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MN/CA9: a potential gene marker for detection of malignant cells in effusions

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Abstract

Many cancers cause malignant effusions. The presence of malignant cells in effusions has implications in diagnosis, tumour staging and prognosis. The detection of malignant cells currently presents a challenge for cytopathologists. New adjunctive methods are needed. Although the effusions provide excellent materials for molecular assay, the available molecular markers are extremely limited, which hinders its clinical application. MN/CA9 has proved to be a valuable marker in many cancers such as lung, breast, colon, kidney, etc. The present study was to evaluate MN/CA9 as a new molecular marker for the detection of cancer cells in pleural effusions. Seventy-one pleural effusions including 59 malignant effusions from patients with cancer, and 12 patients with benign diseases as a control, were subjected to RT-PCR for detection of MN/CA9 gene expression. MN/CA9 gene expression was detected in 53/59 (89.8%) pleural effusions from cancer patients (15/16 for breast cancers, 10/11 for lung cancers, 4/4 for ovary cancers, 2/3 for colon–rectal cancers, 5/6 for cancers of unknown site, 7/8 for mesothelioma and 10/11 for other cancers). Furthermore, MN/CA9 was positive in 13/18 (72.2%) of cytologically negative effusions of cancer patients. MN/CA9 was detected in only 1/12 (8.3%) effusions from the control patients ($p < 0.01$). The sensitivity and specificity of MN/CA9 gene expression were, respectively, 89.8% and 91.7%. Our preliminary results suggest that MN/CA9 could be a potential marker for the detection of malignant cells in effusions. A large-scale study is needed to confirm these results.

Keywords: *Molecular marker, MN/CA9, serous effusion, RT-PCR*

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Introduction

Serous effusions can often be found in patients with cancer (Monte et al. 1987). Malignant effusion, where malignant cells are found, is distinguished from benign effusion which is mainly composed of lymphocytes, macrophages and several rare mesothelial cells. The effusion may be the first manifestation of malignancy of unknown primary sites (Monte et al. 1987). The presence of malignant cells in

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effusions of cancer patients indicates a metastatic disease or a relapse with very poor prognosis and often implies an aggressive treatment such as chemotherapy. Therefore, detection of malignant cells in effusion has importance in diagnosis, disease staging and prognosis. It is clearly important to detect any malignant cells in the fluid with the greatest sensitivity and specificity. The search for malignant cells in effusions is routine work. However, this work is often difficult for the cytologists, either because of scarceness of cells or because of the delicate distinction between malignant cells and inflammatory cells (Bedrossian 1998). The detection of malignant cells currently presents a challenge for cytologists. In about 40% of cases, cytology does not provide a decisive answer as to whether a pleural effusion from a cancer patient is malignant or not (Porcel et al. 2004, Fetsch & Abati 2001). Thus, additional new methods have been evaluated in order to improve the diagnostic sensitivity and accuracy (Fetsch & Abati 2001, Mohanty & Dey 2003).

Recently, the polymerase chain reaction (PCR) technique has been intensively studied for the detection of cancer cells in blood, bone and lymph nodes and it was thought to be more sensitive than the conventional techniques such as cytology and immunocytochemistry (Gerhard et al. 1994, Mori et al. 1998). However, PCR has been less intensively studied in serous effusions, although effusions provide excellent material for PCR analysis. Several studies that have used the PCR technique for the detection of cancer cells in serous effusions have demonstrated it to be a beneficial adjunct to conventional techniques as it generally increases the detection sensitivity of tumour cells in effusions (Okamoto et al. 1998, Yang et al. 1998, Yu et al. 2001, Nagel et al. 2003, Fiegl et al. 2004). However, the available markers are very limited, which hinders clinical applications.

MN/CA9 has proved to be a useful tumour marker. MN/CA9 is not expressed in most normal cells but is expressed in most malignant cells due to a hypoxic reaction in most cancers such as breast, lung, colon, kidney, etc. These cancers are the main cause of the malignant pleural effusions. Therefore, we hypothesise that MN/CA9 might be a new marker for the detection of malignant cells in pleural effusions.

