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Detection of Malignant Cells in Pleural Fluid or Ascites by CD44v8-10/CD44v10 competitive RT-PCR

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Background : *CD44 is a cell surface adhesion molecule which has been implicated in various biologic functions as lymphocyte homing and activation, cellular migration and extracellular matrix adhesion. Over-expression of CD44v8-10 has been found in several cancers and is considered to be associated with tumor progression and metastasis. Recently, a novel molecular method, CD44v8-10/CD44v10 competitive reverse transcription-polymerase chain reaction(RT-PCR) has been developed for detecting cancer cells over-expressing CD44v8-10.*

Methods : *We analyzed from benign and malignant pleural effusion and ascites by CD44 competitive RT-PCR and compared to the conventional cytology.*

Results : *The CD44 competitive RT-PCR analysis showed that all the 24 samples associated with benign disease presented a predominant expression of the CD44v10 transcript (v8-10/v10 ratio : 0.126-0.948), whereas 6 of 7 malignant pleural samples associated with cytology positive cancer expressed the CD44v8-10 transcript (v8-10/v10 ratio > 1.00).*

Conclusion : *These results indicate that CD44 competitive RT-PCR assay is a useful and adjunct to cytological examination in cancer diagnosis, especially in detecting exfoliated cancer cells in pleural effusion.*

Key Words : *Malignant effusion; Malignant ascites; CD44v8-10/CD44v10 competitive reverse transcription-polymerase chain reaction*

Introduction

Many patients with non-small cell lung cancer or gastrointestinal tract cancer have pleural effusion or ascites at diagnosis. Generally, cytological examination is the most common method to detect malignant cells in pleural fluid or ascites; however, the technique is known to miss up to more than 50% of tumors[1,2]. It is crucial to detect malignant cells in pleural fluid or ascites for staging work-up and for decision of treatment strategy, especially in non-small cell lung cancer. Pleural or peritoneal biopsy, which is the "gold standard" for diagnosis, is invasive for routine examination. Rather, pleuroscopic or laparoscopic examination is too invasive[3]. Hence, it is important to develop a sensitive and less invasive diagnostic method to improve the detection of malignant cells in pleural fluid or ascites.

CD44 is a cell surface adhesion molecule implicated in diverse biologic processes, such as lymphocyte activation and homing[4,5], extracellular matrix adhesion[6] and cellular migration[7]. CD44 is composed of at least 20 exons, of which 10 exons can be alternatively spliced into different combinations to make up various isoforms[8,9]. It has been reported that various human malignant tumors have

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been associated with over-expression of CD44 variant isoforms, and widespread attention has been focused on CD44 variant isoforms a candidate marker for detection of cancer[10-14]. However, the variant exons that have been associated with cancer have been detected in corresponding normal tissues in other studies[15,16]. Recently, a novel molecular approach, CD44v8-10/CD44v10 competitive RT-PCR[17], has been developed for the detection of cancer cells over-expressing CD44v8-10. This method is based on the concept that the relative expression of CD44v8-10 transcripts to CD44v10 transcripts can be quantified by using the endogenous CD44v10 transcripts as a source of internal standard competitor RNA. We analyzed from benign and malignant pleural effusion and ascites by CD44 competitive RT-PCR for the detection of cancer cells over-expressing CD44v8-10.

MATERIALS AND METHODS

1. Cancer cell lines

AGS, a human gastric adenocarcinoma cell line(Korean Cell Line Bank, Seoul, South Korea) was maintained in RPMI supplemented with 10% heat-inactivated fetal calf serum(FCS), 100 unit/ml penicillin and 0.1mg/ml streptomycin at 37 °C in an atmosphere containing 5% CO_2.

2. Pleural fluid or Ascites

The pleural fluid or ascites were obtained from 11 patients with malignant disease (5 non-small cell lung cancer, 5 gastric cancer, 1 pancreatic cancer) and 24 patients with benign disease (14 Tbc pleurisy, 10 liver cirrhosis), who were treated at Hanyang Medical Center (Table 1, 2, 3). Cellular components from pleural fluid or ascites were examined by both cytological and competitive RT-PCR analysis. For competitive RT-PCR analysis, 25 ml of pleural fluid or ascites samples obtained from patients were centrifuged at 3,000 rpm for 10 min and the precipitated cells were washed with phosphate- buffered saline (PBS) and stored at -70℃ until use.

