Genetic and Epigenetic Regulation of the Human Prostacyclin Synthase Promoter in Lung Cancer Cell Lines

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Abstract
The importance of the arachidonic acid pathway has been established in colon and lung cancers, as well as in inflammatory diseases. In these diseases, prostacyclin I2 (PGI2) and prostaglandin E2 (PGE2) are thought to have antagonistic activities, with PGI2 exerting anti-inflammatory and antiproliferative activities, whereas PGE2 is proinflammatory and antiapoptotic.

In human lung cancer, prostacyclin synthase (PGIS) and PGI2 are down-regulated, whereas PGE2 synthase (PGES) and PGE2 are up-regulated. Murine carcinogenesis models of human lung cancer reciprocate the relationship between PGIS and PGES expression. PGIS-overexpressing transgenic mice are protected from carcinogen- and tobacco smoke–induced lung tumor formation, suggesting that PGI2 may play a role in chemoprevention. We investigated several potential mechanisms for the down-regulation of PGIS in human lung cancer. Using transcription reporter assays, we show that single nucleotide polymorphisms in the PGIS promoter can affect transcriptional activity. In addition, PGIS expression in several human lung cancer cell lines is silenced by CpG methylation, and we have mapped these sites across the variable number of tandem repeats (VNTR) sequence in the promoter, as well as CpGs within exon 1 and the first intron. Finally, using fluorescence in situ hybridization, we show that human lung cancer cell lines and lung cancer tissues do not have a loss of the PGIS genomic region but multiple copies. These results show that an individual's PGIS promoter haplotype can play an important role in the predisposition for lung cancer and CpG methylation provides an epigenetic mechanism for the down-regulated PGIS expression. (Mol Cancer Res 2007;5(3):295–308)

Introduction
The arachidonic acid pathway produces a variety of bioactive metabolites involved in many different diseases, including cancer and inflammation (1-3). There are three general classes of metabolites in this pathway: leukotrienes, thromboxanes, and prostaglandins. The initial enzymatic step in these different pathways is the release of arachidonic acid by cytosolic phospholipase A from cellular membranes. The eicosanoid pathway then uses the two cyclooxygenase enzymes (COX1 and COX2) to convert arachidonic acid to prostaglandin H2, a relatively unstable intermediate. One metabolic branch point for prostaglandin biosynthesis is the enzymatic conversion of prostaglandin H2 to either prostacyclin I2 (PGI2) by prostacyclin synthase (PGIS) or prostaglandin E2 (PGE2) by PGE2 synthase (PGES). The biological effects of these two prostaglandins are generally antagonistic to each other. PGI2 is anti-inflammatory, a strong vasodilator, an inhibitor of growth of vascular smooth muscle cells, and a potent inhibitor of platelet aggregation (4). In contrast, PGE2 is proinflammatory, induces proliferation, and is antiapoptotic (5). Results from our work and others have highlighted the importance of balance in PGI2 and PGE2 levels in pulmonary diseases, such as pulmonary hypertension and chronic obstructive pulmonary disease (6).

An underlying imbalance in PGI2 and PGE2 levels in human lung cancer tissues has been described (7, 8). PGI2 is markedly lower in lung cancer tissue, and PGIS expression is down-regulated. In contrast, PGE2 level is greatly elevated and PGES expression is up-regulated. A tissue microarray study of human lung cancer showed a strong correlation between the detection of PGIS protein and patient survival (9). Therefore, we hypothesized that transgenic mice overexpressing PGIS from a lung-specific promoter would prevent lung tumor formation. Using this murine model, we showed that PGIS expression significantly decreases lung tumor formation and burden in both chemical carcinogenesis as well as in tobacco-smoke exposure models (10, 11).

Besides the potentially large number of predicted transcription factor binding sites previously described (12, 13), the PGIS promoter is a CpG island (the promoter was originally isolated in a CpG island genomic screen; ref. 14), which contains a 9-bp
variable number of tandem repeats (VNTR) sequence (5'-CCAGCCCCG-3') and several single nucleotide polymorphisms (SNP). Individual PGIS promoter alleles range from three to nine copies of the 9-bp VNTR. The VNTR sequence is predicted to contain tandem binding sites for Sp1 and AP-2 transcription factors, as well as a SNP occurring at the first C within the second repeat (5'→3' relative to the PGIS exon 1). Transient transfection studies in both bovine arterial and human aortic endothelial cells showed that the majority of the PGIS promoter transcriptional activity resides within 230 bp proximal to the initiation methionine codon (13).

Given the role of PGI2 in vascular homeostasis, the correlation of PGIS promoter VNTR number has shown mixed results in several cardiopulmonary diseases (15-18). Initial studies of the association of PGIS promoter polymorphisms in cerebral infarction indicated that promoters with fewer numbers of repeats in the VNTR were found more frequently in cerebral infarction patients. Transcriptional activity assays after transfection into human aortic smooth muscle cells gave increasing activity with increasing numbers of repeats in the PGIS promoters (17). However, PGIS promoter variants were not associated with essential hypertension (18). Finally, VNTR polymorphisms of the PGIS promoter were examined in relation to chronic thromboembolic pulmonary hypertension and not found to be associated with disease. However, patients with at least one “short” VNTR allele (S, less than four repeats) had significantly less 6-keto-PGF1α in urine, suggesting lower PGIS expression from PGIS promoters with a low number of VNTRs. In a larger study, individuals with the SS genotype had significantly higher systolic and pulse pressures (16). All of these studies focused exclusively on the Japanese population and did not further differentiate the SNPs within the PGIS promoter sequence.

Recent studies of human DNA sequence polymorphisms (19) have shown strong correlations with mRNA expression levels in several specific genes, including MDM2 (20), type I...
collagen (*COLIA1*; ref. 21), and *CD209* (22). In each of these studies, SNPs within a predicted Sp1 binding site was correlated with a biological phenotype, specifically accelerated tumor formation, decreased bone density and osteoporosis, and severity of dengue disease, respectively. Several of these studies further showed that these SNPs, within their sequence context, lead to lower Sp1 binding and transcriptional reporter assay activities in vitro (20, 22).

For these reasons, we wanted to understand the potential mechanisms responsible for the down-regulated expression of PGIS in human lung cancer. Our focus has been on the proximal ~230 bp promoter region of the *PGIS* gene. We have accumulated a well-defined set of PGIS promoter sequence variants from pooled human genomic DNA and human lung cancer cell lines. Using the PGIS promoter sequence variants and other molecular techniques, we tested three different mechanisms for regulating PGIS expression: promoter sequence variants and their effect on transcriptional activity, promoter CpG methylation, and fluorescent *in situ* hybridization (FISH) to detect chromosomal loss at the location (20q13) of the *PGIS* gene. Our results show that both genetic and epigenetic factors can play a role in PGIS expression.

