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Reduced expression of the Tslc1 gene and its aberrant DNA methylation in rat lung tumors ☆

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Abstract

TSLC1 gene inactivation due to promoter methylation has been reported in several human cancers. Here, we investigated the expression of the Tslc1 gene and its methylation pattern in 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 maintained without further treatment until they were sacrificed at 25 weeks. Total RNA was extracted from a total of 11 lung adenocarcinomas and their Tslc1 gene expressions were analyzed by real-time quantitative reverse transcription-polymerase chain reaction. Tslc1 expression was significantly reduced in the lung adenocarcinomas compared with three normal lung tissues ($p < 0.05$). Bisulfite sequence analysis of four lung adenocarcinomas and two normal lung tissues revealed that the 5′ upstream region of the Tslc1 gene was highly methylated in the four lung adenocarcinomas, but unmethylated in the two normal lung tissues. These results suggest that aberrant Tslc1 gene methylation may be involved in BHP-induced development of lung adenocarcinomas in rats.
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Keywords: Lung adenocarcinoma; DNA methylation; Tslc1; Rat; Nitrosamine

Loss of heterozygosity (LOH) at chromosome 11q is frequently found in human lung cancers [1,2]. TSLC1 (tumor suppressor in lung cancer 1) has been identified as a tumor suppressor gene on chromosome 11q23.2 in non-small cell lung cancers (NSCLCs) by functional complementation analyses [3,4]. The TSLC1 gene encodes a membrane glycoprotein that belongs to the immunoglobulin superfamily and participates in cell adhesion [4,5]. Recently, the TSLC1 gene has been reported to be inactivated in several human cancers, including NSCLCs [4,6,7], and promoter methylation has been proposed as an important mechanism for the TSLC1 gene inactivation [4,6,7].

Although lung cancer is one of the most common human malignancies, the rate-limiting molecular events in its development are still largely unknown. The experimental model used in the present study features the development of NSCLCs in rats induced by the presence of *N*-nitrosobis(2-hydroxypropyl)amine (BHP) in their drinking water, and is associated with high yields of adenomatous lesions, including adenocarcinomas [8,9]. Since step-by-step monitoring of lung malignancies is possible using this model, we were able to accumulate data on the genetic alterations during the carcinogenesis, including Ki-ras mutations [10], alterations in TGFβ signaling pathway-associated genes [11–13], and alterations to tumor suppressor genes located on human chromosome 3p [14–16]. Recently, we investigated the aberrant DNA

☆ *Abbreviations:* BHP, *N*-nitrosobis(2-hydroxypropyl)amine; RT, reverse transcription; PCR, polymerase chain reaction; LOH, loss of heterozygosity; NSCLC, non-small cell lung cancer.

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methylation patterns of the E-cadherin and Connexin 26 genes in rat lung adenocarcinomas, which are associated with their reduced expressions [17,18]. E-cadherin is a transmembrane glycoprotein that mediates cell–cell adhesion in epithelial cells [19], while Connexin 26 is a member of the Connexin family and forms gap junctions, which are mediated by intercellular channels connecting adjacent cells [20].

Aberrant cell adhesion is considered to be one of the critical events for the invasion and metastasis of cancer cells. Therefore, in order to better understand the involvement of cell adhesion in rat lung carcinogenesis, we investigated the expression of the Tslc1 gene and measured its DNA methylation status in lung adenocarcinomas induced by BHP in rats.

Materials and methods

Animals and treatment. A total of 14 male Wistar rats (5 weeks of age) were obtained from Japan SLC Inc. (Shizuoka, Japan). The animals were housed at 3–5 animals/cage in an air-conditioned room at a constant temperature of 25 °C on a 12-h light–dark cycle. Food and water were given ad libitum throughout the study. After a 1 week acclimation period on a basal diet in pellet form (CF-2 Diet; Clea Japan, Tokyo, Japan), 11 animals received drinking water containing BHP (Nacalai Tesque Co. Ltd., Kyoto, Japan) at a concentration of 2000 ppm for 12 week, followed by drinking water without BHP. In order to obtain normal lung tissues, the remaining three animals were maintained without carcinogen exposure throughout the experimental period. The normal lung tissues were used as a control to eliminate contamination by macroscopically undetected cancerous tissues. All rats were exsanguinated from the abdominal aorta under light ether anesthesia at 25 week after the start of the experiment.