3. RNA Preparation and Competitive RT-PCR

Total RNA was obtained by the thiocyanate, phenol-chloroform method described previously[18]. Total RNA was spectrophotometrically quantified at 260/280 nm and its quality was tested in 1% agarose gel to find intact

Table 1. CD44v8-10/CD44v10 ratio in pleural fluid from benign disease

Patient	Age/Sex	Disease	Cytology	CD44v8-10/CD44v10 ratio	
1	32/F	Tbc	negative	0.219	
2	64/M	Tbc	negative	0.338	
3	32/F	Tbc	negative	0.423	
4	68/M	Tbc	negative	0.448	
5	22/M	Tbc	negative	0.948	
6	27/F	Tbc	negative	0.367	
7	34/M	Tbc	negative	0.587	
8	52/F	Tbc	negative	0.419	Range:0.219-0.948
9	23/M	Tbc	negative	0.429	
10	65/M	Tbc	negative	0.570	
11	21/F	Tbc	negative	0.222	
12	24/M	Tbc	negative	0.706	
13	60/M	Tbc	negative	0.562	
14	82/M	Tbc	negative	0.710	

Tbc: tuberculosis

Table 2. CD44v8-10/CD44v10 ratio in ascites from benign disease

Patient	Age/Sex	Disease	Cytology	CD44v8-10/CD44v10 ratio	
1	72/F	LC	negative	0.168	
2	70/F	LC	negative	0.662	
3	57/F	LC	negative	0.676	
4	73/F	LC	negative	0.126	
5	49/M	LC	negative	0.359	Range: 0.126-0.756
6	72/F	LC	negative	0.330	
7	42/M	LC	negative	0.148	
8	48/M	LC	negative	0.756	
9	41/M	LC	negative	0.264	
10	62/M	LC	negative	0.481	

LC: liver cirrhosis

28S, and 18S RNAs. cDNA was synthesized from 5 μg of total RNA in a 25 μl reaction mixture containing 5 μl of 5 x reverse transcriptase reaction buffer, 200 μM dNTP, 100 μM of random hexamer, 50 units of RNasin, 2 μl of 0.1 M dithiothreitol, and 200 units of Moloney leukemia virus reverse transcriptase. The mixture was incubated at 37 C for 60 min, heated to 95 C for 10 min, and then chilled on ice.The competitive RT-PCR was performed by using the sense primer, S2 (5"-GACAGAATCCCTGCTACCAATA-3") and the antisense primer, AS2 (5"-ATGTGTCTTGGTCTCCTGATAA-3") that were designed to amplify both CD44v8-10 and CD44v10 cDNA, simultaneously, by utilizing sequence identity(17). Five microliters of cDNA were transferred into

Table 3. CD44v8-10/CD44v10 ratio in malignant pleural effusion or ascites

Patient	Age/Sex	Disease	Source	Cytology	CD44v8-10/CD44v10 ratio
1	69/M	AGC	PE	adenocarcinoma	6.49
2	59/F	AGC	PE	adenocarcinoma	1.055
3	42/M	NSCLC	PE	adenocarcinoma	1.69
4	70/M	NSCLC	PE	adenocarcinoma	1.125
5	72/M	NSCLC	PE	adenocarcinoma	5.179
6	67/M	NSCLC	PE	adenocarcinoma	1.354
7	61/M	NSCLC	PE	adenocarcinoma	0.871
1	56/M	AGC	Ascites	adenocarcinoma	2.06
2	62/M	Pancr	Ascites	adenocarcinoma	0.744
3	67/F	AGC	Ascites	adenocarcinoma	0.883
4	58/F	AGC	Ascites	adenocarcinoma	0.385