### Results

The original cloning of the human PGIS promoter was on a DNA fragment containing ~3 kb of sequence 5' to the translational start Met1 (12, 13). We have shown by deletion mapping studies that the proximal ~230-bp region confers maximum transcriptional reporter activity in transfected rat aortic smooth muscle cells (8). This region of the PGIS promoter is rich not only in variety of potential transcription factor binding sites (13) but also in DNA sequence variations (Fig. 1). The *Snu* and *NcoI* sites used for cloning PGIS promoter fragments are indicated with the "*"*, identifying the G→C change to introduce the *NcoI* site.

There are three reported SNPs in dbSNP3 for this region, identified as SNP1, SNP2, and M1 in Fig. 1. M1 is especially interesting as it occurs within the 9-bp VNTR repeat sequence (5’-CCAGCCCGG-3’). Population studies of this region revealed that the VNTR sequence can occur with three to nine copies tandemly repeated (17, 23). The M1 SNP (C→T) always occurs in the second repeat (counting 5’→3’ within the promoter as shown) regardless of the total number of repeats an allele has present. The dbSNP database only reports heterozygosity data for M1 within the context of a four-repeat VNTR allele (SNP rs5582), with the less common SNP C→T having a frequency of ~5%. SNP1 T22→G (SNP rs5580) has the less common T→G change with a frequency of 28% in the populations studied. SNP2 G45→A (SNP rs5581) was reported with a 0.95/0.05 frequency.

To generate an extensive collection of PGIS promoter variants, we isolated and sequenced the region shown in Fig. 1 from 105 independent clones derived from a de-identified pool of human genomic DNA from ~550 individuals. The PGIS promoters were amplified from genomic DNA using PCR, and cloned into pGL3-Basic, a luciferase transcription reporter plasmid. The *NcoI* site created by the PCR reverse primer is ligated in-frame to the initiation Met1 of the luciferase coding region at its *NcoI* site (CCATGG). These constructs closely recreate the PGIS promoter context with its native initiation Met1. The PGIS promoter sequences were scored for the VNTR repeat number and frequency of the three SNPs (Tables 1 and 2). Although the ethnicity of the individuals in this genomic DNA pool is unknown, the VNTR distribution observed is very similar to those previously reported by other investigators (17, 23). The four- and six-repeat VNTR alleles are the most frequently observed, with the odd-numbered VNTR repeat alleles occurring <5% of the time. VNTR alleles with more than seven repeats are extremely rare and have only been observed in the Gabonese population study (23).

The SNP1 and SNP2 distributions were similar to the frequencies reported in dbSNP although their 32 tests were

### Table 1. Population Frequency of VNTR Repeats in the PGIS Promoter

<table>
<thead>
<tr>
<th>VNTR Repeat No.</th>
<th>De-identified Human Genomic Pool, %</th>
<th>French Caucasian (25), %</th>
<th>Japanese (17), %</th>
<th>Tunisian (23), %</th>
<th>Gabonese (23), %</th>
</tr>
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<tbody>
<tr>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
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<tr>
<td>4</td>
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<td>5</td>
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<td>82</td>
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<td>48</td>
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<td>1</td>
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<td>8</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
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NOTE: Data from published studies used the normal individual controls in which they determined ≥100 different alleles. De-identified human genomic pool data were obtained in this study. All pairwise χ2 tests of the VNTR distribution pattern showed significant differences between populations (χ2 test P ≤0.05) except for Tunisian and Japanese or Tunisian and French Caucasian.

Table 2. Observed Frequency of the Two Reported SNPs of the PGIS Promoter from the Human Genomic Pool, and M1 Frequency from the Genomic Pool Compared with Previously Reported Populations

<table>
<thead>
<tr>
<th>SNP</th>
<th>High Frequency (%)</th>
<th>Low Frequency (%)</th>
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<tbody>
<tr>
<td>SNP1 T22→G human genomic pool (rs5580, frequency T/G, 0.72/0.28)</td>
<td>84</td>
<td>16</td>
</tr>
<tr>
<td>SNP2 G45→A human genomic pool (rs5581, frequency G/A, 0.95/0.05)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>M1 C→T (human genomic pool)</td>
<td>61</td>
<td>39</td>
</tr>
<tr>
<td>M1 C→T (French Caucasians; ref. 25)</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>M1 C→T (Tunisian; ref. 23)</td>
<td>71</td>
<td>29</td>
</tr>
<tr>
<td>M1 C→T (Gabonese; ref. 23)</td>
<td>77</td>
<td>23</td>
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significantly different ($P \leq 0.025$), probably due to differences in the populations under evaluation (Table 2). SNP2 was not detected in its minor form, whereas SNP1 T22→G was found with the frequency of 84% to 16%, respectively. Previously reported PGIS promoter studies of the VNTR have not reported frequencies for these SNPs (15, 17, 18, 24). Our pooled genomic DNA study revealed that M1 C→T, the SNP occurring within the VNTR repeat itself, have a frequency of 61% and 39% (Table 2). One study of French Caucasians (25) identified M1 within this population only in the six-repeat VNTR (68% overall of the alleles were six repeats in this population) at an allele frequency of 45% C and 55% T, much more common than expected from the data in dbSNP for the four-repeat allele (95% C; 5% T). M1 frequency data was also reported for the Tunisian and Gabonese populations (23). As found for the frequency distribution of VNTR lengths, the M1 frequency in the Gabonese population is distinct. Thus, the frequency for M1 may vary within the context of how many repeats are within the VNTR sequence of a given allele.

Six different human non–small cell lung cancer lines were characterized for their PGIS expression levels by quantitative reverse transcription-PCR (qRT-PCR) and Western blot analysis (Fig. 2). The non–small cell lung cancer lines were originally derived from a variety of histologic phenotypes in the primary lung tumor (26), although many of the lines were grown from metastasis sites, not the primary tumors. A representative plot of the various cell line qRT-PCR measurements is shown in Fig. 2A. Only H226 had significant mRNA expression ($C_t = 21.0$), with H157 having an intermediate mRNA level ($C_t = 31.4$). The other four cell lines (A549, H322, H460, and H2122) gave PGIS $C_t$ values that were not statistically different from the no template control reactions (average cell line $C_t = 38.4$ versus no template control $C_t = 37.9; P > 0.10$). These four cell lines are indicated as “nonexpressing” PGIS because they did not give qRT-PCR signals above background, and thus are at the current limits of expression sensitivity. Western blot analysis of protein extracts from these cells detected significant expression of PGIS protein in H226, in agreement with the analysis of protein extracts from these cells detected significant amounts of PGIS protein, had two repeat with M1 in its second VNTR repeat. (Because the M1 SNP occurs within the context of the VNTR, the rarer T form will be abbreviated as repeat#M1, e.g., “6M1”; the more common form as repeat#WT, “6wt.”) Both alleles had the common SNP variants at the other locations. Many of the cell lines had distinguishable PGIS promoter sequences by their full allele type (VNTR and SNPs). For instance, H157 had 5wt and 6M1 alleles, although interestingly the 6M1 allele also had the relatively rare SNP2 (G45→A, ~5% occurrence). The 6M1 SNP was not found in any other cell line associated with SNP2 G45→A, suggesting that SNP2 G45→A and 6M1 do not form a common haplotype. The M1 alleles found in other lines were

