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ORIGINAL ARTICLE

The role of pleural fluid MAGE RT-nested PCR in the diagnosis of malignant pleural effusion

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carcinoembryonic antigen; cytology; malignancy; melanoma antigen (MAGE); pleural effusion.

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Abstract

Background: Melanoma antigen (MAGE) genes are expressed in tumor cells, the testis and the placenta. The purpose of this prospective study was to investigate the sensitivity, specificity, and accuracy of the carcinoembryonic antigen (CEA), MAGE reverse transcriptase-nested polymerase chain reaction (RT-nested PCR), and cytology of pleural fluid in the diagnosis of malignant pleural effusion.
Methods: Patients in whom unilateral pleural effusion was identified on chest radiography from January to December 2009 were included in the study. MAGE genes were analyzed by RT-nested PCR using MAGE A1-6 common primers.
Results: Of 81 enrolled patients, 46 were diagnosed as malignant pleural effusion, and 24 were diagnosed as benign pleural effusion. The diagnoses of 11 patients were not confirmed in this study. The diagnostic sensitivity, specificity, and accuracy of MAGE RT-nested PCR were 61.4%, 95.7%, and 73.1%, respectively. The diagnostic sensitivities of cytology and CEA (>5 ng/mL) were 61.4% and 75.0%, respectively. Among 17 patients with negative cytology who had malignant pleural effusion, 12 and 10 patients were positive for CEA (>5.0 ng/mL) and MAGE RT-nested PCR, respectively. However, of five patients with malignant pleural effusion that was not recognized by cytology and CEA, MAGE RT-nested PCR correctly predicted a malignant etiology in only one additional patient (20%).
Conclusions: MAGE RT-nested PCR seems to add little on the combination of conventional methods in the diagnosis of malignant effusion.

Introduction

Malignant pleural effusion is a common clinical problem in patients with malignancy. The diagnosis of a malignant pleural effusion is important in both the management of the effusion and the prognosis of the malignancy.[1] Although pleural fluid cytology is the simplest method of definitively diagnosing malignant pleural effusion, its sensitivity is typically only 50–70%.[2] Closed pleural biopsy is less sensitive than pleural fluid cytology in the diagnosis of malignant pleural effusion.[1] The sensitivity of these methods varies depending on the extent of pleural involvement, the type of primary malignancy, and the volume of pleural fluid tested.[1,3] Thoracoscopy is highly sensitive and is useful to confirm malignant pleural effusion.[1,4] Markers of malignancy could be helpful in predicting malignancy and in suggesting the necessity of thoracoscopy to obtain tissues in patients with suspected malignant pleural effusion and negative pleural fluid cytology. Therefore, previous studies investigated noninvasive tumor markers for the diagnosis of malignant pleural effusion.[5,6]

The melanoma antigen (MAGE) gene is a testicular germ cell tumor antigen first identified among gene coding for tumor regression antigens recognized by cytotoxic T lymphocytes.[7] None of the MAGE genes were expressed in a large

panel of healthy tissues, excluding the testis and placenta.[8] MAGE is expressed in many kinds of malignancies, including lung cancer, stomach cancer, ovarian cancer, and leukemia.[9–12] The use of MAGE reverse transcriptase-nested polymerase chain reaction (RT-nested PCR) in the diagnosis of lung cancer, using specimens from bronchial washing and percutaneous needle aspiration biopsy, has been reported.[13,14] However, the role of MAGE RT-nested PCR in the diagnosis of malignant pleural effusion is not well understood. In this study, we prospectively investigated the diagnostic performances of MAGE RT-nested PCR, carcinoembryonic antigen (CEA), and cytology of pleural effusion in the diagnosis of malignant pleural effusion.

Methods

Patients

This prospective study was performed in patients with pleural effusion at Samsung Medical Center from January 2009 to December 2009. The patients included in this study were 18 years or older, with unilateral pleural effusion on chest radiography. The exclusion criteria were body temperature >38.3°C, peripheral blood leukocytosis (>12 000/μL), bilateral pleural effusion, and contraindication for thoracentesis (prothrombin time international normalized ratio [INR] >1.8 or platelet <50 000/mm^3). The institutional review board of Samsung Medical Center approved this prospective study. Informed consent was obtained from all study subjects prior to intervention. This study was registered at ClinicalTrials.gov (NCT01179685).