AGC: advanced gastric cancer; NSCLC: non-small cell lung cancer; Pancr: pancreatic cancer; PE: pleural effusion

an eppendorf tube containing 200μM dNTP, 1.5 mM $MgCl_2$, 2.5 U *Taq* polymerase and 20 pmoles of primer S2 and AS2. Thirty-five cycles of amplification were performed in a thermocycler (Robocycler 40: Stratagene, La Jolla, CA) at 94 C for 1 min, 56 C for 1 min, and 72 C for 1 min, with a final extension step performed for 10 min at 72 C. To ensure the RNA was of sufficient purity for RT-PCR, a PCR assay with primers specific for the gene β-actin cDNA was carried out in each case. The primer sequences for β-actin primers were as follows: 5"-TGACGGGGTCACCCACACTGTGCCCATCTA-3" and 5"-CTAGAAGCATTGCGGTGGACGATGGAGGG-3". Each series of RT-PCR reactions included mononuclear cell samples from peripheral blood as a negative control and AGS cell samples as a positive control, respectively. The PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining followed by UV transillumination. The resulting images were density scanned and peak intensity of each band corresponding to the amplimers of CD44v8-10 and CD44v10 were then quantified by use of image analyzer, and CD44v8-10/CD44v10 (v8-10/v10) ratio was calculated (Biorad, Fluor-S image analyzer, BMS, USA). All the samples were assessed in 2 independent PCR reactions, and the reported v8-10/v10 ratios represent the mean values from these duplicate determinations.

RESULTS

1. Quantitation of CD44v8-10 variant by CD44v8-10/CD44v10 competitive RT-PCR

To examine the validity of the RT-PCR method as a quantitative assay, 2×10^6 normal mononuclear cells were mixed in separate tests with an increasing number of AGS gastric cancer cells expressing CD44v8-10. The resultant mixtures were subjected to competitive RT-PCR. As shown in Fig. 1 when the v8-10/v10 ratios were plotted against the number of added AGS cells, linear relationships were observed.

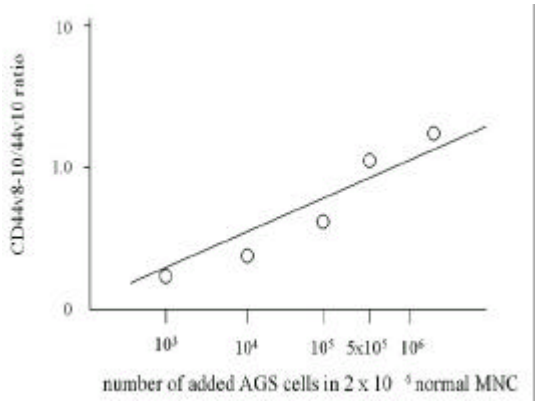

Figure 1. The CD44v8-10/CD44v10 ratios were plotted against the number of added cancer cells on a log-log scale.

2. Competitive RT-PCR for pleural samples

Twenty one samples, 14 from patients with benign pleural fluid and 7 from patients with malignant effusion were analyzed by competitive RT-PCR by using the set of primers, S2 and AS2, that can co-amplify CD44v8-10 and CD44v10. The CD44v10 transcript was predominantly expressed in comparison with the CD44v8-10 transcript in all 14 of the pleural effusion samples from patients with benign disease (v8-10/v10 ratio: 0.219-0.948),

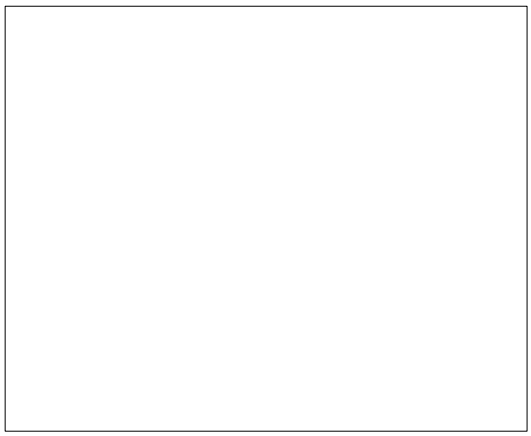

Figure 2. Competitive reverse transcription-polymerase chain reaction(CC-RT-PCR) products were electrophoresed on ethidium bromide-stained 2% agarose gels from benign diseases (tuberculosis and liver cirrhosis). M; 1-kilobase molecular weight markers. The CD44v8-10/CD44v10 ratios for each sample are also shown.