![Figure 2](https://example.com/figure2.png)

**FIGURE 2.** PGIS and PGES expression in human non–small cell lung cancer lines by qRT-PCR and Western blot analysis. **A**, qRT-PCR plot for PGIS mRNA expression in human lung cancer cell lines. H226 expresses significant amount of PGIS mRNA (calculated critical threshold value, $C_t = 21.0$), whereas the PGIS mRNA level in H157 was detectable ($C_t = 31.4$) although ~500-fold lower than in H226. The PGIS $C_t$ values for the nonexpressing cell lines (A549, H322, H460, and H2122) were indistinguishable from the no template control reactions (average cell line $C_t = 38.4$ versus no template control $C_t = 37.9; P > 0.10$). The $β$-actin $C_t$ values for the six cell lines averaged $C_t = 20.8$ and were not statistically different from one another ($P > 0.10$). $C_t$ values are the average of three independent RNA preparations measured in duplicate reactions. **B**, Twelve micrograms of whole-cell protein radioimmune precipitation assay buffer extracts were SDS denatured and separated on a 4% to 20% polyacrylamide gradient gel. After semidry transfer to an Immobilon-P membrane, the PGIS protein was detected using a rabbit polyclonal antibody to bovine PGIS (Cayman), or mouse monoclonal antibody to human $β$-actin (Chemicon). Six different non–small cell lung cancer lines were tested (lanes 1-6: A549, H157, H226, H322, H460, and H2122, respectively).
also not associated with SNP2 G45→A. SNP1 (T22→G) was found associated with all size repeats except four (only one promoter isolated from the cell lines), as expected for a relatively common SNP.

The PGIS promoters characterized from the human genomic pool and lung cancer cell line DNAs resulted in a large collection of PGIS promoter variants in pGL3-Basic, a transcription reporter plasmid. PGIS promoter sequence variants were transfected into A549 and H2122, and the luciferase activities were measured relative to the h-galactosidase transfection control plasmid (Table 3). Promoter variants were chosen to assess the effects of VNTR length changes while keeping the SNP composition constant (T22, G45, and wt C within the VNTR; Fig. 4A) or the SNPs with constant VNTR length (Fig. 4B). Results from these studies are shown in Fig. 4 after transfection into these cell lines, and the relative ordering of promoter strength was similar. Figure 4A examines the transcriptional activity as VNTR repeat number increases within the promoter sequence context of all the SNPs in their most common form (T22 G45 wt). As others have observed, there is an increase in transcriptional activity of the PGIS promoter with increasing number of VNTR repeats. Each VNTR repeat sequence contains a putative Sp1 transcription factor recognition site. One hypothesis explaining this trend is that there is increased Sp1 occupancy with longer VNTRs, resulting in higher transcriptional activity. The transcriptional effect of the SNP1 T22→G and M1 were next assessed within a fixed VNTR repeat length (Fig. 4B). The less frequent form of SNP1, G22, produced lower transcriptional activity in the luciferase reporter assay. The M1 SNP showed a trend toward slightly higher activity in its less common form (M1 T), although the results were not statistically significant at P < 0.05.

As mentioned above, the PGIS promoter was originally isolated in a genomic DNA library screen enriching for regions containing a CpG island (14). Many of the CpG islands have a transcriptional regulatory role through methylation silencing, especially in many cancer types. One method used to detect and map the locations of methylated CpG dinucleotides makes use of the ability of the restriction enzymes to cleave methylated recognition sites containing CpG. By way of example, genomic DNA is treated with either HpaII (only cuts unmethylated CCpGG sites) or MspI (cuts CCpGG regardless of methylation). Once digested, PCR using primers that are outside of these restriction sites is done. If a CCpGG site is methylated, the expected PCR product will be found after HpaII digestion but not MspI. Using this approach, the human lung cancer cell line genomic DNAs were tested for the methylation status at a number of potential CpG methylation sites (Fig. 5). The results showed that H226 (the PGIS expressing cell line) and H157 were unmethylated at all the sites that were queried using the restriction enzymes. All the other cell lines were either partially or fully methylated.

A more sequence-specific approach to mapping CpG methylation uses chemical modification of unmethylated cytosine by bisulfite, and subsequence mapping by DNA sequence analysis (27, 28). Various PCR methods have been developed based on this idea that can map the CpG methylation status of a particular region as well as a more global analysis.

<table>
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<tr>
<th>VNTR Repeat No.</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tbody>
<tr>
<td>VNTR</td>
<td>3wt T22</td>
<td>4wt T22</td>
<td>5wt T22</td>
<td>6wt T22</td>
</tr>
<tr>
<td>6wt G22</td>
<td>6M1 T22</td>
<td>6M1 G22</td>
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These methods rely on the ability of bisulfite to chemically convert cytosines to uridines (C to T change in amplified DNA sequence) but 5-methylcytosines are resistant to this change (mC remains unchanged as C). We used this method to determine the CpG methylation pattern of 21 to 23 CpG dinucleotides spanning the VNTR sequence, exon 1, and the 5' side of intron 1 (Fig. 6A). The results are shown for the common CpG methylation patterns observed for each allele in the PGIS promoter region (Fig. 6B). The PGIS alleles in cell lines H157 and H226 can be differentiated because of their different repeat number in the VNTR (H157, five or six repeats; H226, four or six repeats). Cell lines with no detectable PGIS expression by qRT-PCR (A549, H322, and H2122) showed significant CpG methylation across the region of the PGIS promoter examined, with most CpG dinucleotide being consistently methylated. H226, which expresses PGIS mRNA, was uniformly unmethylated at all sites with <10% CpG methylation found. The VNTR sequence, which contains a CpG site (CCAGCCCpG), had no detectable methylation in either allele of H226. Interestingly, H157 also had a low amount of CpG methylation in this region and expressed a small amount of PGIS mRNA expression by qRT-PCR (Fig. 2B).

