The Absence of Mth1 Inactivation and DNA Polymerase $\kappa$ Overexpression in Rat Mammary Carcinomas with Frequent A:T to C:G Transversions

Eriko Okochi, Shizue Ichimura, Takashi Sugimura and Toshikazu Ushijima

Carcinogenesis Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045

Single nucleotide instability (SNI), an increase in spontaneous point mutation rates (MRs) without involvement of microsatellite instability, is present in rat mammary carcinoma cell lines and human breast cancer cell lines. A:T to C:G transversions, which are generally rare, were frequently observed in two rat mammary carcinoma cell lines and in their primary carcinomas, and were considered to be related to the molecular mechanism of SNI. In this study, two known molecular mechanisms that cause increases of A:T to C:G transversions, inactivation of the MutT mammalian homologue (Mth1) gene and overexpression of the DNA polymerase $\kappa$ (Pol $\kappa$) gene, were analyzed in two rat mammary carcinoma cell lines and 11 rat primary carcinomas. PCR-SSCP analysis revealed no mutations in the entire Mth1 coding region. Quantitative real-time RT-PCR analysis showed that Mth1 mRNA expression was slightly, but significantly, increased in the primary carcinomas ($P$=0.001 using GAPDH for normalization, and $P$=0.002 using histone H4, t-test), contrary to our expectation, and was decreased to 1/2 in the cell lines. The expression of Pol $\kappa$ which is known to be error-prone with frequent A:T to C:G transversions, was rather decreased in the cell lines and primary carcinomas. Inactivation of Mth1 and overexpression of Pol $\kappa$ were unlikely to have caused SNI in the two rat mammary carcinoma cell lines with a high frequency of A:T to C:G transversions, and searching for other unknown molecular mechanisms is important.

Key words: MutT homologue — DNA polymerase kappa — Rat mammary carcinomas — Single nucleotide instability — Transversion

A new type of genomic instability, single nucleotide instability (SNI), was recently found to be present in rat mammary carcinomas and human breast cancers. SNI is characterized by an increase of spontaneous point mutation rates (MRs) without microsatellite instability (MSI). We observed SNI in two of the two rat mammary carcinoma cell lines established from mammary carcinomas induced in lacI-transgenic rats by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), and in five of six human breast cancer cell lines. In human breast cancers, the incidences of other types of genomic instability, MSI and chromosomal instability (CIN), are 0–10% and 8.1–59.3%, respectively. Considering that genomic instability is required for a cell to acquire multiple mutations for carcinogenesis and tumor progression, it was suggested that SNI is involved in a significant fraction of human breast cancers.

In the two rat cell lines, A:T to C:G transversions, which are rarely observed as spontaneous mutations in normal mammary epithelial cells, accounted for 15 and 21% of the total hprt mutations and 25 and 24% of the lacI mutations. Since the frequency of A:T to C:G transversions of the lacI gene was also elevated in the two primary mammary carcinomas of the two cell lines, the molecular abnormality present in the cell lines was suggested to be also present in the primary carcinomas. In the human breast cancer cell lines, two of the five SNI-positive cell lines showed elevated frequencies of A:T to C:G transversions, and it was suggested that a mechanism responsible for SNI in the rat mammary carcinomas could also be responsible for SNI in some of the human breast cancer cell lines.

As for mechanisms that cause increases of A:T to C:G transversions, two molecular abnormalities have been reported. First, inactivations of E. coli MutT and its mammalian homologue (Mth1) are known to lead to accumulation of 8-OH-dGTP. In the bacterial system, the MutT mutator strain shows 100 to 10,000-fold elevation of A:T to C:G transversions. Embryonic stem cells with targeted deletion of the Mth1 gene show an MR twice as high as that in wild-type embryonic stem cells. Second, DNA replication with error-prone DNA polymerases, DinB in E. coli and DNA polymerase $\kappa$ (Pol $\kappa$) in mammalian cells, is known to increase A:T to C:G transversions. In this study, we analyzed the inactivation of the rat Mth1 gene and overexpression of rat Pol $\kappa$ in the two PhIP-induced mammary carcinoma cell lines and 11 primary mammary carcinomas.