Definition

Effusions were considered malignant when one of the following criteria was present: (i) demonstration of malignant cells at cytological examination or in a biopsy specimen; or (ii) pleural thickening or nodularity with fludeoxyglucose (FDG) uptake on chest computed tomography (CT) or positron emission tomography (PET)/CT scans in a patient with a known underlying malignancy that had been pathologically confirmed (clinical diagnosis of malignant pleural effusion).[3] Benign pleural effusion was defined as a specific diagnosis of benign pleural effusion (tuberculous pleurisy, parapneumonic effusion, empyema, and chylothorax) or transudative pleural effusion. The diagnostic criteria of tuberculous pleurisy were as follows: (i) positive *Mycobacterium tuberculosis* culture from pleural fluid; (ii) positive *M. tuberculosis* culture from a pleural biopsy specimen; (iii) chronic granulomatous inflammation with caseation necrosis in a pleural biopsy sample and clinical improvement following antituberculosis chemotherapy; (iv) lymphocyte-dominant exudative pleural effusion, sputum positive for *M. tuberculosis*, and clinical improvement after antituberculosis chemotherapy; and (v) lymphocyte-dominant exudative pleural effusion, pleural fluid adenosine deaminase >45 U/L, and clinical improvement following antituberculosis chemotherapy. Indeterminate pleural effusion was defined as pleural effusion that did not satisfy benign or malignant effusion criteria.

Tumor marker assay

Pleural fluid CEA was measured using a commercially available chemiluminescent immunoassay kit (ADVIA; CENTAUR CEA; Bayer HealthCare, Tarrytown, NY, USA) according to the protocol recommended by the manufacturer.

MAGE RT-nested PCR using A1-6 common primers

For MAGE RT-nested PCR, the specimens were placed in RNA conservative solution and transferred to the laboratory at −20°C and maintained at −70°C until required for mRNA extraction. Total mRNA was extracted from the pleural fluid according to the manufacturer's instructions using an mRNA extraction kit with magnetic capture beads (MAGE Capture Fluid Kit; iC&G Co., Daegu, Korea). RT-nested PCR was performed using common primers for MAGE A1-6.[13,15] In the MAGE gene test, we detected a total of eight subtypes for the *MAGE* gene A1-6 (MAGE A1-A3, A4a, A4b, A5a, A5b, and A6) amplified through two rounds of RT-nested PCR. We used the first PCR products as the template for the second PCR. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used for internal control of PCR. The primer pairs used in RT-nested PCR were as follows; for *MAGE* gene with outer primer pairs (forward GAAGGAGAAGATCTG and reverse TCCAGGTAGTTTTCCTGCAC) and inner primer pairs (forward CTGAAGGAGAAAGATCTGCC(A/T)GTG and reverse CCAGCATTTCTGCCTTTGTGA), and for GAPDH with primer pairs (forward CGTCTTCACCAC CATGGAGA and reverse CGGCCATCACGCCACAGTTT). MAGE and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were amplified using a commercial kit (CancerHunter; iC&G Co., Daegu, South Korea). Distilled water (DW) was used as a negative control. By comparing the results of positive controls, negative controls, and patients corresponding to the molecular weights of the *MAGE* gene, the sample was determined to be negative if a band corresponding to the *MAGE* gene was absent and positive if a band was present (Fig 1).

Statistical analysis

The data are presented as the number (%) or median (range) unless otherwise stated. Diagnostic performances