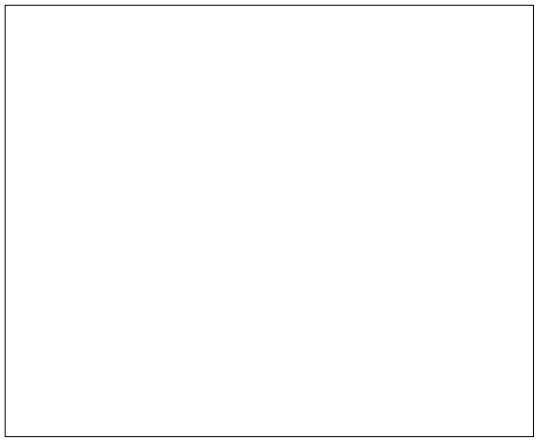

Figure 3. Competitive reverse transcription-polymerase chain reaction (CC-RT-PCR) products were electrophoresed in ethidium bromide-stained 2% agarose gels from malignant pleural effusion and malignant ascites. M; 1-kilobase molecular weight markers. The CD44v8-10/CD44v10 ratios for each sample are also shown.

whereas 6 of 7 samples from patients with malignant cancers (5 non-small cell lung cancer, 2 gastric cancer) presented a predominant expression of CD44v8-10 transcript (Table 1,3). The representative data are shown in Figure 2 and 3. When we place the diagnostic cutoff value for the v8-10/v10 ratio at 1.0, the sensitivity and specificity of competitive RT-PCR were 85.7% and 100%, respectively.

3. Competitive RT-PCR for ascites samples

Fourteen samples, 10 from patients with benign ascites (10 liver cirrhosis) and 4 from patients with malignant ascites (3 gastric cancer, 1 pancreatic cancer) were analyzed as previously described. All the ascites samples from patients with benign disease showed CD44 v8-10/CD44v10 ratio < 1.0 (range: 0.126-0.756). However, only one of four malignant ascites (gastric cancer) revealed > 1.0 (Table 2,3). The representative data are shown in Figures 2 and 3.

DISCUSSION

It has been known that conventional cytological analysis does not have high sensitivity to detect malignant cells in pleural fluid or ascites[1,2]. At present, there is no reliable biological marker that is used for routine clinical examinations. Recently, molecular biological techniques make it possible to detect cancer cells in those samples.

Since it has been reported that a CD44 splice variant containing variant exon 6 could confer metastatic potential on rat pancreatic adenocarcinoma cells[19], a great deal of interest was focused on the CD44 splice variants expression in human malignant tumors. Many investigators reported that the expression of specific CD44 variant exons was detected in human carcinomas, such as bladder cancer, non-small cell lung cancer and breast cancer, indicating that molecular approaches of CD44 splice variant might be a promising tool for cancer diagnosis[12-15].

Other investigators demonstrated that among the CD44 splice variants, CD44v8-10 was predominantly expressed in human colon cancer and non-small cell lung cancer by RT-PCR using the set of primers that are able to amplify all CD44 variant isoforms and DNA sequence analysis[20-21]. However, CD44v10 was also expressed in normal leukocytes, so the discrimination method should be needed. Recently, Okamoto *et al*[17] developed noble competitive RT-PCR with which to quantify the relative expression of CD44v8-10 transcripts to CD44v10 transcripts. The endogenous CD44v10 transcripts, which were identified as the predominant CD44 splice variants in leukocytes, were used as internal standard, competitor RNAs for measuring the expression level of CD44v8-10 transcripts.

When we applied the competitive RT-PCR assay to pleural fluid and ascites, our results showed that the

v8-10/v10 ratios in all the samples associated with benign disease were less than < 1.0, whereas malignant diseases revealed significantly higher than those associated with benign diseases. Although the v8-10/v10 ratio displayed large variation between the benign diseases, the ratios were consistently low in various benign diseases suggesting that the v8-10/v10 ratio might be a useful molecular marker for cancer diagnosis. However, in malignant ascites only one sample showed dominant CD44v8-10. This discrepant result should be elucidated with a large number of samples from ascites. These findings indicate that the CD44v8-10/v10 ratio may be an important molecular marker for cancer diagnosis, especially in exfoliated cells from pleural fluid.

CONCLUSION

CD44 competitive RT-PCR assay is a useful and adjunct to cytological examination in cancer diagnosis, especially in detecting exfoliated cancer cells in pleural effusion. To determine the exact role of CD44 variant in the diagnosis of exfoliated cells from pleural fluid or ascites, further studies with a large number of samples are needed.

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