1 To whom correspondence should be addressed.
E-mail: tushijim@ncc.go.jp
Abbreviations: FBS, fetal bovine serum; hprt, hypoxanthine-guanine phosphoribosyltransferase; DMEM, Dulbecco’s modified Eagle’s medium; BBR, “Big Blue” rat.
MATERIALS AND METHODS

Tumors, cell lines and extraction of DNA/RNA Ten doses of PhIP (75 mg/kg/day of PhIP-HCl) were administered to 6-week-old female (BBR×SD)F₁ rats by gavage, and mammary carcinomas were induced in 15 of 33 rats at 56–69 weeks.15) The normal mammary glands were obtained from three non-treated, age-matched rats by the gland isolation technique.15) Two mammary carcinoma cell lines, PhIP12-1 and PhIP7-4, were established from two of these mammary carcinomas.15) A rat embryonic fibroblast cell line transfected with the lacI gene, “Big Blue” Rat 2, was purchased from Stratagene (La Jolla, CA). PhIP12-1, PhIP7-4, and “Big Blue” Rat 2 were grown in DMEM (GIBCO BRL, New York, NY) supplemented with 10% FBS (JRH Bioscience, San Antonio, TX), penicillin and streptomycin (GIBCO BRL). A primary culture of rat normal mammary epithelial cells (MECs) was prepared as a pool of mammary ducts from six 8-week-old rats, as in previous reports.15, 16) The cells were cultured in an equilibrium mixture of DMEM with 10% fetal calf serum (FCS) and streptomycin (GIBCO BRL). A primary culture of rat normal mammary epithelial cells (MECs) was prepared as a pool of mammary ducts from six 8-week-old rats, as in previous reports.15, 16) The cells were cultured in an equilibrium mixture of DMEM with 10% fetal calf serum (FCS) and keratinocyte basal medium (Clonetics, Walkersville, MD) supplemented with epidermal growth factor (EGF), insulin, hydrocortisone, transferrin, gentamicin and calcium. DNA was extracted by serial extraction with phenol and chloroform and ethanol precipitation,17) and total RNA was isolated using ISOGEN (Nippon Gene, Toyama).

PCR-SSCP analysis and direct sequencing The sequences of the primers used for PCR-single stand conformation polymorphism (SSCP) analysis18) of the rat Mth1 gene and the PCR conditions are summarized in Table I. PCR was performed for 35 cycles in a 5 μl reaction volume with 0.2 μl of [α-32P]dCTP (0.37 MBq/μl; Amersham-Pharmacia Biotech, Uppsala, Sweden) and 50 ng of template DNA, as described.15, 19) After denaturation of the PCR products, the samples were run in a 5% acrylamide gel with or without 5% glycerol.

For direct sequencing, the PCR product amplified using primer sets shown in Table I was purified using a “Micro Spin” S-300 HR Column (Amersham Pharmacia Biotech). Using the purified PCR product as a template, cycle sequencing was performed with a Dye terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, CA), and analyzed with an “ABI PRISM” 310 Genetic Analyzer (Applied Biosystems).

Cloning of partial cDNA of Rat Pol κ Rat cDNA was synthesized from the total RNA of MEC using Superscript II reverse transcriptase (Life Technologies, Rockville, MD). A 651-bp cDNA fragment was amplified using a set of primers based on mouse Pol κ cDNA (GenBank accession number AF163571), 5’-CTGATGGGTGTCCGAATGTCTACT-3’ (sense), 5’-ACATTCCTCTTTTGCTGCAGTATTC-3’ (antisense), and the fragment was directly sequenced (GenBank accession number AB076985).

