Decreased Fidelity in Replicating CpG Methylation Patterns in Cancer Cells

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Abstract
The unmethylated or methylated status of individual CpG sites is faithfully copied into daughter cells. Here, we analyzed the fidelity in replicating their methylation statuses in cancer cells. A single cell was clonally expanded, and methylation statuses of individual CpG sites were determined for an average of 12.5 DNA molecules obtained from the expanded population. By counting the deviation from the original methylation patterns inferred, the number of errors was measured. The analysis was done in four gastric cancer cell lines for five CpG islands (CGI), and repeated six times (total 1,495 clones sequenced). HSC39 and HSC57 showed error rates <1.0 × 10⁻³ errors per site per generation (99.90-100% fidelity) for all the five CGIs. In contrast, AGS showed significantly elevated error rates, mainly due to increased de novo methylation, in three CGIs (1.6- to 3.2-fold), and KATOIII showed a significantly elevated error rate in one CGI (2.2-fold). By selective amplification of fully methylated DNA molecules by methylation-specific PCR, those were stochastically detected in KATOIII and AGS but never in HSC39 and HSC57. When methylation of entire CGIs was examined for five additional CGIs, KATOIII and AGS had frequent methylation, whereas HSC39 and HSC57 had few. KATOIII and AGS had four and eight times, respectively, as high expression levels of DNMT3B as HSC39. These data showed that some cancer cells have decreased fidelity in replicating methylation patterns in some CGIs, and that the decrease could lead to methylation of the entire CGIs. (Cancer Res 2005; 65(1): 11-7)

Introduction
CpG methylation serves as a long-term cellular memory (1). The methylation pattern of each cell is formed during embryonic development and is maintained in adult somatic cells (1, 2). Methylation of CpG islands (CGI) in gene promoters causes silencing of the downstream genes in normal and cancer cells (3–6). In order to maintain the methylated or unmethylated status of CpG sites, hemi-methylated CpG sites are methylated at DNA replication into fully methylated CpG sites, mainly by DNA methyltransferase 1 (DNMT1) that has a high affinity to hemi-methylated CpG sites (1, 5). It is also important for the maintenance of methylation patterns that unmethylated CpG sites are not methylated during DNA replication and in other phases of the cell cycle. The presence of Sp1 binding sites and gene transcription has been implicated as a mechanism to protect CGIs from de novo methylation (1, 7–10).

As for the fidelity in replicating methylation patterns, including both methylated and unmethylated statuses of CpG sites, there had been limited information. The methylated status of exogenous and endogenous sequences was inherited with fidelities of ~94% and 98.8% to 99.9% per site per generation, respectively (11, 12). Recently, taking advantage of methylation analysis by sequencing after bisulfite modification (13), we measured the fidelity in replicating the methylation status of each CpG site in normal cells (14). A single human mammary epithelial cell was expanded to 10⁶ cells, methylation patterns in the expanded cell population were analyzed, and deviation from the inferred original methylation patterns of the two alleles in the starting single cell was analyzed. The fidelity in replicating methylation patterns was in the range of 99.85% to 99.92% per site per generation in promoter CGIs and 99.56% to 99.83% in CGIs outside promoter regions. Fidelities of 99.90% and 99.60% are expected to yield 0.020 and 0.077 methylation errors per site, respectively, in 20 generations.

When a CGI is methylated, almost all CpG sites in the CGI are methylated. The methylation of a normally unmethylated CGI is known to be triggered by methylation of multiple CpG sites in the CGI (15, 16). With the high fidelity observed in normal cells, the probability that multiple CpG sites are methylated is very low, assuming that de novo methylation of proximate CpG sites is not cooperative. However, methylation of CGIs is frequently observed in cancers (6). Moreover, some cancers are associated with frequent aberrant methylation of multiple CGIs, called the CGI methylator phenotype (17, 18). Although there remains a dispute over the presence of the CGI methylator phenotype (19), one possible mechanism for the CGI methylator phenotype is a general decrease in the fidelity in replicating methylation patterns. Alternatively, methylation of CGIs could cluster by a possible growth advantage conferred by methylation of promoter CGIs of tumor suppressor genes. Cancer cells with a long history would be expected to have accumulated more aberrant methylation than cancer cells with a short history. Also, “aberrant” methylation observed in cancer cells could be simply representing methylation present in cancer precursor cells, which is too small a fraction to be detected in normal tissues.