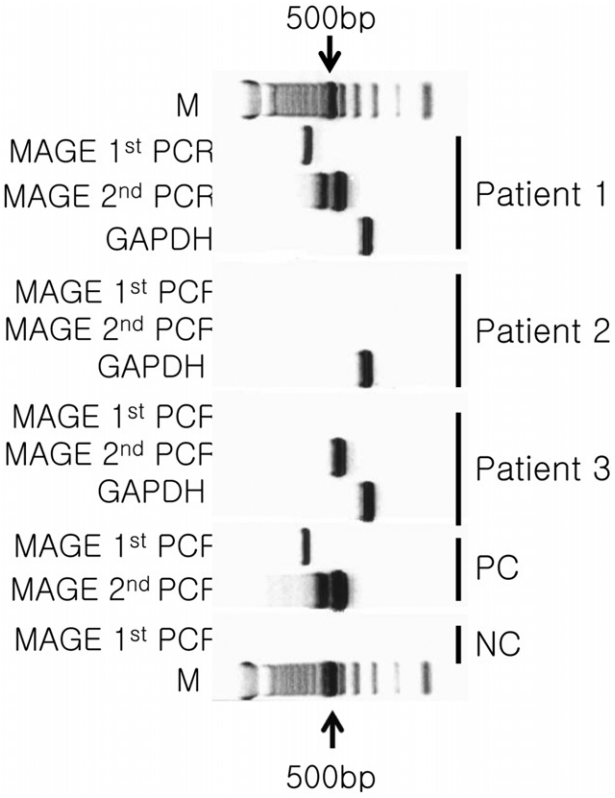

Figure 1 Representative results of the study patients. Melanoma antigen (MAGE) genes were amplified by two rounds (1st and 2nd) of reverse transcriptase-nested polymerase chain reaction (RT-nested PCR) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified by RT-PCR. Patients 1 and 3 displayed positive results for MAGE RT-nested PCR. Patient 2 displayed a negative result for MAGE RT-nested PCR.

analyses were performed for patients confirmed as having benign or malignant pleural effusions and who had available CEA, MAGE RT-nested PCR, and cytology data. When repeated cytologic examinations were performed in study patients, the result of the first cytology was used for the diagnostic performance analysis. We evaluated the sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of CEA, cytology and MAGE RT-nested PCR, for the diagnosis of malignant pleural effusion according to standard definitions. The receiver operating characteristic (ROC) curves were used to assess the best discriminative cutoff value of pleural fluid CEA for the diagnosis of malignant pleural effusion. Generalized estimating equation (GEE) analyses were used to compare the sensitivities of diagnostic methods. A P-value of <0.05 was considered statistically significant. Data was analyzed using PASW statistics 17 (SPSS Inc., Chicago, IL) and R (version 2.11.1).

Results

Patient characteristics

During the study period, 81 consecutive patients with unilateral pleural effusion were enrolled. The median age of these patients was 62 years, and 56 patients (69.1%) were male. Of the enrolled patients, 46 (56.8%) were diagnosed with malignant pleural effusion and 24 (29.6%) were diagnosed with benign pleural effusion (Table 1). The diagnosis of 11 (13.6%) of the 81 patients was not confirmed, despite extensive evaluation. Fourteen patients were excluded from the diagnostic performances analysis; these included the 11 without confirmed diagnoses and three others who were missing pleural fluid CEA data. The modalities used for diagnosing malignant and benign pleural diseases are summarized in Table 1.

Final diagnosis of malignant and benign pleural effusions

Table 2 summarizes the primary origins of the malignant effusions and the final diagnoses of the benign pleural effusions for 67 patients included in the diagnostic performances analysis. Of 44 malignant pleural effusion patients, non-small cell lung cancer was the most common causative factor (n = 34). Of the 23 patients with benign pleural effusion, tuberculous pleurisy was the most common causative factor (n = 18).

Table 1 Baseline characteristics of 81 study patients and diagnostic modalities used for the differentiation of malignant and benign pleural effusions

Characteristic	No. (%) or median (range)
Total patients	81
Age, years	62 (19–89)
Male/female	56 (69.1)/25 (30.9)
Diagnosis	
Malignant pleural effusion	46 (56.8)
First pleural fluid cytology	27 (33.3)
Repeated pleural fluid cytology	4 (4.9)
Pleural biopsy by VATS	6 (7.4)
Clinical diagnosis	9 (11.1)
Benign pleural effusion	24 (29.6)
Positive *M. tuberculosis* culture from pleural biopsy	1 (1.2)
Lymphocyte-dominant exudative pleural effusion with high ADA or positive *M. tuberculosis* culture from sputum	16 (19.8)
Diagnosis of tuberculous pleurisy by VATS	2 (2.5)
Diagnosis of other benign disease by VATS	3 (3.7)
Others	2 (2.5)
Undetermined	11 (13.6)

VATS, video-assisted thoracoscopic surgery.