Real-time RT-PCR Three micrograms of total RNA was treated with DNase I (Life Technologies) and reverse-transcribed with oligo-dT primer (Promega, Madison, WI) and Superscript II reverse transcriptase in 20 μl, and the cDNA solution was diluted to 40 μl with TE (10 mM Tris-Cl, 1 mM EDTA, pH 7.4). Real-time PCR was performed in a 50 μl reaction mixture with 2 μl of the template cDNA, 10 pmol of a primer set, 5 μl of PCR buffer containing 15 mM MgCl₂, 0.2 mM dNTP mixture, 0.5× concentration of “SYBR” Green (BioWhittaker, Walkersville, MD) and 0.4 μl of “AmpliTaq Gold” Taq polymerase (Perkin Elmer, Wellesley, MA). Production of dsDNA was monitored using an iCycler iQ detection system (Bio-Rad Laboratories, Hercules, CA). For normalization of RNA expression levels, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and histone H4 were used. Histone H4 reflects

| Exon | Region | Sequence | GenBank accession no. | 5’-Position | Size (bp) | Anneal (℃) | Mg²⁺ (mM) |
|------|--------|----------|-----------------------|------------|----------|------------|-----------|
| 1    | F      | 5’ AAC AGA CCA CAG GCC AAG C 3’ | D49978     | 129       | 282      | 57         | 1.5       |
|      | R      | 5’ TAT TGT TCC GAC TCT GTC C 3’ | 410        |           |          |            |           |
| 2    | F      | 5’ ATG CCC ATG TCT TCC TTA AC 3’ | D49979     | 30        | 236      | 55         | 1.5       |
|      | R      | 5’ GGT GAT TCT TTT TCT CTA GC 3’ | 265        |           |          |            |           |
| 3A   | F      | 5’ CTC TAG TAC CCA CGC ACT CG 3’ | D49980     | 41        | 808      | 57         | 1.5       |
|      | R      | 5’ CCG CTG GAC ACT GTC AGC AC 3’ | 848        |           |          |            |           |
| 3B   | F      | 5’ CAC TGC AAG TGC TAT GGT CAG 3’ | D49980     | 373       | 266      | 55         | 1.5       |
|      | R      | 5’ GCC ACA ACC TTA AAA CAG 3’ | 638        |           |          |            |           |
| 3C   | F      | 5’ GCC TGT TCC GTG TTT TAG 3’ | D49980     | 607       | 242      | 55         | 1.5       |
|      | R      | 5’ CCG CTG GAC ACT GTC GAC AC 3’ | 848        |           |          |            |           |

F, forward primer; R, reverse primer.

Since an optimal condition for PCR-SSCP could not be obtained at region 3A, after amplifying the entire exon 3, direct sequencing was performed.

502
the number of cells in the S-phase.\(^{20}\) Primer sequences used were as follows: \textit{Mth1}, 5'-GCAAGGTCAGGAGGAGAGAGAGG-3' (sense), 5'-ATCCTACCTTTGATGAGTGTG-3' (antisense); Pol \(\kappa\), 5'-GGACCAAGAAGCCTCAGTC-3' (sense), 5'-TGCCCATCTCTGAGTTCTC-3' (antisense); \textit{GAPDH}, 5'-TGGTAAGGTCGTTGAGTGAA-3' (sense), 5'-AGGGTCTGTTGATGGAACA-3' (antisense); histone H4, 5'-GTGTCAAGCGATCTCGGGG-3' (sense), 5'-GAGCGGTCTGCTGGTGAGTG-3' (antisense). PCR conditions were as follows; 95°C for 10 min, 40 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s. To quantify the number of molecules of a specific gene in a sample, a standard curve was generated using samples that contained 10 to 10\(^7\) copies of the gene. Real-time RT-PCR was repeated five times for each sample and the average and SE were calculated.

RESULTS

Mutation and expression analysis of \textit{Mth1}\(^{19}\) Two PhIP-induced mammary carcinoma cell lines, PhIP12-1 and PhIP7-4, and 11 primary mammary carcinomas were analyzed for mutations of \textit{Mth1}. Exon 1, exon 2, and two regions of exon 3 (region 3B and 3C) were examined by PCR-SSCP analysis, which was performed under at least two conditions so that the sensitivity was high enough to detect the mutated bands.\(^{19}\) Region 3A in exon 3 was analyzed by direct sequencing, since an optimal condition for PCR-SSCP could not be obtained.

