3/35) respectively. By Fisher's exact test, there was significant difference between the two

groups ($P<0.01$). The values of CK19 mRNA from 10 patients with positive cytology were beyond 200 copies/ μl , suggesting relatively good correlation between CK19 mRNA and cytological examination of pleural effusions.

The positive rate of CK19 in the group of malignant pleural effusions was 34.4% (11/32), compared to 5.7% (2/35) in the group of benign pleural effusions. By Fisher's exact test, there was significant difference between the two groups ($P<0.01$). Furthermore, the positive rate of CK19 mRNA was higher than that of CK19 in the malignant group ($P<0.05$) (Table 1).

About diagnostic significance of CK19 mRNA and CK19 in pleural effusions, please see the Table 2.

DISCUSSION

CK19, a kind of texture protein with molecular weight of about 40000, has relatively high expression in some tumor tissues such as lung cancer, breast cancer and etc. which are prone to result in malignant pleural effusions. So CK19 probably possesses higher expression in pleural effusions resulted from these tumors. CK19 mRNA was frequently used as a marker in the detection of circulating tumor cells of epithelial origin and sensitivity of this assay was 1 CK19-mRNA-positive cell per 10^6 mononuclear cells (Yuan *et al.*, 2002). But CK19 mRNA has rarely been investigated in pleural effusions. The diagnostic significance of

CK19 mRNA in pleural fluid was evaluated in our study.

Our data showed that levels of CK19 mRNA in the malignant pleural effusions were significantly higher than those in the benign group ($P<0.01$). On the threshold of 200 copies/ μl , CK19 mRNA possessed relatively high specificity (82.9%) and sensitivity (62.5%). So the level of CK19 mRNA is a good marker in differential diagnosis of benign and malignant pleural effusions. We thought that the relatively high sensitivity of CK19 mRNA was due to the breakthrough of RT-PCR methodology and high expression of CK19 in some tumor cells causing pleural effusions. At the same time, we also found a false-positive rate in the benign group. The reason for the relatively high rate of false-positives may be attributed to pseudogenes, different blood cell separation methods, or illegitimate expression of CK19 (Wong *et al.*, 1997; Kao and Huang, 2002).

Besides, the relatively strong inflammation of benign diseases can induce the expression of CK19 mRNA (Noguchi *et al.*, 1996). But we found that most of the levels of CK19 mRNA in patients with benign lung diseases were within 200 copies/ μl and significantly lower than those in patients with malignant diseases. Using more than a single marker gene could be a potential way to overcome this problem, because there is a little chance of encountering significant illegitimate expression of more than one gene at a time (Lockett *et al.*, 1998). A false-negative rate may be attributed to heterogeneity and diversity of tumor cells, and their dis-

Table 1 Comparison of positive rate of CK19 mRNA and CK19 in pleural effusions

Group	Number	CK19 mRNA			CK19		
		+	–	Positive rate (%)	+	–	Positive rate (%)
Malignant group	32	20	12	62.5 ^{*, Δ}	11	21	34.4 [#]
Benign group	35	6	29	17.1	2	33	5.7

^{*, #} $P<0.01$, vs benign group; ^Δ $P<0.05$, vs CK19

Table 2 Diagnostic significance of CK19 mRNA and CK19 in pleural effusions

Tumor markers	Se (%)	Sp (%)	PPV (%)	NPV (%)	Accuracy (%)	Youden index
CK19 mRNA	62.5	82.9	77.0	70.7	73.1	0.454
CK19	34.3	94.3	84.6	61.1	65.7	0.286

Se: sensitivity, Sp: specificity, PPV: positive predictive value, NPV: negative predictive value

continuous entry into pleural effusions (Mori *et al.*, 1996). Lower than CK19 mRNA, the sensitivity of CK19 was 34.4%, which indicated gene transcription marker may be better than protein marker in differential diagnosis of benign and malignant pleural effusions.

In summary, our primary study indicated that CK19 mRNA is a promising marker for differentiating the characteristics of pleural effusions. But more cases and data are needed to further approve this new molecular marker in situations such as cancer diagnosis, monitoring the outcome of disease, and eventually as a treatment response indicator.

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