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Reduced expression of the E-cadherin gene and its aberrant DNA methylation in hamster pancreatic tumors ☆

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Abstract

The expression of the E-cadherin gene and its DNA methylation status in the 5′ upstream region were investigated in pancreatic duct adenocarcinomas (PDAs) induced by *N*-nitrosobis(2-oxopropyl)amine (BOP) in hamsters. Female Syrian golden hamsters received 70 mg/kg BOP, followed by repeated exposure to an augmentation pressure regimen consisting of a choline-deficient diet combined with DL-ethionine then L-methionine and a further administration of 20 mg/kg BOP. A total of 15 PDAs were obtained, along with total RNA for assessment of expression by real-time quantitative reverse transcription-polymerase chain reaction. The expression of the E-cadherin was significantly reduced in PDAs ($p < 0.05$) compared with normal pancreatic tissue. For the analysis of methylation status, bisulfite sequencing was performed with two normal pancreatic tissues and six tumors. The normal pancreatic tissue was all demethylated in this region of E-cadherin. In contrast, six PDAs were highly methylated, correlating with reduced expression of the E-cadherin. These results suggest that aberrant DNA methylation of the E-cadherin gene may play a role in the development of PDAs induced by BOP in hamsters.

Keywords: Pancreatic duct adenocarcinoma; DNA methylation; E-cadherin; Hamster; Nitrosamine

E-cadherin is a transmembrane glycoprotein that mediates cell–cell adhesion in epithelial cells [1]. Reduced expression of E-cadherin is found in tumor progression and metastasis, while abnormal expression of E-cadherin is involved in tumor invasion and metastasis [2–6]. Since methylation of cytosine residues at CpG dinucleotides can suppress gene expression in mammalian genomes [7–11], it has been suggested that aberrant DNA methylation of the promoter regions of E-cadherin may be the major mechanisms of gene silencing in several tumors, including pancreatic cancers [12–14].

Pancreatic duct adenocarcinoma (PDA) has one of the lowest cure rates of human malignancies [15,16]. In order to understand the underlying mechanisms, we have generated a model for the development of hamster PDAs by *N*-nitrosobis(2-oxopropyl)amine (BOP), mirroring the human situation, and this model gives us the ability to gain a better understanding of the underlying mechanisms [17–19]. Indeed, we have reported several genetic changes in this pancreatic carcinogenesis model. For example, Ki-ras mutations were frequently found in preneoplastic lesions as well as PDAs, while p53 mutations were detected as late events [20–22].

☆ *Abbreviations:* PDA, pancreatic duct adenocarcinoma; BOP, *N*-nitrosobis(2-oxopropyl)amine; PCR, polymerase chain reaction; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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In addition, we have provided evidence that alteration of the Fhit gene may also be involved in the development of PDAs [23]. In contrast, the analysis for epigenetic changes, such as DNA methylation status in the promoter region of genes, has been limited, since only a small amount of genetic information is available for hamsters.

Recently, we determined the 5′ upstream region of the hamster p16 gene using the suppression PCR method combined with gene-specific primers and measured its methylation patterns in PDAs [24]. In the present study, we determined the 5′ upstream region of the hamster E-cadherin gene with this method, and to better understand epigenetic changes in pancreatic carcinogenesis expression of the E-cadherin gene and its DNA, methylation status in hamster PDAs induced by BOP was examined.

Materials and methods

Production of hamster PDAs. A total of 18 female Syrian golden hamsters, weighing approximately 100 g each, were used (Japan SLC). PDAs were induced in 15 animals according to the rapid production model [17–19]. Briefly, BOP (30 mg/kg body weight) (Nakalai Tesque, Kyoto, Japan) was given subcutaneously as initiation, followed by two cycles of augmentation pressure which consisted of choline-deficient diet administration and ethionine–methionine–BOP injection [17–19]. To obtain normal control tissues including the pancreas, the remaining three animals were maintained free from carcinogen exposure throughout the experimental period. All hamsters were sacrificed under light ether anesthesia at 10 week after the beginning of the experiment and the pancreas was immediately excised. Macroscopically apparent tumors were dissected from the surrounding tissue and frozen in liquid nitrogen.

