Differential Expression of CD44 Splice Variants in Malignant and Benign Pleural Effusions

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Tojo, N., Inase, N., Ichikawa, M., Miyazato, I. and Nara, N. Differential Expression of CD44 Splice Variants in Malignant and Benign Pleural Effusions. Tohoku J. Exp. Med., 1996, 179 (4), 273-279 —— Expression of CD44 isoform that contains variant exons (v1-v10) has been implicated in tumor progression and metastasis. Especially, CD44 isoform containing v6 (CD44v6) and that containing v7 (CD44v7) were shown to confer full metastatic behavior on tumor cells. We examined the expression of CD44v6 and CD44v7 in malignant pleural effusions (13 lung cancers and 4 non-lung cancers) and in benign pleural effusions (7 tuberculosis and 3 pneumonia) with Southern blot analysis of reverse transcription (RT)-polymerase chain reaction (PCR) products. CD44v6 was expressed not only in malignant pleural effusions (12 of 13 lung cancers and 4 of 4 non-lung cancers), but also in benign pleural effusions(9/10). In contrast, although expression of CD44v7 was found in most malignant pleural effusions (12 of 13 lung cancers and 4 of 4 non-lung cancers), it was found in only a few cases of benign pleural effusions. These results suggest that the expression of CD44v7 may be correlated with a tumor-specific event such as metastasis or dissemination in malignant pleural effusion, while no such correlation can be found with CD44v6.

CD44 adhesion molecule is expressed in a variety of cells and tissues, including hematopoietic, epithelial, and mesothelial cells. CD44 gene is composed of at least 20 exons, 10 of which can be alternatively spliced. The most abundant basic CD44 isoform, the so-called hematopoietic form (CD44H), is generated by splicing out 10 consecutive exons within the extracellular domain. Primary diversity in CD44 isoforms results from alternative splicing of these 10 exons, which are called variant exons (v1-10). Expression of particular CD44 isoforms may be linked to tumor progression and metastatic potential in breast (Matsumura

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and Tarin 1992; Dall et al. 1995; Iida and Bourguignon 1995), colon (Tanabe et al. 1993; Mulder et al. 1994), and gastrointestinal cancers (Heider et al. 1993a; Harn et al. 1995). In support of these observations, overexpression of CD44 isoform containing exons v6-7 (CD44v6-7) was shown to confer full metastatic potential upon a rat tumor cell line (Rudy et al. 1993). Furthermore, monoclonal antibodies specific to CD44v6 prevented metastatic spread of a rat pancreatic cancer in vivo (Seiter et al. 1993).

In lung cancer, CD44H has been reported to be frequently expressed in non-small cell type, but rarely expressed in small cell type (Penno et al. 1994). As for CD44 isoform containing variant exon (CD44v), CD44v4-7 was expressed in all of 23 cases of localized lung cancer that could be surgically dissected. However, the expression of CD44v, which might contribute to tumor progression and metastasis, has not been examined in advanced lung cancer.

To examine whether CD44v6 or CD44v7 is expressed in advanced lung cancer, we analyzed the cancer cells in malignant pleural effusion with Southern blot analysis of reverse transcription (RT)-polymerase chain reaction (PCR) products. The results showed that although CD44v6 was expressed in both advanced cancer and benign diseases, CD44v7 was almost exclusively expressed in advanced cancer, suggesting that v7 in CD44 might be a tumor-specific exon.

**Materials and Methods**

**RNA extraction from pleural effusion**

Pleural effusion samples were taken from 15 patients with lung cancer (11 adenocarcinoma, 3 squamous cell carcinoma, and 1 small cell carcinoma) and 5 samples with non-lung cancer (stomach, liver, kidney, ovary, and bile duct). All effusions were confirmed by cytologic study to be associated with malignancy. Lung cancers (n = 15) were staged according to the TNM classification (International Union Against Cancer (UICC) 1987) into Stage IIIB (n = 5) and Stage IV (n = 10). As controls, pleural effusions were also collected from 10 patients with benign diseases (7 tuberculosis, and 3 parapneumonic effusions). All samples were centrifuged at 2000 rpm for 10 min, and their pellets were stored at −80°C until use. RNA was extracted with the acid guanidinium thiocyanate-phenol-chloroform technique (Chomczynski and Sacchi 1987).

