Short Communication

Reduced Expression of the Pcdh20 Gene and Its Aberrant DNA Methylation in Lung Adenocarcinomas Induced by N-nitrosobis(2-hydroxypropyl)amine in Rats

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Abstract: Protocadherins are a major subfamily of the cadherin superfamily that play an important role in communication between adjacent cells. To clarify the involvement of Protocadherin 20 (Pcdh20) gene in rat lung carcinogenesis, we investigated the expression of Pcdh20 and its methylation status in rat lung adenocarcinomas induced by N-nitrosobis(2-hydroxypropyl)amine (BHP). Six-week old male Wistar rats were given 2000 ppm BHP in their drinking water for 12 weeks and were maintained without further treatment until they were sacrificed after 25 weeks. Total RNAs were extracted from 7 lung adenocarcinomas, one from each BHP-treated rat, and the expression levels of Pcdh20 were measured using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. Four out of 7 tumors showed reduced expression of Pcdh20, compared with 3 normal lung tissues. For methylation analysis, bisulfite sequencing was performed. The 5' upstream region of Pcdh20 was methylated in 4 adenocarcinomas with reduced expression of Pcdh20, but was unmethylated in normal lung tissue. These results suggest that aberrant methylation of the Pcdh20 gene might be involved in the development of lung adenocarcinomas induced by BHP in rats. (J Toxicol Pathol 2008; 21: 257–260)

Key words: Pcdh20, methylation, rat, lung adenocarcinoma, nitrosamine

The cadherins are a family of cell surface glycoproteins responsible for cell-cell adhesion, cell polarity and tissue morphology1. Protocadherins are a major subfamily of the cadherin superfamily, and more than 60 protocadherins have been identified2,3. A few members of the protocadherin family are suspected to be involved in the development of several tumors, including colon, liver and renal cancers4,5. Among them, aberrant DNA methylation and reduced expression of the protocadherin 20 (PCDH20) gene have been found in non-small cell lung cancers (NSCLCs), suggesting that epigenetic alterations of PCDH20 may play an important role in the development of NSCLCs6. Moreover, restoration of PCDH20 expression in NSCLC cells reduces cell numbers in colony formation and anchorage-independent assays6.

Lung cancer is one of the most common human malignancies. The experimental model used in the present study features the development of NSCLCs in rats given BHP in their drinking water, with high yields of adenomatous lesions, including adenocarcinomas7,8. As it is possible to monitor the step-by-step development of lung malignancies with this model, the molecular mechanisms involved can be readily investigated. Taking advantage of this model, we have been able to accumulate data on genetic alterations during carcinogenesis including Ki-ras mutations9, alterations in genes associated with the transforming growth factor-β signaling pathway10,11 and alterations in tumor suppressor genes located on human chromosome 3p12,15.

Methylation of cytosine residues at CpG dinucleotides is characteristic of suppression of gene expression in mammalian genomes16,17. Cytosine methylation can reduce the binding affinity of transcription factors and influence chromatin structure16–19. It has been suggested that aberrant DNA methylation of the promoter regions of genes is the major mechanism of gene silencing in several tumors16,17.
Recently, we identified aberrant DNA methylation patterns on the genes encoding E-cadherin, p16 and Rassfl1a in rat lung adenocarcinomas, and these methylation patterns were associated with reduced expression of those genes. In the present study, we measured expression of the Pcdh20 gene and its DNA methylation status in the promoter region in lung adenocarcinomas induced by BHP in rats to better understand the involvement of the Pcdh20 gene during lung carcinogenesis.

A total of 10 male Wistar rats were purchased at 5 weeks of age from Japan SLC Inc. (Shizuoka, Japan). All animal maintenance and experimental procedures were in compliance with the ethical rules for animal experimentation of Kinki University. After a one week acclimation period on a basal diet in pellet form (CF-2 Diet; Clea Japan, Tokyo, Japan), 7 animals received drinking water containing BHP (Nakalai Tesque Co. Ltd., Kyoto, Japan) at a concentration of 2000 ppm for 12 weeks and drinking water without BHP thereafter. In order to obtain normal lung tissue, the remaining 3 animals were maintained free from carcinogen exposure throughout the experimental period. All rats were exsanguinated from the abdominal aorta under light ether anesthesia 25 weeks after the start of the experiment.

Upon sacrifice, the lungs were immediately excised, and grossly apparent tumors were dissected from their surrounding tissue. Samples were frozen in liquid nitrogen and stored at −80°C until analysis. Portions of the tumors were fixed in 10% neutral buffered formalin at 4°C until analysis. Portions of the tumors were fixed in 10% neutral buffered formalin at 4°C, routinely processed for hematoxylin and eosin staining and histopathologically evaluated according to diagnostic criteria previously described.

Total RNA was extracted from frozen tissue using an RNeasy Mini Kit (QIAGEN, Hilden, Germany), and first-strand cDNA was synthesized from 0.2 µg of the total RNA using Ready-To-Go Your-Prime First Strand Beads (GE Healthcare UK Ltd., Buckinghamshire, England). To eliminate the possibility of false positives caused by residual genomic DNA, all sample were treated with DNase.

