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Detection of hTERT mRNA, CEA, CA19-9 in Pleural Effusion and Its Clinical Significance

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OBJECTIVE To explore the clinical significance of the human telomerase reverse transcriptase (hTERT) mRNA, CEA and CA19-9 in differential diagnosis of benign and malignant pleural effusions.
METHODS Concentrations of CEA and CA19-9 in pleural effusions were assayed using automated chemiluminescence, and expression of hTERT mRNA was detected by RT-PCR.
RESULTS The positive rates of hTERT mRNA, CEA and CA19-9 expression in the group with malignant effusions were significantly higher compared to the group with benign effusion ($P < 0.05$). The sensitivity (%), specificity (%) and diagnostic accordance rates (%) of the 3 tumor markers were as follows: *i*) hTERT mRNA: 81.8/90.5/86.1; *ii*) CEA: 52.3/92.9/72.1; *iii*) CA19-9: 34.1/90.5/61.6. The positive rates of hTERT mRNA + CEA (%) expression in the pleural effusions were 97.7.
CONCLUSION All of these tumor markers can be helpful for differential diagnosis of pleural effusions. hTERT mRNA had more clinical value in differentiation of the pleural efffusions. CA19-9 is unfit to be as an optimal index. The combined assay of hTERT mRNA and CEA in pleural effusions can further raise the positive detection rate of the tumor markers and can be helpful in producing a diagnosis.

KEY WORDS: pleural effusions, hTERT mRNA, CEA, CA19-9.

Introduction

Pleural effusions are a common consequence of various diseases. With the growth of tumor incidence, the number of cases involving malignant pleural effusions has also significantly increased. Correct evaluation of a pleural effusion has of important clinical significance for an auxiliary diagnosis, treatment strategy, and establishment of a prognosis. At present, clinical diagnosis of a malignant pleural effusion mainly relies on a cytological examination, but the rate of cytological diagnosis ranges only from 40% to 60%[1]. In our study, therefore, an examination of the expressions of 3 tumor markers in pleural effusions, i.e. hTERT, CEA and CA19-9, was conducted, in order to determine their diagnostic value as the tumor markers, and to develop new methods to diagnosis malignant diseases.

Patients and Methods

Patients

Eighty-six pleural effusion specimens were collected from 86 patients with different diseases, who had been hospitalized at the First, the Second and the Third Affiliated Hospitals of Nanchang University, Jiangxi Chest Hospital and Jiangxi Maternal and Child Health Hospi-

tal, during a period from September 2005 to June 2006. All specimens were obtained during the course of the first thoracentesis.

Exfoliocytological examination of the pleural effusion and/or pleural biopsies both were conducted in 44 patients in the group with malignant effusion (25 males and 19 females), with an age range from 16 to 75 years, and an average of 54.7. The malignant pleural effusions were affirmed if the result of one of the examinations was positive. Among the 44 cases, malignant mesothelioma was found in 3, malignant thymoma in 1, lung cancer in 19, breast cancer in 3, gastric cancer in 1, ovarian cancer in 3, liver cancer in 1, B-cell lymphoma in 1 and metastatic adenocarcinoma in 2. There were 10 cases with questionable origins. The 42 patients in the group with benign effusions (22 males and 20 females), with an age range from 16 to 90 years and an average of 46.6, received both the pleural-effusion exfoliocytological examination and biopsy of the pleura. Clinical data, such as negative results from both examinations, and related outcomes of clinical imageology and bacteriology, etc. could be used for final diagnosis of the benign pleural effusions. Among the 42 cases, tuberculous pleurisy was found in 30, pneumonia in 6, heart failure in 4 and renal failure in 2.

Specimen treatment

One sample tube of fresh pleural effusion was taken (50 ml). After conducting the centrifugation of the specimen at 5000 rpm, 4℃, for 5.0 min, 10.0 mL of the fresh supernatant fluid was kept for determination of CEA and CA19-9. Then, the precipitated cells were collected in a 1.5-ml Eppendorf test tube to be treated with DNAse using the following steps. If the effusion was bloody, an equivalent volume (50 ml) of PBS was used for repeated aerated stirring via an air syringe at room temperature. The centrifugation was conducted again, using the same conditions, and then the supernatant fluid was discarded. For apparent sanguineous specimens, the process was repeated once or twice. Pretreatment of all specimens was carried out within 1.0 h.

