行政院國家科學委員會專題研究計畫　成果報告

原發性肺癌 CD44 基因之表現與 DNA 之甲基化的關係(2/2)

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中文摘要

CD44 是位於細胞表面的分子，是細胞外基質 hyaluronan 的受體. 細胞可藉著其表面的 CD44 分子黏附於細胞外基質，淋巴球可藉著其表面的 CD44 分子黏附於淋巴結內之 high endothelial venules，癌細胞亦可藉著其表面的 CD44 分子黏附於基質，造成轉移. 本計劃主持人在前一年度之計劃曾發現，肺腺癌惡化時, CD44v6 之表現會減弱，其機轉尚不明. 本計劃即研究肺癌 CD44 基因之轉錄調控區之甲基化情形，目的是觀察 CD44 基因之表現受到抑制是否與其轉錄調控區中 CpG sites 之甲基化有關. 方法是將 15 個手術切除之肺腺癌組織的 genomic DNA 抽出後，經過 bisulfite 處理，再以 PCR 增量 CD44 基因之轉錄調控區，然後作 DNA 序列分析，找出此調控區中 6 個 CpG sites 之甲基化情形. 結果發現肺腺癌 CD44v6 之表現與其轉錄調控區中 CpG sites 之甲基化之間並無顯著之關係.

ABSTRACT

Methylation of the CD44 promoter at one or more CpG dinucleotides has been proposed as being important in the control of CD44 expression. In our previous study we found CD44 was down-regulated in patients with more advanced stages of pulmonary adenocarcinoma. Therefore in this study we examined the methylation status of the 6 CpG sites in the promoter region upstream the CD44 gene translation initiation site by genomic sequencing using differential base modification by sodium bisulfite. In 15 tumor samples of the surgically resected pulmonary adenocarcinoma, we found there was no consistent relationship between methylation status of any of these 6 CpG sites and the expression of the CD44v6 protein as studied by immunohistochemical staining.

INTRODUCTION

CD44 is a type I transmembrane protein that functions as a major cell surface receptor for hyaluronan (1,2). Experimental evidence indicates a role for CD44 in mediating cell migration (3,4), lymphocyte roling (5), and lymphocyte migration into sites of inflammation (6,7). CD44 has also been implicated in mediating tumor dissemination and metastasis (8,9). While CD44 shows a broad tissue distribution (10,11), expression of the molecule is regulated in certain circumstances, such thymocytes (12) and human B cells (13). Quantitative and qualitative regulation of CD44 expression has also been noted in tumor cells. In non-Hodgkin's lymphoma, a high level of CD44 expression is a negative prognostic factor (14). In an experimental rat model of prostate cancer (15) and in human prostate cancer (16), loss of CD44 expression is correlated with metastasis and a poor prognosis.

Using methylation-sensitive restriction endonucleases, failure to express CD44 in tumor cells has been correlated with methylation of one or more sites within an approximately 600-bp region immediately upstream of the site of CD44 translation initiation in nonmetastasizing fibrosarcoma cell lines (17) and in prostate tumors (18). These observations have led to the suggestion that methylation of CpG dinucleotides within his upstream region might be one mechanism by which CD44 gene expression is regulated.

The region immediately upstream of the site of CD44 translation initiation functions as a promoter (19,20), and is rich in CpGs that could be potentially methylated. In the human, 44 CpGs are clustered in the 650-bp region immediately upstream of the position of translation initiation (19). Only a small part of these CpGs can be detected by restriction-sensitive endonucleases (21).

In our previous study on the CD44 expression in non-small cell lung cancer, we found CD44 expression downregulated in patients with more advanced stages of adenocarcinoma (22). In order to understand the relationship between the down-regulation of CD44 expression and methylation status of CD44 promoter region, we examined the methylation status of the cytosine residues within the CpG-rich regions immediately upstream of the CD44 gene in non-small cell lung cancer by genomic sequencing and correlated the methylation status with CD44 expression of the tumor.

MATERIALS AND METHODS

Specimens

Fifteen samples of pulmonary adenocarcinoma were obtained from surgically resected specimens. The expression of CD44v6 was examined by immunohistochemical staining in our previous study (22).

Genomic DNA Sequencing for Methylation Status of CD44 Promoter Region (23)

Genomic DNA was extracted from lung cancer samples using DNAzol reagent kit.