Lung cancer cell lines were treated with 5-aza-2'-deoxy-2'-deoxycytidine (5-aza-dC) to assess the functional importance for epigenetic silencing of the CpG methylation detected in the PGIS promoter (Fig. 7). In A549 and H2122, both with heavily CpG methylated PGIS promoters, PGIS mRNA expression was increased ~1,000-fold after 48 h treatment with 5 μmol/L 5-aza-dC (as determined by ΔΔCt calculations). H157, which has little CpG methylation of the PGIS promoter and expresses a limited quantity of PGIS mRNA, showed little change in its PGIS mRNA level after 5-aza-dC treatment. Because PGIS promoters in H157 are unmethylated, 5-aza-dC would be predicted to have limited effect on PGIS expression, although it is possible that 5-aza-dC could derepress the expression of another gene, for instance a component of the transcription complex, which is limiting PGIS expression in H157. H226, which also has little CpG methylation but does express a significant amount of PGIS mRNA, was also not affected by 5-aza-dC treatment. Finally, the level of ß-actin mRNA was unaffected in all four cell lines by 5-aza-dC treatment. These results are consistent with CpG methylation causing epigenetic silencing of the PGIS promoter in cell lines with heavily methylated promoter sequences.

One model whereby longer VNTR-containing promoters give increased PGIS expression is due to increased binding of the transcription factor Sp1. However, it has never been shown if Sp1 is capable of binding the PGIS promoter directly. Using chromatin immunoprecipitation, we tested this model by comparing Sp1 binding to the PGIS promoter in either the

FIGURE 4. Transcriptional activity of PGIS promoter variants in luciferase reporter plasmid pGL3-Basic. A, A549 and H2122 were transiently transfected with VNTR variants, keeping the other SNPs constant and in their most commonly occurring form (SNP1 T22, SNP2 G45, and M1 C). Columns, luciferase activity per µ-galactosidase units (transfection control plasmid) after normalizing to the most common PGIS promoter variant (6wt T22, RLU/Z); bars, SD. Bottom tables, P values calculated by t test (two-tailed, nonconstant variance); bold numbers, P < 0.05. Expression of all PGIS promoter constructs was significantly different from pGL3-Basic (P < 0.001). B, A549 and H2122 were transiently transfected with SNP1 and M1 variants keeping the VNTR repeat number constant. Columns, luciferase activity per µ-galactosidase units (transfection control plasmid) after normalizing to the most common PGIS promoter variant (6wt T22); bars, SD. Bottom tables, P values calculated by t test (two-tailed, nonconstant variance); numbers in bold, P < 0.05. The t test comparison of 4wt T22 versus G22 was P < 0.05 in A549 but not in H2122.
expressing cell line H226 or nonexpressing cell line A549 (Fig. 8). Sp1 antibody specifically recovered the PGIS promoter from H226, and the PGIS promoter was not recovered in A549. Interestingly, both the four- and six-repeat PGIS alleles were binding Sp1 in H226, suggesting that either or both alleles are capable of being transcribed. This result is consistent with the importance of Sp1 binding to the PGIS promoter for PGIS mRNA expression.

Genomic instability, causing loss and amplification of large regions of the genome, is common in many cancers (29, 30). In human lung cancer, a catalog of frequently deleted and amplified regions of the genome has been compiled, with chromosome 20q13 often found as an amplified region. We used FISH to specifically examine loss of the PGIS gene, which spans ~75 kbp, by examining its genomic location on a larger scale. A human genome-wide survey of FISH probes was searched and the bacterial artificial chromosome (BAC) clone RPC11-298O6 was identified. DNA sequence using primers flanking the BAC cloning site verified that RPC11-298O6 spans the PGIS gene. The specificity of RPC11-298O6 for FISH was tested on the

FIGURE 5. Mapping methylated CpG dinucleotides in the PGIS promoter by 5-methylcytosine–specific restriction enzymes. A, Ethidium bromide staining of a high-resolution Metaphor agarose gel is shown of the PCR products generated after restriction digestion of cell line genomic DNA with the indicated restriction enzymes. Restriction enzymes chosen include CpG methylation–resistant (MspI), CpG methylation–sensitive (HpaII, BstUI, and HaeII), and three control enzymes (C1, HaeIIl sites in the PGIS promoter but no CpGs; C2, BstUIl; C3, TaqI, CpG methylation sensitive but no sites within the PGIS promoter). The presence of the PCR product in lanes 1, 4, and/or 5 indicates CpG methylation at those sites. For BstUIl, which has two recognition sites, the absence of the PCR product indicates that at least one of the two sites was unmethylated. B, Restriction map of the sites used and tabulated results for the 10 human lung cancer cell lines examined. M, methylated CpG site; U, unmethylated CpG site at the restriction enzyme sites. H157 and H226 were the only two cell lines completely unmethylated.
lymphoblastoid cell line GM09948, which has a normal male karyotype. Analysis was done on metaphase spreads with p3.2 probe recognizing homology to the centromere of chromosome 20 (Cen20) and RPC11-298O6 recognizing homology with a chromosomal band compatible with 20q13.13 (Fig. 9A and B). The fluorescence signals generated by both probes had excellent intensity. The hybridization efficiency estimated for the BAC probe based on analysis of 30 metaphase spreads was 98.3%. RPC11-298O6 was then used to test the presence or absence of the PGIS gene in six different non–small cell lung cancer lines and six human lung adenocarcinoma tissues (Table 4). In all the cell lines examined, there was increased copy number of both RPC11-298O6 and Cen20, although the PGIS to Cen20 ratio of ~1.0 was maintained (Fig. 9A and B). The fluorescence signals generated by both probes had excellent intensity. The hybridization efficiency estimated for the BAC probe based on analysis of 30 metaphase spreads was 98.3%.

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**FIGURE 6.** Methylated CpG dinucleotide mapping of the VNTR of the PGIS promoter: exon 1 and intron 1. **A.** Map of the PGIS promoter, exon 1 and intron 1, analyzed by DNA sequencing after bisulfite treatment of genomic DNA. A total of 21 to 23 CpG sites (asterisk lollipops) were analyzed depending on the VNTR repeats (small black arrows) for a given allele (four to six repeats, with each repeat containing one CpG). Gray box starting with ATG, the exon 1 coding region. **B.** Representation of the CpG methylation patterns observed in individual PCR clones after bisulfite treatment of genomic DNA. The cell line, VNTR repeat number of distinguishable alleles, and number of independent clones observed for each CpG methylation pattern is shown in the first three columns. Crossed boxes, missing repeats in alleles with less than six repeats in the VNTR. Unmethylated (open boxes) and methylated (filled boxes) CpG positions. To show common overall methylation patterns, a few positions are shaded to indicate <100% methylation at those positions (hatched boxes, >50% methylation; light stippled boxes, <50% methylation). Boxes with asterisk, absence of a CpG site that may correspond to an infrequent SNP not previously reported. H157 and H226 exhibited little CpG methylation in the region analyzed compared with the other cell lines.