In this study, we measured fidelity in replicating methylation patterns in four gastric cancer cells, HSC39, HSC57, KATOIII and AGS, using promoter CGIs of five genes, bA305P22.2.3, FLJ32130, a homologue of RIKEN2210016 (RIKEN2210016), E-cadherin and Cyclophilin A. The first four genes were selected because they can be potentially silenced in human gastric cancers (18, 20).

Materials and Methods

Cell Culture and DNA/RNA Extraction. HSC39 and HSC57 were kind gifts from Dr. K. Yanagihara, National Cancer Center Research Institute, and
KATOIII and AGS were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) and the American Type Culture Collection (Manassas, VA), respectively. Human mammary epithelial cells were purchased from Cambrex (East Rutherford, NJ).

For analysis of fidelity in replicating methylation patterns, a single cell in log-phase growth was plated in a well of a 96-well plate. Cells were serially transferred to a well of a 12-well plate and to a 10-cm dish. When the cells grew to the target cell number (5 × 10⁶), they were collected after measuring the actual number of cells. The number of cell generations observed was calculated from the plating efficiencies and the final cell count. The culture was repeated six times for each cell line.

High molecular weight genomic DNA was extracted by serial extraction with phenol/chloroform and ethanol precipitation, and RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan).

**Fluorescence In situ Hybridization.** Chromosome spreads were obtained by using standard protocols (21). BAC clones (RP11-305P22 for bA305P22.2.3, CT8975K-A-635H12 for FLJ32130, RP11-575L7 for RIKEN2210016, RP11-354M1 for E-cadherin, and RP11-105B9 for Cyclophilin A) were obtained from the mapping core group at the Sanger Institute or from Dr. H. Shizuya at the California Institute of Technology. A BAC was labeled with biotin 16-dUTP, using a nick translation kit (Roche, Basel, Switzerland). Probes were mixed after denaturation and then hybridized to the chromosome preparations. After washing, the signals were visualized after incubation with avidin-FITC. The washed slides were counterstained with diamidino-2-phenylindole (VYSIS, Downers Grove, IL).

**Bisulfite Modification and Sequencing.** Sodium bisulfite modification was done according to previous reports (13, 22). After restriction of genomic DNA with BamHI, 500 ng of the DNA were denatured in 0.3 N NaOH. The denatured DNA was dissolved in a solution containing 3.1 mol/L NaHSO₃ (pH 5.3) and 0.5 mmol/L hydroquinone, and the solution underwent 15 cycles of denaturation at 95°C for 30 seconds and incubation at 50°C for 15 minutes. After desalting the sample with the Wizard DNA clean-up system (Promega, Madison, WI), the sample was desulfonated in 0.3 N NaOH. The DNA sample was ethanol-precipitated with ammonium acetate and dissolved in 20 μL of TE buffer.

Each region of a specific CGI was amplified by PCR using 1 μL of the solution and primers common to the methylated and unmethylated DNA sequences (Supplementary Table; refs. 14, 18). PCR products were cloned into pGEM-T Easy vector (Promega) and were cycle-sequenced using an ABI automated DNA sequencer (Applied Biosystems, Foster City, CA). At least 10 clones and more than thrice the gene copy number obtained by bisulfite modification, the PCR product of the CGI of the E-cadherin gene mixed with rat genomic DNA was simultaneously processed. The unconversion rate of cytosines at CpG sites was <0.002. The methylation-specific PCR (MSP) product of the RIKEN2210016 CGI was similarly cloned and three to four clones were analyzed.