Tissue preparation. Upon sacrifice, the lung tissues were immediately excised. Following dissection of grossly apparent tumors from their surrounding tissues, the majority of each tumor was frozen in liquid nitrogen and stored at −80 °C until analysis. A further portion of each tumor was fixed in 10% neutral-buffered formalin at 4 °C, routinely processed for hematoxylin and eosin staining, and evaluated histopathologically according to previously described diagnostic criteria [8,9].

5′ and 3′ RACE for the rat Tslc1 gene cDNA. The primers used for 5′ and 3′ RACE were designed based on the mouse Tslc1 cDNA (GenBank Accession No. AB092414). The 5′ RACE was performed using a 5′ RACE System (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was extracted from the rat normal lung tissues using ISOGEN (Nippon Gene Inc., Toyama, Japan) and 1 μg of the total RNA was reverse-transcribed using the downstream gene-specific primer 5′-TGCCGCGGCCGAGAGGAGCA-3′. A homopolymetric tail was then added to the 5′-end of the resultant cDNA using terminal deoxynucleotidyl transferase and dCTP. Thereafter, PCR amplification was performed with a nested gene-specific primer 5′-GGCC GAGAGGAGCAACAGCA-3′, an abridged anchor primer, and UAP (from the RACE kit).

The 3′ RACE was carried out using a 3′ RACE System (Invitrogen Corporation). First-strand cDNA was generated from 1 μg of the total RNA using a 3′ RACE adaptor primer with a polydeoxythymidylate tail. Subsequent rounds of PCR were performed using nested gene-specific primers 5′-CTGCAGACCCAGCGGTATCTA-3′ and 5′-GAAGTGCA GTATAAACCGCA-3′, and an adaptor primer (from the RACE kit).

The RACE products were subcloned using a TOPO TA Cloning Kit (Invitrogen Corporation) and sequenced with a BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems Japan Ltd., Tokyo, Japan) and an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan Ltd.).

Real-time quantitative RT-PCR. Total RNA was extracted from frozen tissues using ISOGEN (Nippon Gene) and first-strand cDNA was synthesized from 0.2 μg of the total RNA using Ready-To-Go Your-Prime First Strand Beads (Pharmacia, Tokyo, Japan). To eliminate possible false positive caused by residual genomic DNA, all samples were treated with DNase.

Real-time quantitative RT-PCR analysis was carried out using a Smart Cycler 2 System (TaKaRa Bio, Shiga, Japan) and SYBR premix Ex Taq (TaKaRa) according to the manufacturer's protocol. A 1 μl aliquot of the synthesized cDNA was used in the following assay. The primers were designed according to the sequence obtained from the above-described 5′ and 3′ RACE analyses (GenBank Accession No. AB257091) as follows: for Tslc1: 5′-TTATCCTCTGCAAGGCCTAAC-3′ (sense) and 5′-TGTT GAGGCATTTCGTCATC-3′ (antisense); and for glyceraldehyde-3-phosphate dehydrogenase (Gapdh): 5′-TTGTGAAGGTCGGTGTGAA C-3′ (sense) and 5′-ACCTGTAGACCTCGGCACTG-3′ (antisense). The PCR conditions were as follows: 95 °C for 20 s, and 55 cycles of 95 °C for 15 s and 62 °C (for Tslc1) or 55 °C (for Gapdh) for 30 s. To obtain a standard curve for each gene, cDNAs synthesized from normal lung tissues were used. Plots of the PCR amplifications were used to determine the threshold cycle (C_t) values. These C_t values represented the first cycle in which there was a significant increase in fluorescence above the background fluorescence. The initial copy number of the target mRNA was calculated by plotting the C_t value against the input target quantity. The data for the target genes were normalized to the Gapdh data. Each assay was repeated at least twice for confirmation. Data were analyzed statistically using Student's *t*-test.