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

Real-time quantitative RT-PCR analysis using a Smart Cycler II System (TaKaRa Bio, Shiga, Japan) and a SYBR Premix Ex Taq (TaKaRa) was performed according to the manufacturer's protocol. One microliter of the synthesized cDNA was used in the following assay. The primer sets used in this assay were as follows: for E-cadherin, 5′-CTGCAGGTCTCATCATGGC-3′ (sense) and 5′-ACCTG TAGACCTCGGCACTG-3′ (antisense) and for glyceraldehyde-3-phosphate dehydrogenase (Gapdh), 5′-TTGTGAAGGTCGGTGTG AAC-3′ (sense) and 5′-AGGGGTCGTTGATGGCAACA-3′ (antisense). The PCR conditions were as follows: 95 °C for 20 s, 45 cycles of 95 °C for 15 s and 64 °C (for E-cadherin) or 55 °C (for Gapdh) for 30 s. To obtain the standard curve for each gene, a cDNA synthesized from normal lung tissue was used. The amplification plots of the PCR were used to determine the threshold cycle (C_t). The C_t value 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 a plot of the C_t against the input target quantity. The data of the target genes were normalized to Gapdh. Each assay was repeated at least twice for confirmation. Data were statistically analyzed by Student's t test.

Determination of 5′ upstream region of hamster E-cadherin gene. To determine the nucleotide sequence of the 5′ upstream region of the hamster E-cadherin gene, the suppression PCR combined with gene-specific primers was performed as described recently [24]. Genomic DNA was extracted from the liver of a female Syrian golden hamster (Japan SLC, Shizuoka, Japan) using a DNeasy tissue kit (Qiagen, Hilden, Germany). Next, 5 μg of the genomic DNA was digested with *Rsa*I (New England Biolabs, MA, USA). After ethanol precipitation, the digested DNA fragments were ligated with 2 μl of a specific adaptor primer at the *Rsa*I-digested end by T4 ligase (TaKaRa) in a total volume of 30 μl at 16 °C. The adaptor primer sequence was as follows: 5′-GTAATACGACTCACTATAGGGCTCGAGCGGCCG CCCGGGCAGGT-3′ and 5′-ACCTGCCC-3′ [24,25]. The primary PCR was performed with the first adaptor primer P1, 5′-GTAAT ACGACTCACTATAGGGC-3′ [24,25], and the first gene-specific primer R1, 5′-CTGCAGCAGGAACAGGATCG-3′. The conditions for the suppression PCR were as previously described [24]. For the second nested PCR amplification, the first PCR product was diluted 20-fold with distilled water and amplified with the second adaptor primer P2, 5′-TGTAGCGTGAAGACGACAGAA-3′ [24,25] and the second gene-specific primer R2, 5′-AGCAGGAACAGGATCGCG GA-3′. Both gene-specific primers were designed from rat E-cadherin cDNA sequences (GenBank Accession No. AB017696). 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, USA), 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 30 s denaturation at 95 °C, 15 s annealing at 69 °C, and 1 min extension at 72 °C, 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, CA, USA), and sequenced with a BigDye terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems Japan, Tokyo, Japan) and an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan).

Bisulfite sequencing. Bisulfite treatment of genomic DNA was performed as previously described [24,26]. Briefly, genomic DNA was extracted with DNeasy tissue kit (Qiagen) from frozen tissues, and 500 ng of each sample was digested with an appropriate restriction enzyme. The digested DNA was denatured in 0.3 N NaOH, and then 2.9 M sodium bisulfite (Sigma, St. Louis, MO, USA) and 0.5 mM hydroquinone (Sigma) were added and the mixture underwent 15 cycles of 30 s denaturation at 95 °C and 15 min incubation at 50 °C. The sample was then desalted with the Wizard DNA cleanup system (Promega, Madison, WI, USA), and desulfonated 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.

For bisulfite sequencing, PCR was performed with the primer sets as follows; BS-F: 5′-ATTTAGATGAAGAGTAAAGTTTTTTGTA-3′, BS-R: 5′-ACACAAAAATCCCATAACTAACC-3′ (annealing temperature: 57 °C). PCR products were subcloned with a TOPO TA cloning kit (Invitrogen) and sequenced with a BigDye terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems Japan) and ABI PRISM 310 genetic analyzer (Applied Biosystems Japan). For each sample, ten clones were sequenced.

Results and discussion

It has been reported that loss or reduced expression of E-cadherin was detected in several human malignancies [2–6]. In pancreatic tumors, reduced expression of E-cadherin was found in human primary pancreatic carcinomas, correlating with an invasion and metastasis of tumor cells [5,6]. Therefore, it has been suggested that loss or reduced expression of E-cadherin is involved in

the progression of pancreatic tumors [5,6]. In the present study, to assess an involvement of E-cadherin in hamster pancreatic carcinogenesis, we first investigated the expression of E-cadherin in hamster PDAs induced by BOP, using real-time quantitative RT-PCR analysis (Fig. 1). All PDAs used in this study were histologically well differentiated. Fifteen PDAs and three normal pancreatic tissues were examined. Relative expression levels of E-cadherin to Gapdh were significantly decreased in PDAs compared with normal pancreas ($p < 0.05$). These results indicate that reduced expression of E-cadherin may play a role in the development of hamster PDAs induced by BOP.