Materials and methods

Patients and diagnosis of pleural effusions

Pleural effusions from 71 patients were prospectively collected from the Departments of Pneumology and Oncology at the University Hospital Centre of Saint Etienne and the Department of Pneumology at the University Hospital Centre of Lyon, France. All effusions had a reliable diagnosis, documented by cytological analysis, histological analysis of the pleural biopsy (if carried out), and a review of the clinical history and follow-up of the patients. All effusions were subjected to routine cytology examination by Papanicolaou staining. A single experienced cytopathologist performed the examination. The effusions were diagnosed as benign/reactive or malignant by using well-established clinical criteria. A pleural effusion was classified as malignant if malignant cells were demonstrated in the fluid or pleural biopsy. Finally, there were 59 malignant effusions from cancer patients and 12 benign effusions from non-malignant disease caused by infection or cardiac failure, which served as controls. Among the 59 malignant effusions from cancer patients, 18 were cytologically negative. The cancer primary sites of the 59 patients with pleural effusions are given in Table I. This study was approved by an institutional ethics committee.

Table I. Expression of MN/CA9 in pleural effusions.

Diagnosis	Number of effusions	MN/CA
Malignant		
Breast	16	15/16 (93.7%)
Lung	11	10/11 (90.9%)
Ovary	4	4/4 (100%)
Colorectal	3	2/3 (66.7%)
Unknown	6	5/6 (83.3%)
Mesothelioma	8	7/8 (87.5%)
Other[a]	11	10/11 (90.9%)
Total	59	53/59 (89.8%)
Benign	12	1/12 (8.3%)

[a]Other cancers included kidney ($n=1$), prostate ($n=1$), stomach ($n=1$), pancreas ($n=2$), sarcoma ($n=2$), nasopharyngeal ($n=2$) and lymphoma ($n=2$).

Preparation of cells from effusions

Each fluid sample was centrifuged in 50-ml tubes at 300 *g* for 10 min at room temperature. Then, pellets were pooled together, washed once and resuspended in RPMI 1640 medium with 10% fetal calf serum (FCS). Fractions of 900 µl were distributed in 1.5-ml cryotubes containing 100 µl of dimethyl sulfoxide (DMSO) and stored in liquid nitrogen until use. When needed, aliquots were thawed in a water bath at 37°C, then washed once with Ca^{2+}/Mg^{2+} free NaCl solution (0.01 M), and a cell count was performed.

RNA extraction

Total RNA was extracted by using the RNABle® extraction solution (Eurobio, Les Ulis, France) following the manufacturer's instructions. Briefly, 0.2 ml of RNABle was used for a total of 10^6 cells. Cell pellets were homogenised with the RNABle solution (containing guanidium isothiocyanate and phenol), then total RNA was extracted by adding 0.1 volume of chloroform and was precipitated by using 1 volume of isopropanol. Then, samples were washed twice in 75% ethanol and resuspended in RNase-free water. Total RNA was quantified spectrophotometrically at A_{260}.

RT-PCR

Reverse transcription was performed with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Paisley, UK), using 0.5 µg of total RNA and following the manufacturer's instructions.

PCR was performed in 50 µl reaction mixture containing 1 µl of cDNA, 5 µl 10X PCR buffer, 1 µl of 10 mM dNTP mix, 1.5 µl of 50 mM $MgCl_2$, 100 ng of both sense and antisense primers, and 1.25 U of *Taq* polymerase (Eppendorf). We designed two specific primers: β-actin and MN/CA9. The primers for β-actin (507 base pairs) were as follows: sense primer 5′-TAC CAC TGG CAT CGT GAT GGA CT-3′, antisense primer 5′-TCC TTC TGC ATC CTG TCG GCA AT-3′; for MN/CA9 (386 base pairs): sense primer 5′-GGA CAA AGA AGG GGA TGA CC-3′, antisense primer 5′-AAA GGC GGT GCT GAG GTG AA-3′. These primers were designed to span the splice junction so that genomic DNA contamination could be monitored. Primers