**Methylation-specific PCR.** MSP (23) was done using 1 μL of the bisulfite-modified DNA solution and primers specific for methylated (M) or unmethylated (U) sequences (Supplementary Table; refs. 18, 23, 24). The optimal annealing temperature was determined by amplifying DNA of normal human mammary epithelial cells and DNA treated by SssI-methylase (NEB, Beverly, MA). PCR was done for 33 to 35 cycles for analysis of CGI methylation, and a sample was regarded to have aberrant methylation when the PCR product was obtained with the M set primers. To examine the presence of DNA molecules fully methylated in the RIKEN2210016 CGI, PCR was done for 40 cycles in four replications.

**Methylation Pattern Error Rate, Fidelity, and Error Rate of a CpG Site.** All these three parameters were calculated as reported (14). To infer the original methylation patterns in the single starting cell, the bisulfite-sequenced clones were classified according to their methylation patterns. The most common patterns were used to assign starting methylation status for each allele of a particular CGI, and deviations in other bisulfite-derived clones were assigned to a particular allele based on the minimum number of changes required to match the inferred original patterns. Bias in the selection of the original patterns was confirmed to be <20% of the total number of changes required to match the inferred original patterns. Bias in the selection of the original patterns was confirmed to be <20% of the total number of changes required to match the inferred original patterns. Bias in the selection of the original patterns was confirmed to be <20% of the total number of changes required to match the inferred original patterns. Bias in the selection of the original patterns was confirmed to be <20% of the total number of changes required to match the inferred original patterns. Bias in the selection of the original patterns was confirmed to be <20% of the total number of changes required to match the inferred original patterns. Bias in the selection of the original patterns was confirmed to be <20% of the total number of changes required to match the inferred original patterns. Bias in the selection of the original patterns was confirmed to be <20% of the total number of changes required to match the inferred original patterns. Bias in the selection of the original patterns was confirmed to be <20% of the total number of changes required to match the inferred original patterns. Bias in the selection of the original patterns was confirmed to be <20% of the total number of changes required to match the inferred original patterns. Bias in the selection of the original patterns was confirmed to be <20% of the total number of changes required to match the inferred original patterns. Bias in the selection of the original patterns was confirmed to be <20% of the total number of changes required to match the inferred original patterns. Bias in the selection of the original patterns was confirmed to be <20% of the total number of changes required to match the inferred original patterns.
Results

Analysis of Fidelities in Replicating Methylation Pattern. A single cell of HSC39, HSC57, KATOIII, and AGS was expanded to 5.0 ± 0.2, 7.1 ± 0.2, 2.9 ± 0.0, and 5.6 ± 0.1 (mean ± SE) × 10^6, respectively. Plating efficiencies during the two transfers were 96 ± 2%, 86 ± 3%, 92 ± 2%, and 95 ± 1%, respectively. Based on these values, the number of cells that should have been produced at the time of harvest was calculated as 5.4, 9.5, 3.4, and 6.2 × 10^7 (final cell count per plating efficiency per plating efficiency), and they were estimated to have undergone 22.3, 23.1, 21.6 and 22.5 generations, respectively. The copy numbers of the five CGIs in the four cancer cell lines were analyzed by FISH (representative results in Fig. 1; summarized in Table 1).

The methylation status of each CpG site was determined by bisulfite sequencing of promoter CGIs of bA305P22.2.3, FLJ32130, RIKEN2210016, E-cadherin, and Cyclophilin A (representative results in Fig. 2). Based on the observed errors and copy numbers obtained by FISH, MPERs, fidelities, and error rates were calculated (Table 1). Two cell lines, HSC39 and HSC57, showed error rates smaller than 1.0 × 10⁻³ errors per site per generation for all the five CGIs, which were in the same range as our previous data in normal cells (14). In contrast, AGS had significantly elevated error rates for bA305P22.2.3 (3.2-fold compared with HSC57, \( P < 0.05 \)), RIKEN2210016 (5.7-fold, \( P < 0.01 \)) and E-cadherin (1.6-fold, \( P < 0.01 \)). KATOIII showed an elevated error rate for RIKEN2210016 (1.9-fold compared with HSC57, \( P < 0.05 \)) and tended to show an elevated error rate for bA305P22.2.3 (2.2-fold, \( P = 0.06 \)).