Table 2 Final diagnosis of the 67 study patients who were included in the diagnostic performances analysis

Diagnosis	No. (%)
Malignant pleural effusion	44 (65.7)
Non-small cell lung cancer	34 (50.7)
Adenocarcinoma	28 (41.8)
Squamous cell carcinoma	2 (3.0)
Large cell neuroendocrine carcinoma	1 (1.5)
NSCLC NOS	3 (4.5)
Small cell lung cancer	1 (1.5)
Metastatic ductal carcinoma, breast primary	2 (3.0)
Metastatic adenocarcinoma, stomach primary	1 (1.5)
Mesothelioma	1 (1.5)
Metastatic hepatocellular carcinoma	1 (1.5)
Metastatic papillary carcinoma, thyroid primary	1 (1.5)
Metastatic peritoneal serous carcinoma	1 (1.5)
Angiosarcoma	1 (1.5)
Metastatic adenocarcinoma, gall bladder primary	1 (1.5)
Benign pleural effusion	23 (34.3)
Tuberculous pleurisy	18 (26.9)
Parapneumonic effusion	2 (3.0)
Chronic empyema	1 (1.5)
Chylothorax	1 (1.5)
Transudate	1 (1.5)

NSCLC NOS, non-small cell lung cancer not otherwise specified.

Diagnostic performances of cytology, MAGE RT-nested PCR, and CEA

The diagnostic performances of cytology, MAGE RT-nested PCR, CEA, and combinations of these methods were analyzed in 67 patients (Table 3). The median pleural fluid CEA value was 3.7 (0.5–13 287.0) ng/mL and ROC curve analysis revealed that the diagnostic performance of pleural fluid CEA was in the favorable range. The area under the curve was 0.907 for pleural fluid CEA and the optimum cutoff value for diagnosis was 5.0 ng/mL for 75.0% sensitivity and 100% specificity.

Diagnostic sensitivity did not differ significantly among the three methods (P = 0.296), with pleural fluid CEA (>5.0 ng/mL) providing the highest sensitivity (75.0%). Among 17 patients with negative cytology that were confirmed as malignant pleural effusion, 12 and 10 patients were positive on CEA (>5.0 ng/mL) and MAGE RT-nested PCR, respectively. However, the diagnostic sensitivities of cytology and MAGE RT-nested PCR were the same, 61.4%. Regarding the combination of cytology and MAGE RT-nested PCR, the diagnostic sensitivity, specificity, and accuracy were 84.1%, 95.7%, and 88.1%, respectively. The combination of cytology and CEA gave diagnostic sensitivity, specificity, and accuracy of 88.6%, 100%, and 92.5%, respectively. For the combination of cytology, CEA, and MAGE RT-nested PCR, the diagnostic sensitivity, specificity, and accuracy were 90.1%, 95.7%, and 92.5%, respectively. Among five patients with malignant pleural effusion that were not recognized by cytology and CEA, MAGE RT-nested PCR correctly predicted a malignant etiology in only one additional patient.

Diagnostic sensitivities of cytology, MAGE RT-nested PCR, and CEA in patients with primary lung cancer

The diagnostic sensitivities of cytology, MAGE RT-nested PCR, and CEA assay in a subgroup analysis of patients with primary lung cancer or other malignancies are summarized in Table 4. In patients with primary lung cancer, the diagnostic sensitivity did not differ significantly among the three methods (P = 0.092), with the pleural fluid CEA assay being the most sensitive (85.7%), followed by cytology (65.7%) and MAGE RT-nested PCR (62.9%). In patients with malignancies other than primary lung cancer, the diagnostic sensitivity did not differ significantly among the three methods (P = 0.105), with MAGE RT-nested PCR being the most sensitive (55.6%), followed by cytology (44.4%) and CEA (33.3%).