Determination of the 5′ upstream region of the rat Tslc1 gene. To determine the nucleotide sequence of the 5′ upstream region of the rat Tslc1 gene, suppression PCR combined with gene-specific primers was performed as described previously [21,22]. Genomic DNA was extracted from rat lungs using a DNeasy Tissue Kit (Qiagen, Hilden, Germany), and 5 μg of the genomic DNA was digested with *Rsa*I (New England Biolabs, Beverly, MA). After ethanol precipitation, the digested DNA fragments were ligated with 2 μl of specific adaptor primers at the *Rsa*I-digested ends using T4 ligase (TAKARA, Kyoto, Japan) in a total volume of 30 μl at 16 °C. The adaptors were as follows: 5′-GTAATACGACTCACTATAG GGCTCGAGCGGCCGCCCGGGCAGGT-3′ and 5′-ACCTGCCC-3′ [21,22]. The primary PCR was performed with the first adaptor primer P1 (5′-GTAATACGACTCCTATAGGGC-3′) [21,22] and the first gene-specific primer R1 (5′-TGCCGCGGCCGAGAGGAGCA-3′). The conditions for the suppression PCR were as previously described [22]. For the second nested PCR amplification, the first PCR product was diluted 20-fold with distilled water and amplified with the second adapter primer P2 (5′-TGATGCGTGAAGACGACAGAA-3′) [21,22] and the second gene-specific primer R2 (5′-GGCCGAGAGGAGCAACAGCA-3′). Both gene-specific primers were designed from the mouse Tslc1 cDNA sequences (GenBank Accession No. AB092414). The PCR amplification was carried out in a total volume of 20 μl containing 1 μl of each gene primer, 200 μM of each dNTP, 1× PCR buffer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA), and 0.5 U of AmpliTaq Gold (Perkin-Elmer) under the following reaction conditions: primary denaturation for 10 min at 95 °C, 35 cycles of denaturation at 95 °C for 30 s, annealing at 69 °C for 15 s, and extension at 72 °C for 1 min, and a final extension for 10 min at 72 °C. The amplified product was separated in a 1% agarose gel containing 0.05 μg/ml ethidium bromide, extracted from the gel, subcloned using a TOPO TA Cloning Kit (Invitrogen), and sequenced with a BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Japan) and an ABI PRISM 310 genetic analyzer (Applied Biosystems, Japan).

Bisulfite sequencing. Bisulfite treatment of genomic DNA was performed as previously described [17,18]. Briefly, genomic DNA was extracted from frozen tissues using a DNeasy Tissue Kit (Qiagen), followed by denaturation of 500 ng of each sample in 0.3 N NaOH, and then 2.9 M sodium bisulfite (Sigma, St. Louis, MO). Subsequently, 0.5 mM hydroquinone (Sigma) was added and the mixture was subjected to 15 cycles of denaturation at 95 °C for 30 s and incubation at 50 °C for 15 min. The sample was then desalted with a Wizard DNA Cleanup System (Promega, Madison, WI) and desulfonated by treatment with 0.3 N NaOH at room temperature for 4 min. After ethanol precipitation with ammonium acetate, the DNA was dissolved in distilled water.

For bisulfite sequencing, PCR was performed with the primer set of rat-Tslc-F (5′-GGGGAAGTAAAGGTTGAAATTTAA-3′) and rat-Tslc-R (5′-AACAACACAATACTCACCATAT-3′) with an annealing temperature of 60 °C. The PCR products were subcloned using a TOPO TA Cloning Kit (Invitrogen), and then sequenced with a BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Japan) and an ABI PRISM 310 genetic analyzer (Applied Biosystems, Japan). For each sample, 10 clones were sequenced.

Results and discussion

Inactivation of the TSLC1 gene has been reported in human cancers [3,4,6,7,23–27]. Since LOH at 11q23 occurred in these tumors, the TSLC1 gene appeared to be the major inactivation target in tumors with loss of 11q23 [28]. Recently, it was reported that aberrant promoter methylation of the TSLC1 gene was detected in 44% of NSCLCs, 32% of prostatic cancers, 29% of hepatocellular carcinomas, and 27% of pancreatic cancers [4,6,7,23]. Moreover, loss of expression of the TSLC1 gene was found in tumors, such as esophageal, gastric, and pancreatic cancers, in association with promoter methylation [24–26]. In contrast, mutations of the TSLC1 gene were rare in tumors [4]. Therefore, it has been suggested that promoter methylation is an important mechanism for TSLC1 gene inactivation in these cancers [3,4,6,7,23–28].