No shifted bands or mutations were detected in any of the samples in exon 1 and three regions of exon 3 (Fig. 1A). In exon 2, shifted bands were observed in five primary carcinomas (Fig. 1A). SSCP analysis along with DNA from the non-cancerous portion of the same animals showed the same shifted bands (data not shown), and direct sequencing showed that the shifted band was due to a silent base change (ACA to ACG, Thr at codon 94; Fig. 1B). These results showed that the shifted band was due to a polymorphism present within the out-bred colony of SD rats used for this study.

Expression levels of \textit{Mth1} mRNA were analyzed by the quantitative real-time RT-PCR method in the two PhIP-induced mammary carcinoma cell lines and nine primary carcinomas. In the two cell lines, the \textit{Mth1} expression level showed a tendency to be decreased compared with the normal mammary glands, a 651-bp rat cDNA fragment by RT-PCR using a set of primers based on mouse Pol \(\kappa\) cDNA (GenBank accession number AF163571), the internal 511-bp fragment was directly sequenced (GenBank accession number AB076985). The nucleotide sequence showed 73% and 87% identity to human and mouse homologues, respectively.

Based on the rat Pol \(\kappa\) cDNA sequence, a primer set for real-time RT-PCR analysis was designed. The Pol \(\kappa\) expression levels in the cell lines were found to be decreased, and those in the primary carcinomas were also decreased compared with the normal mammary glands,

\[ P = 0.001 \text{ using GAPDH for normalization, and } P = 0.002 \text{ using histone H4}, \text{t-test}. \]

\[ P = 0.001 \text{ using GAPDH for normalization, and } P = 0.002 \text{ using histone H4}, \text{t-test}. \]

Explanations of \textit{Pol} \(\kappa\)\(^\text{19}\) First, a partial rat cDNA sequence of the \textit{Pol} \(\kappa\) gene was determined in order to design primers for quantitative PCR of rat \textit{Pol} \(\kappa\). After amplifying the sequence a 651-bp rat cDNA fragment by RT-PCR using a set of primers based on mouse \textit{Pol} \(\kappa\) cDNA (GenBank accession number AF163571), the internal 511-bp fragment was directly sequenced (GenBank accession number AB076985). The nucleotide sequence showed 73% and 87% identity to human and mouse homologues, respectively.

Based on the rat \textit{Pol} \(\kappa\) cDNA sequence, a primer set for real-time RT-PCR analysis was designed. The \textit{Pol} \(\kappa\) expression levels in the cell lines were found to be decreased, and those in the primary carcinomas were also decreased compared with the normal mammary glands,

\[ P = 0.001 \text{ using GAPDH for normalization, and } P = 0.002 \text{ using histone H4}, \text{t-test}. \]
but the decrease was not significant ($P=0.2$ using GAPDH, and $P=0.1$ using histone H4; Fig. 3).

**DISCUSSION**

Potential molecular abnormalities underlying SNI, which is considered to play important roles in rat and human mammary carcinogenesis, were analyzed in this study using characteristic increases of A:T to C:G transversions in SNI as a clue. However, $Mth1$ mutations were absent in the two PhIP-induced mammary carcinoma cell lines and eleven primary PhIP-induced mammary carcinomas. $Mth1$ expression was slightly, but significantly, increased in the primary carcinomas compared with the three normal mammary glands, and was decreased to 1/2 in the cell lines compared with a pool of MECs from four rats. However, considering the redundant expression levels of most enzymes in a cell, it seems unlikely that a decrease by 1/2 of $Mth1$ expression caused SNI, in which MRs were increased 6- to 8-fold. Actual, a recent publication showed that even complete inactivation of $Mth1$ in embryonic stem cells induced only an approximately 2-fold increase of MRs. Moreover, $Mth1$ expression was increased in the primary mammary carcinomas, including the two primary carcinomas from which the cell lines were derived and in which SNI was suggested to be present. It is known that expression of human MutT homologue