Table 3 Diagnostic performances of cytology, melanoma antigen reverse transcriptase-nested polymerase chain reaction (MAGE RT-nested PCR), carcinoembryonic antigen (CEA), and combined approaches

Diagnostic methods	Sensitivity	Specificity	Accuracy	PPV	NPV
Cytology*	27/44 (61.4)	23/23 (100)	50/67 (74.6)	27/27 (100)	23/40 (57.5)
MAGE RT-nested PCR	27/44 (61.4)	22/23 (95.7)	49/67 (73.1)	27/28 (96.4)	22/39 (56.4)
CEA >5.0 ng/mL	33/44 (75.0)	23/23 (100)	56/67 (83.6)	33/33 (100)	23/34 (67.6)
Cytology + MAGE RT-nested PCR	37/44 (84.1)	22/23 (95.7)	59/67 (88.1)	37/38 (97.4)	22/29 (75.9)
Cytology + CEA >5.0 ng/mL	39/44 (88.6)	23/23 (100)	62/67 (92.5)	39/39 (100)	23/28 (82.1)
MAGE RT-nested PCR + CEA >5.0 ng/mL	37/44 (84.1)	22/23 (95.7)	59/67 (88.1)	37/38 (97.4)	22/29 (75.9)
Cytology + MAGE RT-nested PCR + CEA >5.0 ng/mL	40/44 (90.1)	22/23 (95.7)	62/67 (92.5)	40/41 (97.6)	22/26 (84.6)

*Only data from the first cytological examination were included in the analysis. Data are presented as the number/total number (%). CEA, carcinoembryonic antigen; MAGE RT-nested PCR, melanoma antigen reverse transcriptase-nested polymerase chain reaction; NPV, negative predictive value; PPV, positive predictive value.

Table 4 The diagnostic sensitivity of cytology, melanoma antigen reverse transcriptase-nested polymerase chain reaction (MAGE RT-nested PCR), and carcinoembryonic antigen (CEA) in patients with primary lung cancer and other malignancies

Histology	Cytology	MAGE RT-nested PCR	CEA >5.0 ng/mL
NSCLC	23/34 (67.6)	21/34 (61.8)	29/34 (85.3)
Adenocarcinoma	22/28 (78.6)	18/28 (64.3)	26/28 (92.9)
Squamous cell carcinoma	0/2 (0)	1/2 (50.0)	1/2 (50.0)
Others	1/4 (25.0)	2/4 (50.0)	2/4 (50.0)
SCLC	0/1 (0)	1/1 (100)	1/1 (100)
Primary lung cancer	23/35 (65.7)	22/35 (62.9)	30/35 (85.7)
Other malignancy	4/9 (44.4)	5/9 (55.6)	3/9 (33.3)

Data are presented as the number/total number (%). CEA, carcinoembryonic antigen; MAGE RT-nested PCR, melanoma antigen reverse transcriptase-nested polymerase chain reaction; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer. Others, large cell neuroendocrine carcinoma and non-small cell lung cancer not otherwise specified.

Discussion

In this prospective study, diagnostic sensitivities did not differ significantly among the three methods examined. The pleural fluid CEA assay was the most sensitive method (75.0%) as a single test for the diagnosis of malignant pleural effusion. In this study, MAGE RT-nested PCR did not prove more efficient than the combination of cytology and CEA in the diagnosis of malignant effusion. However, the diagnostic sensitivity (55.6%) of MAGE RT-nested PCR as a single test was highest in patients with malignant pleural effusion from other malignancies.

In the diagnosis of malignant pleural effusion, cytological examination is convenient because it is noninvasive. However, in some patients strongly suspected of having malignant effusion, repeated cytology findings may give negative results. In these patients, more aggressive diagnostic modalities, such as thoracoscopic biopsy, may be considered. The markers of malignancy could be helpful in predicting malignancy and in suggesting the necessity of further surgical interventions to obtain tissues in patients with suspected malignant pleural effusion and negative pleural fluid cytology. In previous studies, pleural fluid CEA was the best tumor marker for diagnosing malignant pleural effusion.[5,6,16] Other tumor markers, including cytokeratin 19 fragments and neuron-specific enolase, were less sensitive.[16] In this study, the pleural fluid CEA was the most sensitive test for malignant pleural effusion. Serum CEA may be helpful in the differential diagnosis of non-small cell lung cancer in combination with other tumor markers. The use of serum CEA in diagnosing malignant pleural effusion has been previously evaluated.[16–18] Serum CEA shows high specificity (93–98%) for diagnosis of malignant effusion but the sensitivity was low (33–68%).[16–18]