In all cell lines examined, there was increased copy number of both RPC11-298O6 and Cen20, although the PGIS to Cen20 ratio of ~1.0 was maintained (Fig. 9C-E). H157 had approximately five copies of PGIS and Cen20, whereas the other cell lines had approximately three copies of each. Five of the six human lung adenocarcinoma tissue samples showed a disomic number of copies for PGIS and Cen20 (approximately two per cell) although one tissue had ~3-fold gain in the PGIS gene (approximately six copies per cell; Fig. 9F). These results are in agreement with genome studies indicating a trend toward amplification of 20q13 (29, 30). Our experiments addressed the PGIS genomic region specifically and suggest that the decrease in PGIS expression in human lung cancer is not the result of loss of the PGIS gene.

**Discussion**

The arachidonic acid pathway and its metabolites play a central role in inflammation, vascular and pulmonary diseases, and cancer. A consistent finding in many of these different conditions is the down-regulation of PGIS and up-regulation of PGES. We previously showed that survival is correlated with detectable PGIS expression in lung tumor samples (9). In this work, we sought to identify and understand potential mechanisms leading to the observed down-regulation of PGIS expression in human lung cancer. We investigated three different means of down-regulation of PGIS expression in human lung cancer cell lines and tissues—the transcriptional
effect of the PGIS promoter DNA sequence polymorphisms, the role of CpG dinucleotide DNA methylation within the promoter, and allelic loss of the PGIS gene. From genome studies of human DNA sequence polymorphisms, the PGIS promoter has a number of SNPs identified as well as a VNTR located adjacent to the initiation methionine of the PGIS protein. The VNTR sequence contains predicted Sp1 and AP-2 transcription factor binding sites. We assessed the transcriptional activities of a wide range of observed PGIS promoter variants. In cell lines, increasing number of VNTR repeats resulted in higher transcriptional activity. These data support a model whereby increasing tandem transcription binding sites facilitates their binding and ability to increase transcription. In addition, we tested different SNPs within a constant VNTR framework of the promoter. The relatively common SNP1 (T22→G) gave lower transcriptional activity when present as G compared with T at a constant VNTR repeat number. This is the only SNP that introduces a new potential CpG dinucleotide site for methylation silencing. The M1 SNP (C→T), which occurs within the VNTR, had a trend toward increasing the PGIS promoter transcriptional activity. The presence of the M1 SNP (C→T) in either form is still predicted to be a Sp1 binding site. Finally, SNP2 (G45→A) gave a significant increase in transcriptional activity within the five-repeat VNTR context. One hypothesis that may explain the effect of the SNPs on transcriptional activity is a generalized increase in transcription factor access to the PGIS promoter with reduced G-C content. Alternatively, as shown for the CpG island of the c-myc promoter (31), the PGIS promoter may form a DNase hypersensitive site and/or G-quadruplex structure, which could be altered by the different SNPs and/or VNTR repeat lengths.

Recently, several papers have examined the role of PGIS promoter VNTR variations and methylation in colorectal cancer (32, 33). Results comparable with our PGIS studies of murine and human lung cancer have been observed in a murine model and human colorectal cancer. A large case-control study of adenomatous and hyperplastic colon polyps showed an increased risk for these polyps in individuals when both PGIS promoter alleles have VNTR repeat lengths less than six (odds ratio, ~1.9; ref. 33). In addition, Frigola et al. (32) described the hypermethylation of the CpG dinucleotides in the PGIS promoter in colorectal cancer. Seven colon cancer cell lines were hypermethylated, and there was no detectable PGIS mRNA expression in the four lines tested. Fifteen paired normal and tumor colorectal cancer tissues were examined, with 10 showing partial or hypermethylation of the promoter and low relative PGIS expression (tumor tissue versus its normal counterpart). Four tumor samples were unmethylated and had increased expression of PGIS in the tumor tissue. One tissue sample was hypermethylated but had increased PGIS expression. As we have shown in lung cancer, the PGIS promoter CpG methylation status is correlated with low PGIS expression and seems to be a frequent event in colorectal cancer.

**FIGURE 7.** Induction of PGIS mRNA expression in CpG-methylated cell lines by 5-aza-dC treatment. The indicated cell lines were treated with 5 μmol/L 5-aza-dC for 48 h, and total RNA was prepared. Quantitative RT-PCR was used to measure the level of PGIS and β-actin mRNAs from 100 ng of total RNA per reaction. The calculated critical threshold value (Ct) was determined from three independent experiments, each measured in duplicate. A total PGIS expression value was estimated using the average of five different primer-probe sets that spanned the gene. The induction of PGIS mRNA by 5-aza-dC in A549 and H2122 was ~1,000-fold and was statistically significant (****, P < 0.0001). The PGIS Ct values of untreated A549 and H2122 were not statistically different from the no template control reactions.
We have shown that different PGIS promoter alleles have different transcriptional activities in our reporter assay. We showed for the first time the effect that PGIS promoter SNP and VNTR length variants have on its transcriptional activity. In addition, hypermethylation of CpG dinucleotides found within the PGIS promoter CpG island region is associated with decreased expression. The down-regulation of PGIS expression by CpG methylation can be reversed by 5-aza-dC, an analogue of deoxycytidine that cannot be methylated. Finally, the transcription factor Sp1 was found associated by chromatin immunoprecipitation with the PGIS promoters found in H226, a PGIS-expressing lung cancer cell line. The degree of CpG methylation within the specific sequence context of the PGIS promoter SNPs and VNTRs could yield a wide range of transcriptional activity for the PGIS gene. In particular, does an individual’s inherited PGIS allelotype provide a predisposition marker for developing lung cancer or other pulmonary diseases due to lower mRNA expressing PGIS promoters or as a signal for CpG hypermethylation? Mapping CpG methylation in microdissected lung tissue samples from lesions/tumors in lung cancer, pulmonary hypertension, and chronic obstructive pulmonary disease may provide evidence for a more generalized epigenetic mechanism for the down-regulation of PGIS expression. Our future studies will investigate these relationships in human lung diseases in which our mouse studies have shown a protective effect of PGIS overexpression in decreasing lung cancer and pulmonary hypertension in this model system (6, 10).