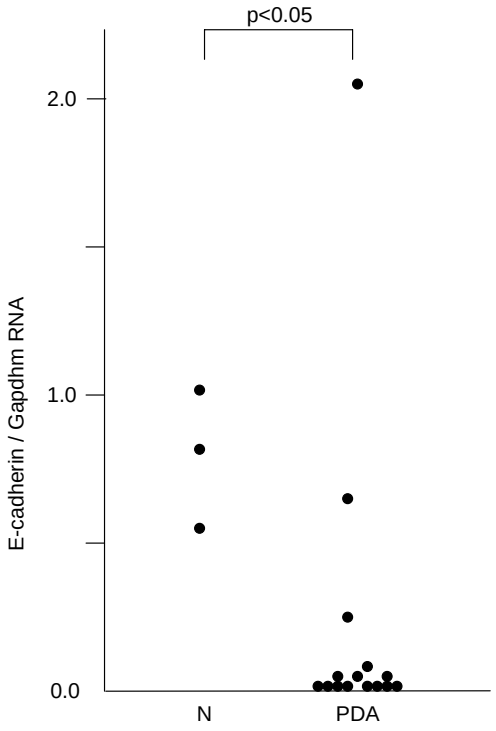

Fig. 1. Expression levels of E-cadherin mRNA relative to Gapdh mRNA. N, normal pancreas; PDA, pancreatic duct adenocarcinoma.

It has been considered that hypermethylation around the promoter region may be an important mechanism to inactivate E-cadherin in human malignancies [2,3]. Hypermethylation of CpG sites around the E-cadherin promoter region significantly correlated with reduced expression of E-cadherin in human hepatocellular carcinomas [3]. Aberrant DNA methylation of E-cadherin was also reported in human pancreatic tumors and a relationship was demonstrated between methylation frequency and tumor progression [14]. In the present study, to investigate DNA methylation status of the E-cadherin gene, we determined the sequence of the 5′ upstream region of hamster E-cadherin gene by suppression PCR combined with gene-specific primers [24]. This method is a highly effective and rapid technique for determining the 5′ upstream region and is applicable to the investigation of methylation status in various hamster tumors [24]. The nucleotide sequence of this region of the hamster E-cadherin is shown in Fig. 2 (GenBank Accession No. AB221485). Based on this sequence, we then performed a bisulfite sequence analysis to measure methylation status at the same region (between nt −370 and 17), which contains 28 CpG sites, in PDAs. The six PDAs with a marked reduction of E-cadherin expression were used in this analysis. Two normal pancreatic tissues showed a demethylated status in this region. In contrast, all six PDAs were highly methylated (Fig. 3), correlating with a reduced expression of E-cadherin. Therefore, these results suggest that the reduced expression of E-cadherin due to aberrant methylation may play a role in the development of PDAs induced by BOP in hamsters.

In conclusion, we found a reduced expression of E-cadherin and aberrant methylation of its CpG sites in hamster PDAs induced by BOP. Recently, we reported aberrant methylation patterns of the 5′ upstream region of the p16 gene in hamster PDAs [24]. The hamster model of pancreatic carcinogenesis is useful for understanding the development of human pancreatic cancer [17–19]. Therefore, to further clarify an