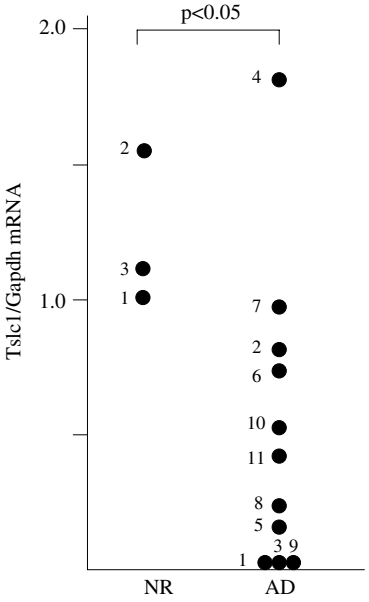

Fig. 1. Expression levels of Tslc1 mRNA relative to Gapdh mRNA. NR, normal lung tissue; AD, adenocarcinoma.

In the present study, we initially assessed the involvement of the Tslc1 gene in rat lung carcinogenesis by investigating Tslc1 gene expression in rat lung adenocarcinomas induced by BHP. A total of 11 lung adenocarcinomas were obtained, one from each animal treated with BHP. Three normal lung tissues from untreated rats were used as controls, in order to eliminate the possibility that non-cancerous portions of BHP-treated rats may include small microscopic lesions that were undetectable macroscopically, such as adenocarcinomas, as well as hyperplasias and adenomas. The results of the real-time quantitative RT-PCR analysis for the 11 adenocarcinomas and three normal lung tissues are shown in Fig. 1. The relative expression levels of Tslc1 to Gapdh were significantly decreased in the adenocarcinomas compared with the normal lung tissues ($p < 0.05$).

Next, we investigated the DNA methylation status of the Tslc1 gene by determining the sequence of the 5′ upstream region of the rat Tslc1 gene by suppression PCR combined with gene-specific primers [21,22]. The nucleotide sequence of this region of the rat Tslc1 gene is shown in Fig. 2 (GenBank Accession No. AB257090). Based on this sequence, we subsequently performed a bisulfite sequence analysis to measure the methylation status of the same region (between nt-273 and 20), which contained 23 CpG sites, in rat lung adenocarcinomas. A total of four adenocarcinomas with markedly reduced Tslc1 gene expression were used in this analysis, and compared with two normal lung tissues as controls. The results revealed that this region was demethylated in the two normal lung tissues. In contrast, all four adenocarcinomas were highly methylated in this region, in parallel with the reduced expression of Tslc1 (Fig. 3). Therefore, these results suggest that reduced Tslc1 gene expression due to aberrant methylation may be involved during lung carcinogenesis induced