Materials and Methods

Biological Materials

Human lung cancer cell lines were obtained from B. Helfrich (University of Colorado Comprehensive Cancer Center Tissue Culture Core, Denver, CO). Tissue culture cells were maintained at 37°C in 5% CO₂ using RPMI 1680 with glutamine, 1% concentration of penicillin-streptomycin, and 10% FCS (complete RPMI medium; Mediatech, Herndon, VA and Invitrogen, Carlsbad, CA). Cell lines were split every 3 to 4 days to ~25% confluence. 5-Aza-2’-dC (Sigma, St. Louis, MO) was added at 5 μmol/L concentration, 24 h after splitting the cell lines to complete medium, and the cells were treated for 48 h. Restriction and DNA modifying enzymes were from New England Biolabs (Beverly, MA), and DNA constructions were transformed into subcloning efficiency DH5α (Invitrogen). Chemicals and reagents were from Sigma-Aldrich (St. Louis, MO) or Pierce (Rockford, IL). High-resolution agarose gel electrophoresis of PCR products was done using 2% Metaphor Agarose (Cambrex, East Rutherford, NJ), 1 × Tris-borate EDTA buffer, and 1-mm combs to form the sample wells. DNA oligonucleotides were synthesized by IDT, Inc., (Coralville, IA) and purified by high-performance liquid chromatography.

FIGURE 8. Chromatin immunoprecipitation of SpI binding to the PGIS promoter in H226, a PGIS mRNA–expressing cell line. Chromatin immunoprecipitation of soluble chromatin extracts from A549 or H226 using rabbit anti-human Sp1 IgG (lanes 1 and 3) or normal rabbit IgG (lanes 2 and 4). Negative controls included immunoprecipitation Protein G Sepharose beads alone (lane 5), DNA extraction buffer from beads (lane 6), and a no template control PCR reaction (lane 9). Lanes 7 and 8, recovered genomic DNA from ~10% of the immunoprecipitation extract as input DNA controls from A549 and H226, respectively.

PGIS Protein Expression Analysis

Total protein extracts from the human lung cancer cell lines were prepared in radioimmunoprecipitation assay buffer containing protease inhibitors (Sigma). Protein concentration was measured using the micro–bicinchoninic acid assay using bovine serum albumin as the standard (Pierce). Ten micrograms of total protein from each cell line was separated on a precast 4% to 20% polyacrylamide gel (Cambrex) and transferred to Immobilon-P filters (Millipore, Billerica, MA) for Western blot analysis. After blocking the filter with 5% skim milk in 1 × PBS + 0.05% Tween 20, polyclonal rabbit antihuman PGIS (gift from Dr. David Dewitt, Michigan State University, East Lansing, MI), rabbit anti-human PGIS (Cayman, Ann Arbor, MI), or mouse monoclonal anti-human β-actin (Chemicon, Temecula, CA) were added at 1/1,000 dilution and rocked overnight at 4°C. After rinsing the filter five times in 1 × PBS + 0.05% Tween 20, secondary donkey anti-rabbit IgG conjugated with horseshard peroxidase (Amersham, Piscataway, NJ) was added at 1/20,000 dilution at room temperature for 1 h. The filter was washed five times in 1 × PBS + 0.05% Tween 20, and PGIS was detected using SuperSignal West DuraSubstrate Detection reagent (Pierce).

PGIS mRNA Expression Analysis by qRT-PCR

Total RNA was prepared from the human lung cancer cell lines by first lysing the cells directly in 1 mL TRIzol reagent (Invitrogen), and the RNA was separated into the aqueous phase after addition of chloroform. The RNA was further purified using the RNeasy Mini kit (Qiagen, Valencia, CA). RNA was quantitated using UV absorption, and quality was verified by analyzing 100 ng on a BioAnalyzer (Agilent, Santa Clara, CA). One hundred nanograms of total RNA were reverse transcribed and quantitated using the iScript One-Step RT-PCR reagent (Bio-Rad, Hercules, CA) in 25 μL final volume with commercially available primer-probe sets for β-actin and PGIS (Applied Biosystems, Foster City, CA) using the manufacturer’s conditions. Data were collected on either an ABI7500 or iCycler real-time PCR machine (Applied Biosystems or Bio-Rad, respectively). Five different primer-probes sets were used to quantitative PGIS expression spanning exons 1-2, 2-3, 3-4, 7-8, or 8-9, and used to give a calculated gene expression average.

PCR Amplification of the Human PGIS Promoter

The PGIS promoter region is ~85% G-C rich and required custom PCR conditions. The cloning strategy made use of a naturally occurring StuI site, 232 bp 5’ to the translational methionine start codon (Met1), and the alteration of the G→C residue adjacent to the Met1 creating an unique NcoI (Fig. 1). The primers used (5’ PGIS F1, 5’-TGACATTTTCCCCCAGG-CCTGAGCTGC-3’; PGIS Neo R2, 5’-TACGGCCCAAG-CCATGCGGGCGT-3’; StuI and NcoI sites are underlined)
also had high calculated $T_m$ values to facilitate amplification (68-72°C). Reaction conditions were as recommended by the polymerase supplier but included 1× Q reagent (Qiagen), 100 ng of human genomic DNA, and cycle variables: 1-min preheat, 94°C; supplier-recommended enzyme activation step, 94°C; and 40 cycles between 1 min, 94°C and 2 min, 72°C. Advantage 2 Polymerase (Clontech, Mountain View, CA) and Hot Star Polymerase (Qiagen) were the most robust enzymes tested under these conditions. After genomic DNA amplification, the resulting DNA product was digested with StuI and NcoI, and ligated into SmaI and NcoI digested pGL3-Basic (Promega, Madison, WI), a standard luciferase transcription reporter plasmid. One hundred five independent clones derived from the human genomic pool DNA and at least eight independent promoter clones from each cell line were sequenced.

DNA Sequence Determination and Analysis

One-quarter scale Big Dye v1.1 reactions (ABI, Foster City, CA) were run with ~180 ng plasmid DNA and 10 pmol sequencing primers for 30 cycles using the recommended protocol in the presence of 0.5× Q reagent (Qiagen) with an