Fig. 2. Expression of $Mth1$ mRNA in rat mammary carcinoma cell lines and primary tissues. mRNA levels were quantified by the real-time RT-PCR method, and normalized to GAPDH (A) or histone H4 (B). The RT-PCR was repeated five times, and the resultant averages±SE are shown. Cell culture; no. 1, MEC, no. 2 and 3, mammary cancer cell line, PhIP7-4, PhIP12-1, respectively. Primary tissues; no. 4–6, normal mammary glands obtained from three non-treated, age-matched rats, no. 7–15, primary mammary carcinomas, PhIP2-2#2, PhIP4-3#2, PhIP7-4#1, PhIP10-1#1, PhIP12-1#1, PhIP12-3#1, PhIP14-3#1, PhIP16-1#1 and PhIP16-2#1.

Fig. 3. Expression of $Pol\kappa$ mRNA in rat mammary carcinoma cell lines and primary tissues. mRNA levels were quantified by the real-time RT-PCR method, and normalized to GAPDH (A) or histone H4 (B). Samples are the same with those in Fig. 2.
is increased in human renal-cell carcinomas and human lung cancer cell lines, reflecting the elevated oxidative stress.\textsuperscript{21,22} The increase of \textit{Mth1} expression in the rat primary mammary carcinomas might also have reflected the elevated oxidative stress. The expression levels of \textit{Pol \kappa} in the two cell lines and nine primary carcinomas were decreased compared with the rat MEC and normal mammary glands, respectively, contrary to our expectation, but decreased compared with the rat MEC and normal mammary glands. Furthermore, contrary to our expectation, but decreased compared with the rat MEC and normal mammary glands, respectively, contrary to our expectation, but decreased compared with the rat MEC and normal mammary glands. Therefore, these results excluded \textit{Mth1} insufficiency and \textit{Pol \kappa} overexpression as possible causes of SNI.

Insufficiency of nucleotide excision repair (NER) is known to increase induced mutations when cells are exposed to exogenous mutagens that need NER, but it does not lead to increases in spontaneous mutations.\textsuperscript{23,24} This is why NER insufficiency was not initially consid-
phisms. *Proc. Natl. Acad. Sci. USA*, **86**, 2766–2770 (1989).

19) Ushijima, T., Hosoya, Y., Suzuki, T., Sofuni, T., Sugimura, T. and Nagao, M. A rapid method for detection of mutations in the lacI gene using PCR-single strand conformation polymorphism analysis: demonstration of its high sensitivity. *Mutat. Res.*, **334**, 283–292 (1995).

20) Lee, P. J., Washer, L. L., Law, D. J., Boland, C. R., Horon, I. L. and Feinberg, A. P. Limited up-regulation of DNA methyltransferase in human colon cancer reflecting increased cell proliferation. *Proc. Natl. Acad. Sci. USA*, **93**, 10366–10370 (1996).

21) Okamoto, K., Toyokuni, S., Kim, W. J., Ogawa, O., Kakehi, Y., Arao, S., Hiai, H. and Yoshida, O. Overexpression of human mutT homologue gene messenger RNA in renal-cell carcinoma: evidence of persistent oxidative stress in cancer. *Int. J. Cancer*, **65**, 437–441 (1996).

22) Kennedy, C. H., Cueto, R., Belinsky, S. A., Lechner, J. F. and Pryor, W. A. Overexpression of hMTH1 mRNA: a molecular marker of oxidative stress in lung cancer cells. *FEBS Lett.*, **429**, 17–20 (1998).

23) Bol, S. A., van Steeg, H., Jansen, J. G., Van Oostrom, C., de Vries, A., de Groot, A. J., Tates, A. D., Vrieling, H., van Zeeland, A. A. and Mullenders, L. H. Elevated frequencies of benzo(a)pyrene-induced Hprt mutations in internal tissue of XPA-deficient mice. *Cancer Res.*, **58**, 2850–2856 (1998).

24) Marionnet, C., Benoit, A., Benhamou, S., Sarasin, A. and Stary, A. Characteristics of UV-induced mutation spectra in human XP-D/ERCC2 gene-mutated xeroderma pigmentosum and trichothiodystrophy cells. *J. Mol. Biol.*, **252**, 550–562 (1995).