for β-actin were also used in order to check RNA integrity and the efficiency of the RT step. Positive (SKRC-52 cell line) and negative controls (PCR mix without cDNA) were included in each round of PCR. After a denaturing step at 94°C for 3 min, PCR was performed with denaturing temperature at 94°C for 1 min, annealing temperature at 57°C for 1 min and extension at 72°C for 1 min. Thirty-five cycles were performed. PCR products were separated by electrophoresis on a 1.5% agarose gel. DNA fragments were visualised and photographed under UV light with ethidium bromide staining. The expected band for MN/CA9 and β-actin was identified by a co-migration of a DNA marker ladder electrophoresed in an adjacent lane. In addition, the fidelity of PCR amplification was confirmed by the sequencing of representative PCR products.

Statistical analysis

MN/CA9 gene expression between the effusions of cancer patients and control patients was compared by using the Fisher's exact test. The level of statistical significance was set at $p < 0.05$. Diagnostic performance of cytology and MN/CA9 gene expression was assessed by sensitivity and specificity.

Results

Results of MN/CA9 expression in the whole population of effusions are shown in Table I. Positive MN/CA9 gene expression is demonstrated in Figure 1. MN/CA9 gene expression was detected in 53/59 (89.8%) pleural effusions from cancer patients (15/16 for breast cancers, 10/11 for lung cancers, 4/4 for ovary cancers, 2/3 for colorectal cancers, 5/6 for cancers of unknown sites, 7/8 for mesothelioma and 10/11 for other cancers). MN/CA9 was only detected in 1/12 (8.1%) effusions from control patients ($p < 0.01$).

Eighteen serous effusions were negative for the tumour cell by cytological examination. Fourteen (72.2%) of these effusions was positive for MN/CA9 gene expression.

The sensitivity and specificity of MN/CA9 were, respectively, 89.8% and 91.7%. The sensitivity and specificity of cytology were, respectively, 70% and 100%.

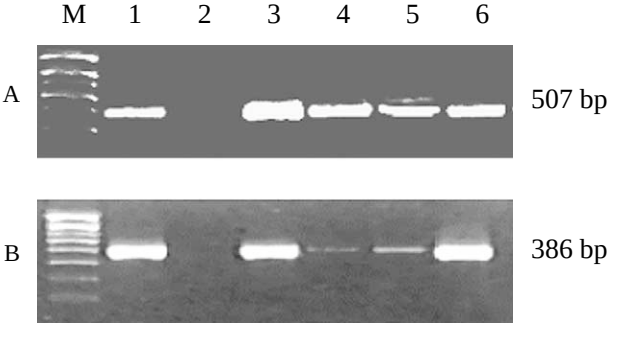

Figure 1. MN/CA9 gene expression in effusions. M, molecular marker; A, β-actin; B, MN/CA9; 1, a positive control; 2, a negative control; 3, 4, 5, 6, four different effusions of a cancer patient positive for MN/CA9.

Discussion

Serous effusions provide excellent material for RT-PCR assay. The choice of molecular markers is critical for this assay. We aimed to evaluate MN/CA9 as a marker that could improve cytological diagnosis in pleural effusions. Thus, we focused our study on a rapid, easy, inexpensive and sensitive technique that could be performed in most hospital laboratories. Basic RT-PCR strategy seemed to be the best choice in terms of sensitivity, specificity, flexibility and cost (To et al. 2003).