FIGURE 9. FISH analysis for the PGIS genomic region in human lung cancer cell lines and lung cancer tissue sections. RPC11-298O6 BAC clone used for FISH studies contains ~200 kb of DNA from chromosome 20q13.13 with overlapping ends homologous to neighboring genes of PGIS, KCNB1, and B4GALT5. A. Analysis was done in metaphase spreads of the lymphoblastoid cell line GM 09948, which has a normal male karyotype, to ascertain the chromosomal location of the PGIS FISH probe RPC11-29806 and Cen20 probe (p3.3). The Cen20 probe recognized homology with the centromere of chromosome 20 and the RPC11-29806 probe recognized homology with a chromosomal band compatible with 20q13.13. The fluorescence signals generated by both probes had excellent intensity. The hybridization efficiency estimated for the BAC probe based on analysis of 30 metaphase spreads was 98.3%. B. Chromosome 20 idiogram and partial metaphase showing hybridization to Cen20 and 20q13.13. C. Metaphase and interphase cell of NSCLC cell line A549 showing three copies of PGIS (red signals) and Cen20 (green signals). D. Metaphase and interphase of non–small cell lung cancer line H226 showing four copies of PGIS (red signals) and Cen20 (green signals). E. Non–small cell lung cancer line H157 showing, respectively, seven and six copies of PGIS and Cen20 in a metaphase cell, and six copies of PGIS and four copies of Cen20 in an interphase cell. F. Formalin-fixed, paraffin-embedded tissue section of a non–small cell lung cancer adenocarcinoma showing large nuclei with 5 to 10 copies of PGIS and 3 to 7 copies of Cen20 (Tissue sample S99-1551 H#2 6654; Table 4).
annealing temperature of 72°C. DNA sequencing primers were designed to have a higher melting temperature and annealing site 60 to 70 nucleotides away from the cloning sites used in pGL3-Basic and a modified pUC19 (see below). The sequencing primers used were as follows: pGL3-F2, 5'-TCGATAGTAC-TCGATAGTAC-3'; pGL3-R2, 5'-GGCGTATCTCTTCATAGCCCTTATGC-3'; pGL3-Basic, 5'-GGCGTATCTCTTCATAGCCCTTATGC-3'; pGL3-R2, 5'-GGCGTATCTCTTCATAGCCCTTATGC-3'; M13 Univ (−71), 5'-GTGCG-TGCAAGGGCATAAGTTGGTGTAAC-3'; M13 Rev (−61), 5'-GTGCGTGAATTGTGAGCGGATAAC-3'; M13 Rev (−61), 5'-GTGCGTGAATTGTGAGCGGATAAC-3'; M13 Rev (−61), 5'-GTGCGTGAATTGTGAGCGGATAAC-3'; M13 Rev (−61), 5'-GTGCGTGAATTGTGAGCGGATAAC-3'. After alcohol precipitation, the reactions were run on an ABI 310 Genetic Analyzer capillary electrophoresis and the chromatograms were read manually to ensure correct interpretation of the high G–C–rich DNA sequence. DNA sequencing was completed by the DNA Sequencing Core of the Barbara Davis Center for Childhood Diabetes (Aurora, CO).

**Cell Line Transfection and Transcription Reporter Assays**

Human lung cancer cell lines were split into six-well tissue culture plates at ~50,000 cells per well in complete RPMI medium. After 1 to 2 days, the cell lines were transfected using 6 μL LT-1 (Mirus, Madison, WI) lipid reagent using 1 μg of pGL3-Basic constructs and 1 μg of pSV-βGal (Promega) as an internal transfection control, as recommended. After an additional 2 days, cell protein lysates were prepared in 400 μL 1× Reporter Lysis buffer (Promega) by scraping, and frozen at −20°C. The total protein concentration was determined by micro–bicinchoninic acid assay (Pierce), β-galactosidase activity using 2× reaction buffer with o-nitrophenyl-β-D-galactopyranoside (Promega), and luciferase levels with Luciferase Reporter Assay (Promega). Luciferase values were calculated relative to the β-galactosidase activity in the same extract, and then normalized to the six-repeat VNTR PGIS promoter construct. Assays were run in triplicate and averaged over at least four independent experiments.

**Mapping of Methylated CpG Dinucleotide sites in the PGIS Promoter**

Genomic DNA was isolated from frozen cell pellets from each of the human lung cancer cell lines using DNeasy kit (Qiagen). Two approaches were used to map methylated CpG dinucleotide sites in the PGIS promoter. First, restriction enzymes of known sensitivity to CpG methylation were used to digest 1 μg of genomic DNA before PCR amplification with CI PGIS F1/PGIS Nco R2 primers. CpG methylation, which blocks restriction digestion, will still generate the expected PGIS promoter fragment after PCR. Second, bisulfite treatment of genomic DNA converts cytosine to uridine but is insensitive to 5-methylcytosine modification. Five micrograms of genomic DNA were treated with alkaline sodium bisulfite as described by Liu et al. (28). After treatment, the DNA was purified using the Wizard DNA clean-up kit (Promega), desulfonated with sodium hydroxide, and concentrated by alcohol precipitation. DNA was dissolved in deionized water and used for PCR amplification by bisulfite-treated DNA sequence specific primers (PGIS bisulfite NI F2: 5'-CCTGGAAAATTTATTTTGGGAGTGGG-3'; PGIS bisulfite NI R3: 5'-CCGAAAACCTTAAACTACAAACC-3'). The PCR amplification conditions involved an additional 1-min annealing step at 52.5°C between denaturation and extension, in reaction buffer without 1× Q reagent. These primers are designed to give asymmetrically cohesive ends for directional cloning into pUC19 containing a unique EcoNI restriction site in-frame with β-galactosidase coding region. Thus, insertion of a PCR DNA fragment gives blue-to-white screening in DH5α. Approximately 20 independent clones were sequence from each cell line, and the results of the methylation at the 21 to 23 different CpG sites tabulated into common methylation patterns. Note that each VNTR repeat contains a single CpG dinucleotide, which accounts for the difference in sites found.

**Chromatin Immunoprecipitation of Sp1 Binding to the PGIS Promoter**

Eight 10-cm dishes of tissue culture cells at 50% to 80% confluence (~20 million cells) were used for each experiment. The cells are fixed by adding 1% formaldehyde (final concentration) directly into complete medium and placed on a platform shaker for 5 min at room temperature. Fixation is stopped by adding 0.1 mol/L glycine (final concentration) for 5 min. Each plate is quickly rinsed twice with 10 mL 1× PBS, and the cells from each plate are scraped into 0.9 to 1 mL 1× PBS. Cells from two plates are pelleted in 2 mL microtube, quick frozen in dry ice, and stored at −80°C. The four fixed cell pellets (eight plates equivalent) are resuspended in 2 mL sonication buffer with protease inhibitors [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% SDS, 0.1% Triton X-100] and transfer to a chilled 15 mL sterile Falcon tube. The fixed cells were sonicated on ice with a Branson Sonicator 450 using the microtip probe at 50% power for two sets of 3 × 10-s bursts with a ~5-min pause between sets. The sonicate was spun at 16,000 × g, 4°C, for 30 min in 2 mL microtube to remove cellular debris.