Semi-quantitative RT-PCR analysis was performed as described previously. PCR amplification was carried out in a reaction volume of 20 µl containing 1 µM of each gene primer, 200 µM of each dNTP, 1 × PCR buffer (Applied Biosystems Japan Ltd., Tokyo, Japan), 0.5 U of AmpliTaq Gold (Applied Biosystems Japan Ltd.) and 0.5 µl of synthesized cDNA mixture. A primer pair for PCR amplification was designed against the rat Pcdh20 sequence (NCBI accession numbers XM_224417; Table 1). Rat glyceraldehyde-3-phosphate dehydrogenase gene was used as an internal control gene (NCBI accession number AF106860). For each gene, multiple cycles of PCR amplification were tested. The cycle number at which the sample with the highest expression level reached an amplification plateau was determined, and a cycle number smaller than this was adopted for the analysis. Amplified products were then separated on 2% agarose gels containing 0.05 µg/ml ethidium bromide.

Bisulfite treatment of genomic DNA was performed as previously described. Briefly, genomic DNA was extracted from frozen tissue using a DNeasy Tissue Kit (QIAGEN), 500 ng of each sample was denatured in 0.3 N NaOH and then 2.9 M sodium bisulfite (Sigma, St. Louis, MO, USA) and then 0.5 mM hydroquinone (Sigma) was added. The mixture underwent 15 cycles of 30 s of denaturation at 95°C followed by 15 min incubation at 50°C. The sample was then desalted using the Wizard DNA cleanup system (Promega, Madison, WI, USA) and desfonulated by treatment with 0.3 N NaOH at room temperature for 5 min. After ethanol precipitation with ammonium acetate, DNA was dissolved in distilled water.

PCR was performed with the primer set for bisulfite sequencing (NCBI accession no: NW 047454; Table 1). PCR products were subcloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and sequenced using a BigDye terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems Japan Ltd.) and an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan Ltd.). For each sample, eight clones were sequenced.

A total of 7 of the adenocarcinomas obtained were histologically well-differentiated (Fig. 1). To assess the expression levels of Pcdh20 in adenocarcinomas, we first performed semi-quantitative RT-PCR analysis on the 7 adenocarcinomas and 3 normal lung tissues. Representative results are shown in Fig. 2. In four out of the 7 adenocarcinomas (57.1%), the expression levels of Pcdh20 in adenocarcinomas, we first performed semi-quantitative RT-PCR analysis on the 7 adenocarcinomas and 3 normal lung tissues. Representative results are shown in Fig. 2. In four out of the 7 adenocarcinomas (57.1%), the expression levels of Pcdh20 were reduced compared with those in normal lung tissues.

Next, we measured the DNA methylation status of the 5' upstream region of Pcdh20 (between nucleotides −247 and 185), which contains 19 CpG sites. Representative results of the bisulfite sequencing analysis of Pcdh20 are shown in Fig. 3. Methylation was observed in the 5' upstream region of Pcdh20 in all four adenocarcinomas in which the expression level of Pcdh20 was reduced. By contrast, in normal lung tissue and one tumor sample (AD1), in which Pcdh20 expression was not reduced, this region was

| Table 1. The Primers Used for RT-PCR-SSCP and Bisulfite Sequencing Analyses |
|-----------------------------|-----------------------------|-----------------------------|
| Assay | Primer | Primer sequence | Annealing temperature (°C) |
| RT-PCR | Pcdh20 | 1F : 5' – GGCATGAATGCAGTCATAGC – 3' | 63 |
| Bisulfite sequencing | Pcdh20 | BS-F : 5' – GGTTAGTTAAGGTTAGTAGTTATG – 3' | 52 |

° C
In human NSCLC cell lines, 10 out of 19 lines (52.6%) showed loss of PCDH20 mRNA expression. After treatment with 5-aza 2'-deoxycytidine, expression of PCDH20 was induced in non expressed cell lines, demonstrating that aberrant DNA methylation of the PCDH20 gene correlated inversely with expression of the gene. Indeed, hypermethylation of this PCDH20 promoter was frequently observed in 32 out of 59 primary NSCLC tissues of humans (54.2%), suggesting that hypermethylation of PCDH20 may be a factor in the carcinogenesis of NSCLCs.

In the present study, we determined that aberrant DNA methylation of Pcdh20 is present in rat lung adenocarcinomas induced by BHP, and this correlates with reduced expression of Pcdh20. Previously, we also reported that reduced expression is due to aberrant DNA methylation patterns of cell-cell adhesion-related genes, such as E-
cadherin, connexin26 and Tslc1, in this type of tumor. Therefore, this suggests that disturbance of the cell-cell adhesion system may play important roles in the development of lung adenocarcinomas induced by BHP in rats. To better understand the function of cell-cell adhesion-related genes in BHP-induced lung carcinogenesis, the genetic and epigenetic statuses of these genes in preneoplastic lesions, such as hyperplasias and adenomas, should be further studied.

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References