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-497                                                    gtacaca cacacacaca
-480  cacacacaca cacacacacc ctaaaatgtt ttttttaat tcttaagtca aagtaaacca
-420  aaccaaacaa acctctccga ccaaacacaa taaagaaaaa cgaaataaaa acctagatga
-360  agagtaaagt cctttgtaac tccaagttac aatggctggg ctgggattcg aacgaatgtg
-300  aactaggaag tctccttagg ccggccccgt gccacaaact acaattaaga gtggaggaag
-240  tcgaggaccg tgaggctcct tggctgccac ctgcgggtgc gtcctcagcc aatcagtggc
-180  gccgggggcg gtgccttcag cgcacctggc ggccgcagcc tctgcctggc tcagtggagt
-120  ggggagcagc ggcgtctcgg tgagttctcc gcaagttctg ctggacgcca gcccagccta
 -60  acccggccct gcccgaccgc acccgagttc cgtgtttgtt cggcgcccgc ccggccagcc
   1  atgggatccc tgtgccgcag cctctccgcg atcctgttcc tgct
```

Fig. 2. Nucleotide sequence of the 5′ upstream region of the hamster E-cadherin gene as far as 497 bp upstream of the translation initiation site. The translation initiation codon is shown in boldface, and the restriction site for *Rsa*I is underlined.

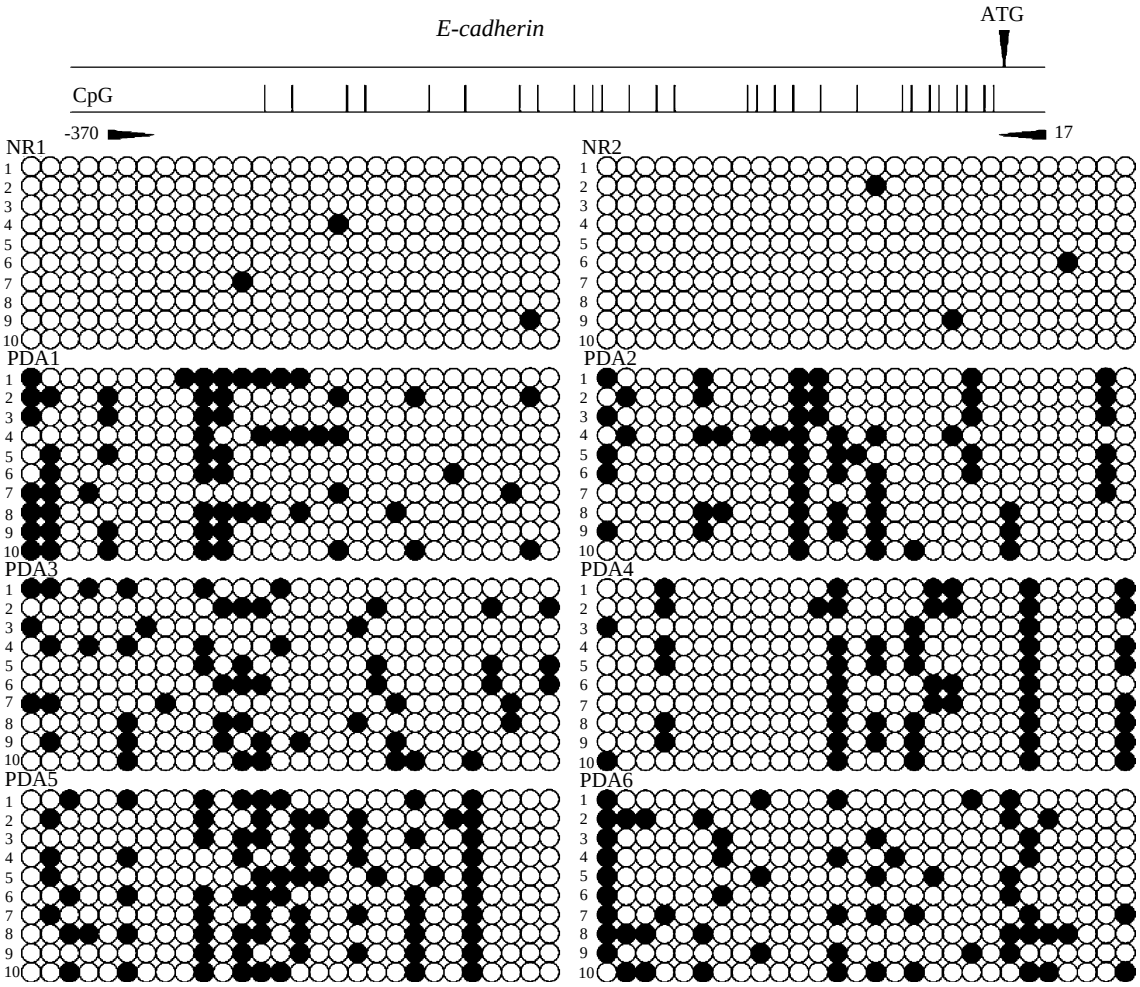

Fig. 3. Results of the methylation analysis of the 5′ upstream region of the hamster E-cadherin gene by bisulfite sequencing. The density of CpG sites gradually decreases with increasing separation from the initiation codon in the upper diagram. A pair of primers for bisulfite sequencing are shown. Methylated CpG sites are shown by closed circles and unmethylated CpG sites are shown by open circles. NR, normal pancreas; PDA, pancreatic duct adenocarcinoma.

involvement of epigenetic events in pancreatic carcinogenesis, more studies of DNA methylation status of the 5′ upstream region of other genes and gene expression are required.

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