Protein G Sepharose 4 Fast Flow (100 μL of slurry; Amersham) was added to each 2 mL sonicate and rotated at 4°C for several hours; the sonicate was preclared by spinning at 16,000 × g, 4°C, for 30 min. An equivalent input DNA was saved (100 μL; 10% immunoprecipitation) at −20°C. Either 5 μL (5 μg) normal rabbit IgG or rabbit anti-human Sp1 (both from Upstate, Charlottesville, VA) was added to each ~1 mL sonicate and rotated at 4°C

| Table 4. Mean Copy Number per Interphase Nuclei and SD of RPC11-29806 (PGIS Gene) and p3.2 (Cen20 Probe) |
|-----------------|-----------------|-----------------|
| SR-PGIS Gene, Mean (SD) | SG-CEP, Mean (SD) | PGIS/CEP 20 Ratio |
| NSCLC Cell Line | | |
| A549 | 3.20 (0.49) | 2.89 (0.44) | 1.10 |
| H460 | 3.05 (0.33) | 2.84 (0.52) | 1.07 |
| H157 | 5.08 (0.95) | 4.86 (0.87) | 1.05 |
| H226 | 3.85 (0.65) | 3.65 (0.94) | 1.05 |
| H322 | 3.09 (0.40) | 2.01 (0.44) | 1.54 |
| H2122 | 3.09 (0.29) | 2.00 (0.28) | 1.54 |
| Human Lung AC Tissue | | |
| S99-1551 H2 | 6.03 (0.23) | 2.74 (0.89) | 2.20 |
| S97-6124 B 2688 | 1.91 (0.64) | 1.78 (0.63) | 1.07 |
| S97-3456 3 3503 | 1.80 (0.40) | 1.64 (0.48) | 1.07 |
| S97-13203 J 3483 | 2.51 (0.92) | 2.06 (0.80) | 1.22 |
| S98-3061 GG 3941 | 2.80 (1.19) | 2.06 (0.89) | 1.36 |
| VA96-1335 A 5525 | 1.74 (0.52) | 1.43 (0.54) | 1.22 |

NOTE: Data were determined in 100 cells from each non–small cell lung cancer line and human lung adenocarcinoma tissue samples. Abbreviations: NSCLC, non–small cell lung cancer; AC, adenocarcinoma.
overnight. To immunoprecipitate the antibodies, 50 μL slurry of Protein G Sepharose 4 Fast Flow per immunoprecipitation was added and rotated at 4°C for several hours. The beads were rinsed five times with 1 mL sonication buffer for 15 min each at 4°C, and then quickly rinsed twice with 1 mL sonication buffer + 150 mmol/L NaCl (300 mmol/L total) at room temperature. To recover the antibody-bound DNA, the beads were resuspended in 120 μL deblocking buffer (sonication buffer + 0.1 mol/L NaHCO3 + 1% SDS containing 10 μg/mL RNase; Roche, Indianapolis, IN), and incubated overnight at 65°C. Proteinase K (Qiagen) was added (800 μg/mL) and incubated at 65°C for 1 h. The DNA was purified using Qia-Quick DNA kit (Qiagen) and resuspended in 30 μL final volume. One microliter of recovered DNA was used in each PCR reaction as described above for amplification of the PGIS promoter from genomic DNA using genomic primers CI PGIS NI F2 (5'-CCACATTTTCCCCAGGCTGAGCTGC-3') and PGIS Intron1 RI R6 (5'-CCGCGGAGAAGGAGAAGATC-3').

Fluorescence In situ Hybridization Studies
A search of human genomic FISH clones for PGIS gene in 20q13.13 revealed a BAC RPC11-29806, which was obtained from Children’s Hospital Oakland Research Institute. The BAC clone was purified using a large-construct DNA kit (Qiagen) and the ends were DNA sequence verified to span the PGIS gene. A chromosome 20 centromeric plasmid (p3.2) was used to verify hybridization of RPC11-29806 to the appropriate region of correct chromosome. One microgram of the BAC clone was labeled by nick translation using the Nick Translation kit (Vysis, Des Plaines, IL), and dUTP nucleotides were conjugated with SpectrumRed, coprecipitated with 10× (v/v) human Cot-1 and salmon sperm DNA, and dissolved in 50% formamide hybridization mix. The centromeric plasmid p3.2 was similarly labeled with SpectrumGreen-conjugated dUTPs, coprecipitated with salmon sperm DNA, and dissolved in 65% formamide hybridization mix.

Cell lines were harvested after mitotic arrest with colcemid (0.05 μg/mL) for 2 h. Hypotonization was done with 0.075 mol/L KCl, and a 3:1 mixture of methanol and glacial acetic acid was used for fixation. Slides were dropped and aged at room temperature for 4 days. Human lung adenocarcinoma tissue samples were H&E-stained sections with selected areas and blank sections. Initially, the slides were incubated for 2 h at 65°C, deparafinized in Hemo-De (Fisher, Pittsburgh, PA), and washed in 100% ethanol for 5 min. The slides were washed in 2× SSC at 75°C for 20 min and digested in 0.25 mg/mL proteinase K/2× SSC at 45°C for 20 min. Slides were washed in 2× SSC for 5 min and dehydrated in an ethanol series.

Dual-color FISH experiments were done according to protocols previously described (34). For studies in cell lines, prehybridization treatment consisted of dehydration of the slides in ethanol, brief wash in 70% glacial acetic acid, and another dehydration. The slides were then digested in 0.008% pepsin/0.01 mol/L HCl at 37°C for 4 min, and fixed in 1% formaldehyde for 10 min at room temperature. Pretreatment of formalin-fixed, paraffin-embedded tissue sections used 2× SSC at 46°C and proteinase K at 37°C for 10 to 15 min each and posthybridization washes in 1.5 mmol/L urea/0.1× SSC. A probe set was prepared combining 200 ng of the RPC11-29806 probe and 80 ng of the control probe p3.2 in dual-color FISH assays, warmed for 1 min at 37°C, and applied to the selected hybridization area, which was covered with a 15-mm circular glass coverslip and sealed. Probe and chromosomal DNAs were denaturated for 8 min at 80°C and then incubated at 37°C for 24 h.

Posthybridization washes were done with 50% formamide/2× SSC and 2× SSC at 46°C (3 × 6 min washes each). Subsequently, the slides were washed in 2× SSC/0.1% NP40 at 46°C for 6 min and in 2× SSC at room temperature for 1 min. Chromatin was counterstained with 4',6-diamidino-2-phenylindole (0.3 μg/mL in Vectashield Mounting Medium, Vector Laboratories, Burlingame, CA). Analysis was done on an Olympus fluorescence microscope using single interference filter sets for green (FITC), red (Texas red), and blue (4',6-diamidino-2-phenylindole), as well as dual (red/green) and triple (blue, red, green) filters.

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References


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