Major instruments and reagents

Instruments used included a PCR amplifier (PTC-100 type), electrophoresis apparatus (ECP3000), ultra violet spectrophotometer (756PC type), gene genius bio imaging system and automated chemiluminesence detector (Bayer. ACS: 180'SE). The reagents were the CEA and CA19-9 reagents matched with the automated chemiluminesence detection (ACS: 180.SE). The main RT-PCR reagents were as follows: Trizol reagent, ribonuclease inhibitor (Invitrogen Co., Ltd.), Oligo (dt) 15 Primer, MMLV Reverse Transcriptase (Promega Co.), dNTP, PCR kit (Beijing Tianwei Shidai Co.). PRIMER PREMIERS 5.0 software was used for self-design of the PCR primer, and the hTERT primer serial (product 412 bp, GEN ID: 7015) was as follows, i.e. *i*) the plus sense: 5'-GGT ATG CCG TGG TCC AGA AGG-3' (Seq No 2228-2248); *ii*) the anti-sense: 5'-GCG TGG GTG AGG TGA GGT GT-3' (Serial No. 2639-2620). The β-actin primer serial (product 500 bp) was as follow, i.e. the plus sense: 5'-CTA CAA TGA GCT GCG TGT GG-3', the anti-sense: 5'-AAG GAA GGC TGG AAG A GT GC-3'. All primers were synthetized by the Shanghai Sangon Biological Engineering Technology & Services Co. Ltd.

RT-PCR determination of hTERT mRNA

The procedure included extraction of total RNA, reverse transcription (RT) into cDNA, and PCR amplification with the RT as the template (annealing temperature: 60 ℃). Then a 2.0% of agarose gel electrophoresis was used for appraisement of the total RNA and the size of cDNA amplification fragment. Ethidium bromide staining was employed for displaying the results. See the results in Fig. 1 and 2.

Automated chemiluminescence detection of CEA and CA199

The ACS 180.SE (Bayer, US) automated chemiluminescence detector was used to automatically detect the protein level of CEA and CA19-9 in 60 pleural effusion cases.

Statistical analysis

The results were expressed as $\bar{x} \pm s$ to indicate the mean value, and the t test and χ^2 test were conducted. The size of test was $\alpha = 0.05$.

Results

CEA, CA19-9 and hTERT mRNA expression in pleural effusions

Table 1 shows that the CEA and CA19-9 levels of the group with malignant effusion were significantly higher compared to the group with benign effusion. There was a statistically significant difference between the 2 groups ($P < 0.05$). Table 2 indicates that positive expression of hTERT mRNA was significantly higher in exfoliated cells of the group with malignant pleural effusion compared to the group with benign effusion. Again there was a significant difference between the 2 groups ($P < 0.05$).

Diagnostic value of CEA, CA19-9 and hTERT mRNA expression in benign and malignant pleural effusions

Values of the CEA of over 20 μg/L and of the CA19-9 of over > 37 U/ml, and the hTERT mRNA expression of exfoliated cells were used as criteria to assign a positive malignant pleural effusion, and for diagnostic appraisement of benign and malignant pleural effusions. Comparisons were conducted between the tumor markers, the tumor marker and the group of combined tumor markers, and between the groups of tumor markers.

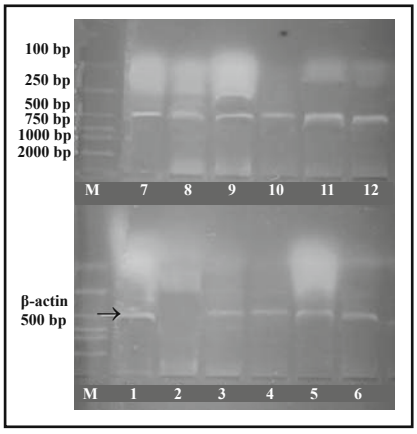

Fig.1. RT- PCR detection of β-actin electro-pherogram. M, the DL2000 marker; 1, positive control; 2, negative control; 3~9, specimens of malignant pleural effusions; 10~12, specimens of benign pleural effusions; all expressions shown, except 2.

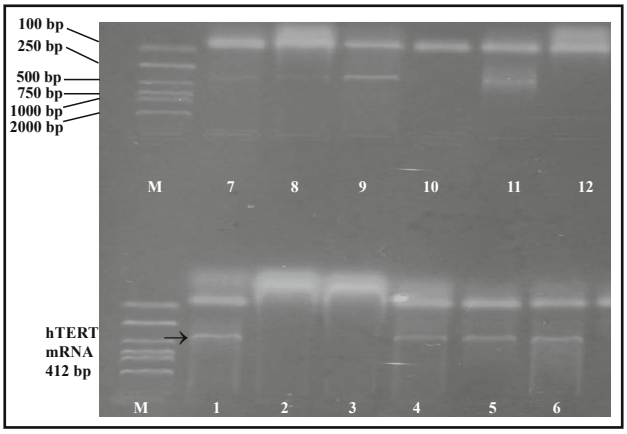

Fig.2. hTERT mRNA electropherogram. M, the DL2000 marker; 1, positive control; 2, negative control; 3~9, specimens of malignant pleural effusions; 10~12, specimens of benign pleural effusions.

Table 1. The CEA and CA19-9 levels in the groups with benign and malignant pleural effusions ($\bar{x}$ ±s).

Effusion group	Cases	CEA (μg/L)	CA19-9 (U/ml)
Malignant	44	106.58 ± 156.41*	122.99 ± 172.37*
Benign	42	3.44 ± 5.69	21.98 ± 43.28

*, compared to the group with benign effusion; *, $P < 0.05$.