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-300          g tacaggctct cctccttcgg ggaagcaaag gctgaaattt aacgtaaaca
-240  tatttgcata ggcccctccc ctcagccccg cccctaggtg gcgcgggcgc gcggccgaac
-180  gctagcgcca gggggcgggg tgggggaggg gggctagtcc tctgagagct gggttgggct
-120  cgcggtgcca cgattggtcg ctcccgactc cgccttcacc gcatctcatt agcatctcat
-60   tagctgtccg ctcgggctcg ggaggcagcc accgctgcca gtctgaggca ggtgcccgac
  1  atggcgagta ctgtgctgcc gagcggatcc cagtgtgcgg cggcagcggc tgtggcggcg
 61  gcggcggcgc ctccagggct ccggctccgg ctcctgctgt tgctcctctc ggccgcggca
121  ttgatcccca cag
```

Fig. 2. Nucleotide sequence of the 5′ upstream region of the rat Tslc1 gene as far as 291 bp upstream from the translation initiation site. The translation initiation codon is shown in boldface, and a restriction site for *Rsa*I is underlined.

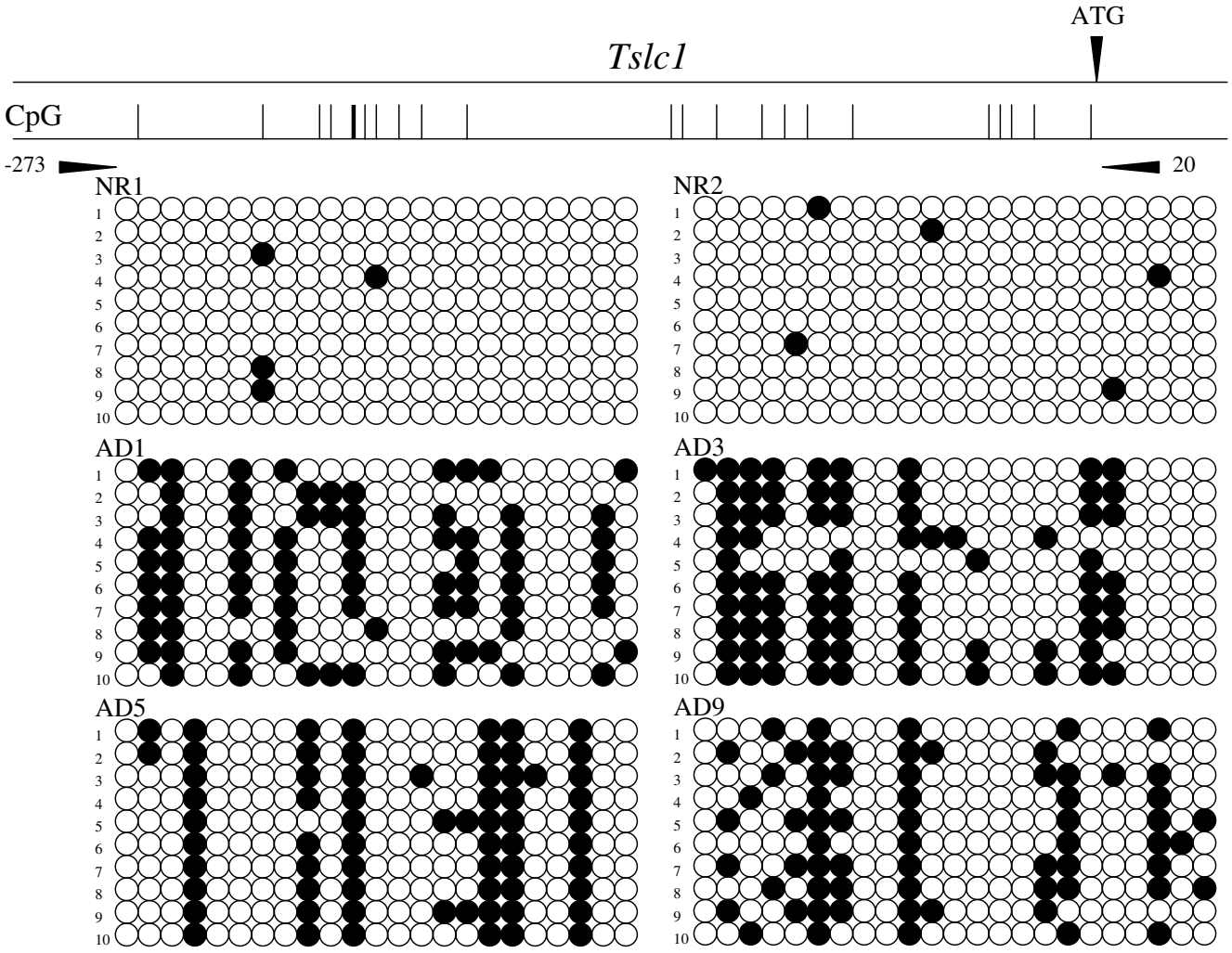

Fig. 3. Results of the methylation analysis of the 5′ upstream region of the rat Tslc1 gene evaluated by bisulfite sequencing. For each sample, 10 clones were sequenced. The primer pair used for the bisulfite sequencing are indicated by arrowheads. Methylated CpG sites are shown by closed circles and unmethylated CpG sites are shown by open circles. NR, normal lung tissue; AD, lung adenocarcinoma.

by BHP in rats. The involvement of LOH of the Tslc1 gene remains to be clarified.

Recently, it has been reported that other TSLC1 cascade genes, such as the DAL-1 gene, are also methylated in human lung cancers, similar to TSLC1, indicating that methylation of TSLC1 and/or DAL-1, leading to loss of their expression, is an important event in the pathogenesis of lung cancers [29]. To further understand the involvement of the Tslc1 cascade in rat lung carcinogenesis, the alterations to Dal-1 should be studied.

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