MAGE genes are expressed only in tumor cells, the male reproductive organs, and the placenta; there is no expression in other normal tissues.[8–12] MAGE RT-nested PCR with MAGE A1-6 common primers has been found to be useful in patients with primary lung cancer.[13,14,19] However, the role of MAGE RT-nested PCR in combination with other diagnostic modalities was not reported. Here, we investigated the diagnostic performances of cytology, CEA assay, MAGE RT-nested PCR, and the combinations of these methods. Of 17 patients with malignant effusion who displayed a negative result on the initial cytological examination, 10 patients showed a positive MAGE RT-nested PCR result. However, among five patients with malignant pleural effusion that were not recognized by cytology and CEA, MAGE RT-nested PCR correctly predicted a malignant etiology in only one additional patient (20%).

In previous studies, the diagnostic sensitivity of MAGE RT-nested PCR differed in relation to the lung tumor histology,[14,19] with the diagnostic sensitivity of MAGE RT-nested PCR in the sputum, bronchial washing fluid, and pleural fluid, higher in patients with squamous cell carcinoma than in those with adenocarcinoma.[19] The diagnostic sensitivity of MAGE RT-nested PCR on tissue samples from percutaneous needle aspiration was 100% in patients with squamous cell carcinoma and 80% in patients with adenocarcinoma.[14] In this study, lung adenocarcinoma was the most common histology of malignant pleural effusion from primary lung cancer (80.0%), with lung squamous cell carcinoma present in only 5.7% of those with malignant pleural effusion from primary lung cancer. Thus, the relatively low sensitivity of MAGE RT-nested PCR can be explained, at least in part, by the distribution of tumor histology. However, MAGE RT-nested PCR had a higher diagnostic sensitivity than CEA and cytology in patients with malignancies other than primary lung cancer. Further studies should examine the diagnostic sensitivity of MAGE RT-nested PCR in relation to tumor origin and histology in patients with malignant pleural effusion.

In this study, the diagnostic specificity of MAGE RT-nested PCR was 95.7%. False positive results were reported for one

patient with chronic empyema. False positive results have also been reported in a previous study using tissue samples from percutaneous needle aspiration biopsy in patients with pulmonary tuberculosis and nonspecific inflammation.[14] The reasons for the false positive results of MAGE RT-nested PCR are unclear.

This study has several limitations. First, the diagnosis of pleural effusion was not confirmed in 11 patients with malignancy (eight for primary lung cancer and three for other malignancies) due to rapid disease progression (n = 3), failure to follow-up (n = 2), and initiation of palliative chemotherapy for documented other metastatic disease (n = 6). Since the pleural effusion observed in patients with malignancies is not always malignant, we excluded these 11 patients in the diagnostic performances analysis. This situation reflects the real practice of unilateral pleural effusion in patients with underlying malignancy. However, the relatively large number of missing diagnoses could have had an impact on the diagnostic performances analysis. In addition, CEA data was missing for three patients as a result of the study being performed in a busy emergency department. One patient was confirmed as tuberculous pleurisy and two patients were confirmed as malignant pleural effusion from primary lung cancer and renal cell carcinoma. However, missing data for CEA may have influenced the diagnostic performances analysis. Second, the distribution of lung cancer histology was not even, with 80% of the lung cancer cases being adenocarcinoma, and the number of study patients relatively small. The observed sensitivity of MAGE RT-nested PCR might have been influenced by the uneven distribution of tumor histology in this study. Third, the diagnostic sensitivity of cytology is contained in the investigation item despite judging effusions as malignant when malignant cells were demonstrated by cytological examination. Therefore, the diagnostic performance of cytology may have been overestimated. Thus, our data should be interpreted conservatively.

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Disclosure

No authors report any conflict of interest.

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