Table 2. Comparison in positive hTERT mRNA expression in the exfoliated cells between the groups with benign and malignant pleural effusions.

Effusion group	Cases	Positive cases	Negative cases
Malignant	44	36	8
Benign	42	4	38

$\chi^2 = 45.14$; $P < 0.005$.

Table 3. Appraisement of the diagnostic criteria detected in the pleural effusions.

Group	Markers	Sensitivity (%)	Specificity (%)	Diagnostic accordance rate (%)	Youden Index
1	hTERT mRNA	81.8[a]	90.5[c]	86.1[b]	0.723
2	CEA	52.3[b]	92.9[c]	72.1[b]	0.452
3	CA19-9	34.1[a]	90.5[c]	61.6[a]	0.246
4	hTERTmRNA + CEA	97.7	83.3	90.7	0.81
5	hTERTmRNA + CA19-9	95.5	81.0	88.4	0.765
6	hTERTmRNA + CEA + CA19-9	97.7	78.6	88.4	0.763
7	CEA + CA19-9	59.1	88.1	73.3	0.472

a, refers to the ratio from a comparison between the tumor marker and the group of combined markers, $P < 0.05$; b, refers to the comparison between the marker and the groups of combined markers, except the Group 7, $\chi^2 \geq 21.25$, $P < 0.0001$; c, indicates the comparison between the marker and all groups of combined markers, Fisher's exact probability and two-sided test, $P > 0.05$.

Discussion

Presently, diagnosis of malignant pleural effusions mainly relies on exfolio-cytological examinations, but a positive rate of only 40% to 60% is reached[1]. This is mainly because pleural effusion production by malignant tumors is a complicated process requiring a correlation between the positive rate of an exfoliocytological examination and the related factors, such as the types, sites and harvest of the specimens from the primary tumor. At the same time, it also is influenced by the pathologist's experience in film reading. Owing to a high sensitivity headed for pleural effusion tumor-marker (TM) detection, as well as a convenient method, more and more attention has been paid to methods for differential diagnosis of pleural effusions. Therefore looking for rational TMs and their combination continue to be a research topic of concern. Although the sensitivity of CEA and CA19-9 is not very high for diagnosis of malignant pleural effusions, a combined detection of CA and CA-19-9 with other TMs is usually carried out for differential diagnosis, owing to their favorable specificity[2,3].

Telomerase (TLMA) is, at present, regarded as an available TM with the most common expression for diagnosis of malignant pleural effusions[4]. hTERT is the catalytic subunit of TLMA, and it is the rate-limiting factor for TLMA activity. It has a close relationship with

activity of the TLMA, and with oncogenesis and progression of the cancer[5-7].

Our results showed that there is value in all 3 TMs for differential diagnosis of both benign and the malignant pleural effusions, conforming to reports from China and overseas[8-10]. Among the 3 TMs, hTERT mRNA is obviously superior to CEA, CA19-9 and combined CEA + CA19-9 detection. Because of the low sensitivity and limited value of CA19-9 in diagnosis of malignant pleural effusions, our study suggests that solitary detection of CA19-9 is not a preferred index, and that hTERT mRNA expression is of value for differential diagnosis of pleural effusions. The positive detection rate and diagnostic accordance rate, by combining hTERT mRNA and CEA or/and CA19-9 detection, were superior compared to a solitary detection. In addition the sensitivity of hTERT mRNA detection was greater compared to combined CEA + CA19-9 detection. Among the 3 TMs, hTERT mRNA has the most favorable diagnostic value and a combination detection with CEA or/and CA19-9 may further improve the diagnostic sensitivity indicating that the combined detection of hTERT mRNA + CEA provides the best in diagnosis of pleural effusions.

It was also shown in our study that in the 44 cases with malignant pleural effusion, positive expression of hTERT mRNA was found in 36, but was absent in the other 8 cases. Possible reasons for a report of "false negatives" may include *i*) in one of the 8 cases with a "flase negative" specimen, the patient suffered a relapse after regional chemotherapy, so the number of tumor cells had decreased in the effusion to a level that was too little for detection. *ii*) owing to the nature of the TLMA expression mechanism without telomerase dependence, TLMA was negative in some tumor cells, and no hTERT gene was expressed[11]. *iii*) it has been found that in various common tumors, the expression of TLMA activity was found in only approximately 85~90% of the tumor cells[11]. Therefore it is difficult to reach a 100% positive rate of hTERT mRNA expression of exfoliated cells in malignant pleural effusions. The optimal sensitivity might be closer to 85~90%.

In 42 cases with benign pleural effusion, positive hTERT mRNA expression was found in 4 cases (9.5%). All of these patients suffered from tuberculous pleurisy. Based on the cytological classification of these cases, the percentage of lymphocytes in the effusions was ≥ 85%, which may explain the positive TLMA expressions due to a portion of activated lymphocytes among the large number of lymph cells[12]. In order to establish a more convenient and preferable differentiation between tubercular and the malignant pleural effusions, further studies on better combinations of the markers are needed.

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