Bisulfite Modification

DNA (1 ug) in 50 ul was denatured by NaOH (final conc: 0.2 M) for 10 min at 37oC. 30 ul of 10 mM hydroquinone and 520 ul of 3M sodium bisulfite at pH5, both freshly prepared, were added and mixed, and samples were incubated at 50oC for 16 hours. Modified DNA was purified using the Wizard DNA purification resin and eluted into 50 ul of water. Modification was completed by NaOH (final conc: 0.3 M) treatment for 5 min at room temp, followed by ethanol precipitation.

PCR Amplification of Bisulfite-Modified DNA (19,21,23,24)

Selected regions of the 450-bp upstream regulatory region were amplified by PCR after bisulfite conversion. PCR products were purified on agarose gels using Viogen DNA purification kit. Sequencings were performed using automatic sequencing analyzer and the BigDye Terminator sequencing kit.

Results

The methylation status of the 6 CpG sites within the 450-bp upstream regulatory region of CD44 gene was examined on 15 resected samples of adenocarcinoma, and was shown

in Table 1. The methylation status was determined by examining the relative sensitivity of the respective cytosine residues to modification by sodium bisulfite under conditions where nonmethylated cytosine residues are converted to uracil in single-stranded DNA, while methylated cytosine residues remain unreactive (21,23,24). We studied 6 CpG sites between –447 and translation initiation. All 6 CpG sites were examined for the plus (sense) and minus (antisense) strand. Table 2 shows that the expression of CD44v6 was not regulated by the methylation status of any of the 6 CpG sites studied.

Table 1. CD44v6 expression shown by immunohistochemical stain and methylation status at 6 CpG sites in the upstream regulatory region of CD44 gene in 15 patients with pulmonary adenocarcinoma

No.	Histology	CD44v6 Expression	Methylation Status at CpG Site					
			3	4	5	6	7	8
9601	AD	+	u	u	u	u	u	u
9629	AD	+	m	m	m	m	u	u
9630	AD	+	m	m	m	m	u	m
40	AD	+	u	m	m	u	u	u
52	AD	+	u	m	m	u	u	u
55	AD	+	m	m	m	m	m	m
57	AD	+	m	m	m	m	u	m
59	AD	+	m	m	m	u	u	m
9631	AD	+	u	u	m	u	u	u
43	AD	+	u	u	u	u	u	u
51	AD	-	u	u	u	u	u	u
9640	AD	-	u	m	m	m	u	u
9644	AD	-	m	u	u	u	u	u
33	AD	-	u	m	u	m	m	m
49	AD	-	u	m	m	u	u	u

AD: adenocarcinoma
u: unmethylated
m: methylated

Table 2. Percentage of methylation at 6 CpG sites within the upstream regulatory region of the CD44 gene in 15 patients with pulmonary adenocarcinoma

	% of CpG Site Methylated					
	3	4	5	6	7	8
Adenocarcinoma showing (+) CD44 v6 staining, n=10	50	70	80	40	10	40

Adenocarinoma showing (-) CD44 v6 staining, n=5	20	40	40	40	20	20

Discussion

Gene methylation has long meen known to contribute to transcriptional repression (25). Methylation may act either directly by inhibiting the binding of transcription factors to methylated CpG-containing binding sites (26) or more indirectly by biinding factors that recognize methylated sequences and act via localized histone deacetylation to inhibit the binding of factors necessary for transcription initiation or progression (27,28). Based on a correlation between CD44 expression and the methylation of *HPAII* or *HhaI* sites within a region of approximately 600 bp upstream of the position of initiation of CD44 translation – a region that is known to contain the CD44 promoter (19,20) – methylation of CpGs within this region has been suggested to act to control CD44 expression (17,18). The experiments in our study were designed to examine this point in more detail.

Analysis of the methylation status of the 6 CpGs in the 450-bp upstream region of CD44 translation initiation site was determined by genomic sequencing using differential base modification by sodium bisulfite. Our study showed that in human pulmonary adenocarcinoma, there was no consistent relationship between methylation status of the CD44 promoter region and CD44v6 expression.

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Self-Assessment of Project Success

We did not find a positive correlation between DNA methylation of any of the 6 CpG sites in the upstream promoter region and CD44 v6 expression. The results could mean:

1. There is actually a positive correlation between DNA methylation within the promoter region and CD44 expression. We failed to document the correlation because: (A) it involves other CpG sites; (B) the correlation involves other isoforms of CD44 molecules; (C) it involves methylation status of the downstream, not upstream, regulatory region of the CD44 gene; and (D) the sample size is too small.

2. The CD44 expression is not regulated by DNA methylation status in the upstream promoter region, but by other mechanisms such as splicing sites selection, metalloprotease activity, the activation or inhibition of other oncogenes.

3.