The errors observed were mainly due to methylation of unmethylated CpG sites, but demethylation of methylated sites was also observed. In RIKEN2210016, a DNA molecule methylated at 9 of 19 CpG sites was observed in AGS (arrowhead, Fig. 24). This showed that AGS, and possibly KATOIII, had decreased fidelities in replicating methylation patterns, that the decrease was prominent in CGIs of bA305P22.2.3 and RIKEN2210016, and that the decrease could lead to methylation of nearly half of all the CpG sites.

### Table 1. Fidelities in replicating methylation patterns in cancer cell lines

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cell line</th>
<th>No. copies</th>
<th>MPER ± S D (no. errors per site per observed generations)</th>
<th>Fidelity (% per site per generation)</th>
<th>Error rate (× 10⁻³ errors per site per generation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bA305P22.2.3</td>
<td>HSC39</td>
<td>2</td>
<td>0.012 ± 0.007</td>
<td>99.95</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>HSC57</td>
<td>5</td>
<td>0.022 ± 0.012</td>
<td>99.90</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>KATOIII</td>
<td>4</td>
<td>0.046 ± 0.024</td>
<td>99.78</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>AGS</td>
<td>3</td>
<td>0.078 ± 0.050*</td>
<td>99.64</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>HMEC</td>
<td>2</td>
<td>0.023 ± 0.016</td>
<td>99.89</td>
<td>1.1</td>
</tr>
<tr>
<td>FLJ32130</td>
<td>HSC39</td>
<td>3</td>
<td>0.000 ± 0.000</td>
<td>100.00</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>HSC57</td>
<td>4</td>
<td>0.007 ± 0.005</td>
<td>99.96</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>KATOIII</td>
<td>3</td>
<td>0.011 ± 0.014</td>
<td>99.95</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>AGS</td>
<td>2</td>
<td>0.006 ± 0.006</td>
<td>99.97</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>HMEC</td>
<td>2</td>
<td>0.000 ± 0.000</td>
<td>100.00</td>
<td>0.0</td>
</tr>
<tr>
<td>RIKEN2210016</td>
<td>HSC39</td>
<td>3</td>
<td>0.010 ± 0.002</td>
<td>99.95</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>HSC57</td>
<td>3</td>
<td>0.015 ± 0.010</td>
<td>99.93</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>KATOIII</td>
<td>3</td>
<td>0.028 ± 0.012*</td>
<td>99.87</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>AGS</td>
<td>2</td>
<td>0.087 ± 0.031†</td>
<td>99.60</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>HMEC</td>
<td>2</td>
<td>0.018 ± 0.005</td>
<td>99.91</td>
<td>0.9</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>HSC39</td>
<td>3</td>
<td>0.023 ± 0.005</td>
<td>99.90</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>HSC57</td>
<td>3</td>
<td>0.022 ± 0.002</td>
<td>99.90</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>KATOIII</td>
<td>3</td>
<td>0.019 ± 0.004</td>
<td>99.91</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>AGS</td>
<td>1</td>
<td>0.036 ± 0.009‡</td>
<td>99.84</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>HMEC</td>
<td>2</td>
<td>0.022 ± 0.012‡</td>
<td>99.89</td>
<td>1.1</td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>HSC39</td>
<td>2</td>
<td>0.011 ± 0.006</td>
<td>99.95</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>HSC57</td>
<td>3</td>
<td>0.008 ± 0.008</td>
<td>99.96</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>KATOIII</td>
<td>5</td>
<td>0.003 ± 0.003</td>
<td>99.98</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>AGS</td>
<td>2</td>
<td>0.015 ± 0.008</td>
<td>99.93</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>HMEC</td>
<td>2</td>
<td>0.032 ± 0.017‡</td>
<td>99.85</td>
<td>1.5</td>
</tr>
</tbody>
</table>

NOTE: MPERs were calculated from the observed number of errors, and the average in six clonal populations is shown along with the SD. Fidelity and error rate were calculated by the equation described in the Materials and Methods. MPERs in KATOIII and AGS were compared with that of HSC57 by the t test.