**Southern blot analysis of reverse transcriptase (RT)-polymerase chain reaction (PCR) products**

Approximately 1 µg of total RNA was used to synthesize first strand oligo(dT) primed complementary DNA (cDNA) at 37°C for 1 hr with Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT) (GIBCO/BRL, Gaithersburg, MD, USA). Polymerase chain reaction was performed in triplicate under the following condition; 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, 35 cycles. Four primers (S1, AS2, AS3, and AS4) were designed to be located in exon 5, exon
Fig. 1. Schematic representation of the human CD44 gene. There are 20 exons encoding the human CD44 gene. The splice variant lesion (exon v1 to v10) is inserted within the extracellular domain. The positions of PCR primers S1, AS2, AS3, and AS4 specific for the exons 5, 10 (v6), 11 (v7), 16 and the oligonucleotide probe P specific for the exon 5 are shown.

6, exon 10(v6), and exon 11(v7), respectively (Screaton et al. 1992) (Fig. 1).

PCR products were separated on 2% agarose gels, transferred to nylon membrane filters (Hybond N+ membrane, Amersham, Buckinghamshire, UK), and then hybridized with $^{32}$P-end-labelled CD44 oligonucleotide probe located in exon 5 (Fig. 1). Moreover, to exclude non-specific detection of v6 and v7 expression, the same S1-AS2 PCR products were hybridized with AS3 primer located in exon 10(v6) and AS4 primer located in exon 11(v7), respectively. The sequences of the sense and antisense primers and oligonucleotide probes are as follows;

S1      ; 5'-TCCCAGACGAGACACGATCCCTGGGA-3' (24 mer)
AS2     ; 5'-TTCCAGATCCATGAGGATCGCGAG-3' (24 mer)
AS3     ; 5'-AGTCCAGGACTGTCCCTCTGG-3' (21 mer)
AS4     ; 5'-CGCGATATCCCTCATGCGATCTGTG-3' (21 mer)
Probe   ; 5'-TAGCGGGATTCTGTCGATG-3' (21 mer)

RESULTS

V6 and v7 expression was not in agreement in 3(2 lung cancers and 1 non-lung cancer) of 30 cases. In these three cases, CD44v6 isoforms were expressed by the S1-AS3 PCR sample products hybridized with the probe located in exon 5, but not by the S1-AS2 PCR sample products hybridized with AS3 primer located in exon v6. In 2 of the 3 cases, CD44v7 isoforms were expressed only by the S1-AS4 PCR sample products hybridized with the probe located in exon 5. These three cases were regarded as non-specific bands, and were not included in the results.

Expression of CD44H in pleural effusions of lung cancer, non-lung cancer, and benign diseases

The primers’ S1 and AS2 amplified not only CD44H isoform (167bp) but also all isoforms of CD44 regardless of the number of inserted exons. CD44H isoforms were expressed in 13/13 of lung cancers, 4/4 of non-lung cancers, and 10/10 of
benign diseases (Figs. 2, 3).

Expression of CD44v6 in pleural effusions of lung cancer, non-lung cancer, and benign diseases