Many previous studies have been published concerning tumour markers in the differential diagnosis of pleural effusions. The traditional tumour markers (CEA, CA15-3, CA19-9, CA125) have gained some attention in pleural effusions in several recent studies (Porcel et al. 2004, Shitrit et al. 2005). They concluded that the addition of tumour markers was useful for the diagnosis of malignancy (Porcel et al. 2004, Shitrit et al. 2005, Light 2004). Telomerase was studied for the diagnosis of malignant pleural effusions (Yang et al. 1998). However, contamination of lymphocytes, which could express the telomerase, may weaken its diagnostic value (Lee 2005). New markers such as human mammaglobin/mammaglobin B and mucin were explored for the detection of malignant cells in effusions (Yu et al. 2001, Fiegl et al. 2004). More recently, we found that some new markers (Ep-CAM, E-cadherin and MGB1) could be useful for the detection of cancer cells in effusions (Passebosc-Faure et al. 2005). However, the major problem with these markers is the lack of sensitivity when the marker is used alone. The combination of a panel of markers is required to attain a relatively high sensitivity (Porcel et al. 2004, Passebosc-Faure et al. 2005, Shitrit et al. 2005). The search for a powerful marker is needed. MN/CA9 gene expression has been documented in many cancers and MN/CA9 is considered as a cancer-related marker (Potter & Harris 2003). MN/CA9 is expressed in cancer cells due to hypoxic conditions, allowing cancer cells to survive and retain their clonogenic potential. MN/CA9 has been intensively studied as a promising diagnostic and prognostic marker in lung, breast, colon, kidney, uterus and cervical cancers, etc. (Liao et al. 1997, Chia et al. 2001, Ivanov et al. 2001, Hedley et al. 2003, Kim et al. 2004, Kivela et al. 2005, Kowalewska et al. 2005). These cancers are the major cause of malignant pleural effusions. MN/CA9 was also used to detect cancer cells in lymph nodes and in the circulation (McKiernan et al. 1999, Li et al. 2001). Therefore, to test this marker for the detection of cancer cells in effusions is theoretically reasonable.

We found that about 89.8% of pleural effusions of cancer patients were positive for the MN/CA9 marker. The sensitivity was superior even to cytology. However, we experienced a weak positivity in the control group that could be due to possible weak expression of MN/CA9 of some kinds of cells in effusions. Our results suggest that MN/CA9 may be a highly sensitive and rather specific marker for malignant cells in pleural effusions. Compared with the previous findings of traditional markers, MN/CA9 is among those that present highest sensitivities. For example, the sensitivity of CEA ranged from 35 to 63.6% (Shitrit et al. 2005). This may be explained by the fact that MN/CA9 is a specific marker for diagnosing malignancy in many kinds of tumours while some traditional markers like CEA were relatively limited to a specific organ. The sensitivity of traditional markers depends on the origin of the cancer site. Moreover, the RT-PCR technique is more sensitive than the traditional techniques.

We are interested in the RT-PCR technique of determining MN/CA9 in cytological samples (Li et al. 2006). Recent data suggest that the molecular detection of cancer cells in effusions could have a prognostic value (Buhr et al. 1997, Lloyd et al. 2006).

We studied MN/CA9 expression in a subgroup of cytologically negative effusions of cancer patients. We found that 72.2% of cytologically negative malignant effusions were positive for MN/CA9. Fiegl et al. found 30.9% positivity in cytologically negative effusions when mammaglobin was used, while the positivity increased to 39% when mammaglobin-B was used (Fiegl et al. 2004). The differences were influenced by the choice of molecular markers. We agree that the molecular assay can significantly increase the detectability of malignancy in effusion specimens of cancer patients. As pointed out by Light, the marker may help to select the patients with cytologically negative effusion for further invasive examination (Light 2004). In the meantime, these patients may also benefit from adjunctive treatment since the presence of malignant cells in effusion indicates a metastatic disease. Our preliminary results suggest that the MN/CA9 mRNA assay could be considered as a potential new clinical field of application for the analysis of pleural effusions.

Conclusion

This study demonstrates that MN/CA9 is a potential marker for the detection of malignant cells in pleural effusions, and that a RT-PCR test of MN/CA9 could be a helpful adjunct to cytological examination for the diagnosis of malignancy in effusion.

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