* \( P < 0.05 \).

† \( P < 0.01 \).

‡ Described in our previous report (14).
Figure 2. Representative of bisulfite sequencing. Unmethylated sites (○) and methylated CpG sites (●), and nucleotide positions of the CpG sites in the PCR product are shown above. Brackets, original methylation patterns inferred; right, numbers of deviation from them. A, RIKEN2210016. In HSC39 and HSC57, methylation patterns were stable, and original patterns were easily inferred in some cultures (culture #2 of HSC39 and #1 of HSC57). In KATOIII and AGS, methylation patterns were unstable, and it was difficult to infer the original methylation patterns. Especially, in AGS, a DNA molecule methylated in 9 of 19 CpG sites was present (arrowhead). B, E-cadherin. Methylation patterns were stable in all the four cell lines.
Emergence of Fully Methylated DNA Molecules in Cell Lines with Decreased Fidelity. The decreased fidelity produced DNA molecules methylated at nearly half of all the CpG sites. Considering that "seeds of methylation" could trigger methylation of the entire CGI (15, 16), we further analyzed the presence of fully methylated DNA molecules (Fig. 3A). Since the number of fully methylated DNA molecules was expected to be extremely small, we adopted a strategy of selective amplification of methylated DNA molecules by MSP (25). MSP primers used recognized three CpG sites located 5′ to the region analyzed by bisulfite sequencing and three 3′ CpG sites within the region (Fig. 3B). Stochastic amplification of methylated DNA molecules was specifically observed for KATOIII (10 positive reactions out of 24 reactions) and AGS (9 positive reactions out of 24 reactions), showing that zero to a few DNA molecules were contained in these reactions. All the PCR products were sequenced, and 16 CpG sites between MSP primers were fully methylated for all the clones sequenced (representative results in Fig. 3B).

Accumulation of aberrant methylation of the entire CGIs was examined, using eight additional promoter CGIs that can be methylated in human gastric cancers (Fig. 3C). None and one CGI were aberrantly methylated in HSC39 and HSC57, respectively, whereas five and seven CGIs were methylated in KATOIII and AGS, respectively. The frequency of aberrant methylation was in accordance with the decreased fidelity in replicating methylation patterns.

Gene Expression Levels of the Analyzed Genes and DNMTs. Considering that gene transcription has been implicated as one of the mechanisms to protect a CGI from de novo methylation (1, 15, 16, 26), expression levels of the five genes were measured by quantitative reverse transcription-PCR (Fig. 4). In KATOIII and AGS, in which decreased fidelity was observed for hA305P22.2.3, but not for FLJ32130, the former gene had higher expression levels than the latter. KATOIII and AGS had RIKEN2210016 expression levels comparable to those of HSC39 and HSC57. Therefore, the decreased fidelity was unlikely to be due to low expression levels.

Most of the errors in the methylation patterns in CGIs were considered to be due to de novo methylation. Therefore, expression levels of two de novo methyltransferases, DNMT3A and DNMT3B, and the maintenance methyltransferase, DNMT1, were also measured. It was shown that DNMT3B was expressed at 4- to 8-fold higher levels in the two cell lines with low fidelities, KATOIII and AGS, and that DNMT3A was not expressed at all in KATOIII.

Discussion

It was shown here that KATOIII and AGS gastric cancer cell lines had decreased fidelities in replicating methylation patterns, which was prominent in specific CGIs, such as those of hA305P22.2.3 and RIKEN2210016. The decreased fidelity was mainly due to methylation of unmethylated CpG sites, and led to emergence of fully methylated DNA molecules. This is the first experimental evidence that some cancer cells have decreased fidelity in replicating methylation patterns compared with other cancer cell lines and normal cells (14). Sequencing an average of 12.5 clones for each CGI in each cell line and repeating six experiments corresponded to analysis of 30,398 CpG sites in 1,495 clones, and this enabled to detect statistically significant increases at 1.6- to 3.2-folds.