The primers’ S1 and AS3 amplified only CD44 isoform containing v6 (CD44v6). CD44v6 was expressed in 12/13 of lung cancers, 4/4 of non-lung cancers, and 9/10 of benign diseases. In contrast to the situation observed with benign diseases, the bands in most of the lung cancers increased both in number and in intensity. The levels of CD44v6 varied in size and number among individual lung cancers, and no correlations were observed with different lung cancer types, including adenocarcinoma, squamous cell carcinoma, and small cell carcinoma. In non-lung cancers, however, bands were only weakly detectable (Figs. 2, 3).
Fig. 3. Southern blot analysis of RT-PCR products from pleural effusions of non-lung cancers and benign diseases. The PCR products obtained with CD44-specific primers S1 and AS2 (Total), S1 and AS3 (v6), and S1 and AS4 (v7) were separated on 2% agarose gels and transferred to nylon membrane filters. Filters were hybridized with oligonucleotide probe to the constitutional exon probe P (see Fig. 1). In Total, the 167bp band present in all cases corresponds to the expected CD44H amplification product. In v7, variant bands obtained with benign disease samples were detected in 2 of 10 cases.

**Expression of CD44v7 in pleural effusions of lung cancer, non-lung cancer, and benign diseases**

The primers’ S1 and AS4 amplified only CD44 isoform containing v7 (CD44v7). While CD44v7 was expressed in 12/13 of lung cancers and 4/4 of non-lung cancers, it was expressed in only 2/10 of benign diseases, suggesting that it is almost exclusively expressed in cancer. The levels of CD44v7 varied in size and number among individual lung cancers and non-lung cancers, and were not correlated with different pathological types of lung cancers and non-lung cancers (Figs. 2, 3).

**Discussion**

In the present study, we examined the expression of CD44H, CD44v6, and
CD44v7 in malignant and benign pleural effusions. We observed the differential pattern of CD44v expression. Although CD44H and CD44v6 were expressed in both malignant and benign pleural effusion, CD44v7 was mainly expressed in malignant pleural effusion.

This result does not conflict with the previous report that all the cases of lung cancer cells express CD44v4-7 (Washimi et al. 1994). CD44v6 bands in lung cancers, increased in both number and intensity compared with those non-lung cancers while no such increases were noted in CD44v7 bands. The causes of malignant pleural effusions of lung cancers were mainly due to dissemination, while those of the non-lung cancers were due to metastasis from remote tumors. These differences may influence the different patterns of CD44v6 expression.

Expression of CD44v6 and CD44v7 in normal human tissues was observed in squamous epithelium of epidermis, tonsil, pharynx, and glandular epithelium of the pancreatic duct, but was nearly absent in other epithelial and non-epithelial tissues (Heider et al. 1993b). The normal mesothelial cell line expressed CD44v, but not CD44v6 or CD44v7 (Jackson et al. 1994). In a lymphohematopoietic system, lymphocytes and macrophages highly expressed CD44H, but usually did not express CD44v (Heider et al. 1993b). However, both B and T lymphocytes as well as macrophages transiently expressed CD44v after antigen stimulation or in postnatal period of rat (Arch et al. 1992). Furthermore, Arch et al. (1992) reported that lymph node cells expressed only CD44v6. Since cellular components in infectious pleural effusion include activated lymphocytes and macrophages, it is reasonable that expression of CD44v6 was detected in infectious pleural effusions. On the Southern blot analysis, there were more than four CD44v6 bands in most cases of lung cancers, while there were less than three in benign diseases. Metastatic cells in pleural effusion might have a splice disorder, and there were several splice variants in each pathological type and case. Whereas lymphocytes in benign conditions might be a physiologically regulated system, splice variants might be limited to a few defined products.

There was a marked difference in CD44v7 expression between malignant and benign pleural effusions. CD44v7 was found in most patients with malignant pleural effusion and in only one patient with pneumonia. These results suggested that CD44v7 may be almost exclusively expressed in malignant pleural effusions, although its functional role in tumor progression and metastasis remains to be elucidated. Cellular components in malignant pleural effusions consist of mesothelial cells, lymphocytes, macrophages, and granulocytes, in addition to cancer cells. Although, we cannot entirely exclude the possibility that non-tumor cells express CD44v7, the infrequency of the expression of CD44v7 in benign pleural effusions supports the postulation that v7 may be a cancer-specific exon.

References

1) Arch, R., Wirth, K., Hofmann, M., Ponta, H., Matzku, S., Herrlich, P. & Zöller, M.