The number of fully methylated DNA molecules was very small, in the range of zero to a few per reaction tube. For their MSP, 500 ng of genomic DNA, which corresponds to 1.5 × 10⁵ gene copies, were treated with bisulfite, and 1 of 20 of it was used. Our protocol of bisulfite treatment is known to reduce copy numbers to 1 of 20 to 30 due to DNA degradation (data not shown). This calculates as 250 to 375 copies of template DNA having been present in one reaction of MSP, and that the fraction of fully methylated DNA molecules was 0-1/250-375 (0-0.4%). Although the fraction was very small, a cell could gain a growth advantage and expand clonally if the full methylation was induced in a promoter CGI of a tumor suppressor gene. It can be noted that mutations, although their frequency is very low, have a significant impact on carcinogenesis. The decreased fidelity was also considered to be related to the frequent methylation of entire CGIs in KATOIII and AGS.
Decreased fidelity was prominent in the CGIs of bA305P22.2.3 and RIKEN2210016. In AGS, Cyclophilin A was most abundantly expressed, followed by bA305P22.2.3, FLJ32130, RIKEN2210016, and E-cadherin. Although absence of transcription is considered to be important for gene silencing (1, 15, 16, 26), the decreased fidelity in cancer cells was shown to affect even actively transcribed genes like bA305P22.2.3 and FLJ32130. Sp1 binding sites are also known to be important to protect CGIs from being methylated (7–9). There are five putative Sp1 binding sites for both bA305P22.2.3 and RIKEN2210016, whereas there are one, three and four sites for FLJ32130, E-cadherin, and Cyclophilin A, respectively. Therefore, no direct correlation was observed between the number of Sp1 binding sites and the resistance to decreased fidelity. Accordingly, promoter CGIs affected by decreased fidelity in cancers seemed to be determined by some other factors than gene transcription and Sp1 binding sites. The number of chromosomes obtained by FISH did not show any clear correlation with the fidelity, and thus histone modifications or other chromatin structures were likely to be involved.

As for the role of overexpression of DNA methyltransferases in induction of CGI methylation, reports that support the role (27–29) and those that do not (15, 30) are available. Since the decreased fidelity observed in this study was mainly due to high susceptibility to de novo methylation, we examined expression levels of DNA methyltransferases, DNMT3A, DNMT3B, and DNMT1. Although DNMT1 expression was not different in cell lines with and without the decreased fidelity, DNMT3B expression was 4- to 8-fold higher in the two cell lines with the decreased fidelity. Therefore, we could not rule out the overexpression of DNMT3B as a mechanism for the high susceptibility to de novo methylation in KATOIII and AGS, but that of DNMT1 was unlikely.

The error rates obtained here were examined in six independent cultures for each CGI in each cell line, and all the differences obtained were statistically significant and led to appearance of fully methylated DNA molecules. Possible selection bias of the original methylation patterns was confirmed to be <20% of the total number of errors, as in our previous study (14). However, our assay system neglects interaction among neighboring CpG sites for the purpose of simple calculation, and calculates the number of errors in one replication from the number of errors accumulated during a culture. The number of errors in one replication can be underestimated by the presence of a mechanism that corrects methylation errors, and can be overestimated by the presence of an error in the early phase of culture. A method recently developed by Laird et al. (31) neatly measures methylation errors in one replication, and reports larger amounts of errors than this report.

In summary, this study showed that some cancers have decreased fidelity in replicating methylation patterns, mainly due to de novo methylation, that the decreased fidelity was prominent in some specific CGIs, and that the decrease could lead to full methylation of a CGI.

Acknowledgments

Received 8/13/2003; revised 9/22/2004; accepted 10/22/2004.
Grant support: Ministry of Health, Labour and Welfare, Japan Grant-in-Aid for the Third Term Comprehensive 10-year Strategy for Cancer Control.

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We thank Dr. H. Shizuya for providing BAC clones and Drs. E. Okochi-Takada and M. Mihara for their critical discussion.
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