DNA Methylation Patterns of the Calcitonin Gene in Human Lung Cancers and Lymphomas

Stephen B. Baylin², Jo W. M. Höppener, André de Bustros, Paul H. Steenbergh, C. J. M. Lips, and Barry D. Nelkin

INTRODUCTION

Recent studies of DNA extracted from human tumors, including overall quantitation of genomic DNA methylation (1) or restriction enzyme analysis to map methylation patterns for individual genes (2–4), have documented patterns of hypomethylation in tumor tissues as compared to surrounding normal tissues. The functional significance of this methylation difference between normal and neoplastic tissues is not yet known, and the specific regions involved within individual genes have not generally been established. In the present report, we now show that in human lung cancers, especially SCLC* and lymphomas, there are multiple sites of CCGG methylation in the 5'-region of the CT gene as compared to methylation of only one specific site in multiple types of non-neoplastic adult tissues examined. We show that absence of methylation at this latter site correlates with the high expression of the CT gene in medullary thyroid carcinoma. However, methylation of this site and others in the 5'-'region is now shown to occur in tumors such as SCLC, which have features of neuroendocrine differentiation and which do express low levels of CT and CT mRNA.

MATERIALS AND METHODS

Cell Culture Lines. All established human cell culture lines, including medullary thyroid carcinoma cells (5, 6) and lung cancer cells (7–12), have been described previously, and conditions of growth and maintenance were exactly as detailed in these publications.

DNA Probes. The 5'-flanking region of the human CT gene, used as a probe in this study, was subcloned from a genomic cosmid clone, which contains the entire coding region for the CT gene and 5'-' and 3'-flanking regions (13). A 4.0 kilobase Bgl II fragment, spanning the Bgl II site shown in Fig. 1, includes exons 1–3 and extends approximately 1.0 kilobase into the 5'-flanking region of the gene. This Bgl II fragment was subcloned into the Bam HI site of pBR322. From this subclone, we prepared a 1.9 kilobase Sph 1 fragment, spanning the unique Sph 1 site in pBR322 at position 565 to an Sph 1 site within the 5'-region of the Bgl II insert shown to contain exon 1 regions [by hybridization to CT gene mRNA and comparison to published sequence data (4)] and portions of the 5'-flanking region, as shown in Fig. 1. This fragment was shown to be free of repeated DNA sequences by the fact that: (a) as shown in "Results," in studies of genomic human DNA, it hybridizes only to single copies of predicted restriction enzyme fragments contained in the clone from which it was prepared, including the 4.0 kilobase Bgl II fragment; and (b) it did not give a hybridization signal to nick translated total placental DNA which would be expected to identify high copy repeat sequences and not single copy genes under the conditions used. This 1.9 kilobase Sph 1 fragment was subcloned into the Sph 1 site of pBR322.

DNA Hybridizations. DNA was extracted from cell cultures and tumor tissue samples by a published technique (15), and 5 µg were digested with either Msp I (10 units/µg DNA) or Hpa II (2–4 units/µg DNA) for 12–16 h, electrophoresed on 1% agarose gels, transferred to nitrocellulose, and hybridized with the 5'-CT gene probe at 60°C for 48–72 h under conditions described previously (16). The probe was labeled by random priming using Klenow polymerase (17) to a specific activity of 0.5 to 1 x 10^10 cpm/µg DNA, and 0.5 to 2.0 x 10^9 cpm were used for each hybridization. The final wash for the filters was performed at 65°C in several changes of 0.25x standard saline citrate:0.5% sodium dodecyl sulfate for 2 to 3 h, and autoradiography was carried out at ~70°C for 24–96 h with intensifying screens. Hind III digested bacteriophage λ DNA was used as a molecular weight marker.

RESULTS

Mapping of CCGG Sites in the MTC Gene and Pattern of Methylation in Normal Tissue. In the rat (18) and human (13, 14, 16, 19), the calcitonin gene codes for multiple peptides, including CT and calcitonin gene related peptide. Through a post-transcriptional alternative processing step, separate mRNA species can be generated from the CT gene for these two peptides which have common 5'-regions (exons 1–3 of the gene) but separate 3'-regions coding either for CT (exon 4) or calcitonin gene related peptide (exons 5 and 6).

We have subcloned, as outlined in "Materials and Methods," the 5'-flanking region of the CT gene, inclusive of exon 1, as a 1.7 kilobase Bgl II-Sph I subclone (Fig. 1). We have used this 5'-probe to map CCGG sites in and around the CT gene, and we find them to be frequent in the 5'-region (Fig. 1). Restriction of genomic DNA with the enzyme Msp I, which cuts at CCGG sites (5), identifies sites 2 through 5 (Fig. 1), generating 3 fragments of sizes 1.0, 0.6, and 0.5 kilobases (Fig. 2A, lane 1) which can be detected with the Bgl II-Sph I 5'-probe but not with cDNA probes specific for exons 4, 5, and 6. Msp I sites 2, 3, 4, and 5 were oriented by showing that a portion of the Bgl II-Sph I fragment, spanning the Bam HI to Sph I region, hybridized to the 1.0 and the 0.6 kilobase fragments but not...
the 0.5 kilobase fragment, generated by Msp I digestion of genomic DNA. Also, restriction analyses with all of the enzymes depicted fit the orientation of Msp I sites shown, including identification of predicted fragments in double digests of genomic DNA with Hpa II plus Bgl II, Hpa II plus Bam HI, Hpa II plus Sph I, and Bgl II plus Bam HI.

The position of site 6 fits recently published sequence data (14). Site 5 (Fig. 1) was further documented by restricting genomic DNA from liver, kidney, lung, spleen, thyroid, and peripheral WBC with Hpa II, which, like Msp I, cuts at CCGG sites, but not when the internal C is methylated (20, 21). A pattern of hybridization is detected which results from failure of the enzyme to cut due to methylation at CCGG site 5 (Fig. 1). This methylation results in diminution of the 1 kilobase fragment seen with Msp I digestion, retention of the 0.6 and 0.5 kilobase fragments, and appearance of a new 2 kilobase fragment (Fig. 2A, lane 2) which spans a region from CCGG sites 4 to 6 (Fig. 1). The orientation of this 2 kilobase fragment was confirmed by identification of predicted patterns upon double digestion of genomic DNA with Hpa II and Bam HI and failure of the fragment to digest with Bgl II (see Fig. 1).

Hypomethylation of the CT Gene in MTC. We next compared the status of CT gene methylation at CCGG sites in DNA from normal tissues to that of DNA from MTC, a tumor which comes from the CT secreting C-cells of the thyroid gland (22) and which continues to contain high levels of both calcitonin (23) and calcitonin mRNA (13, 16, 19). As shown in Fig. 2A (lanes 3 and 4) and Table 1, CCGG site 5 in the 5′-region of the CT gene (Fig. 1) is either highly (Fig. 2A, lane 3) or more partially (Fig. 2A, lane 4) hypomethylated in all 10 MTC tissues and one established culture line (5, 6) as compared to normal thyroid tissue from the patients harboring the tumors (Fig. 2B, lane 2), and to normal tissues from other individuals (Fig. 2B, lanes 6–9; Fig. 3, lane 4; Fig. 3B, lanes 6–9). This result is not surprising in light of the frequently described positive relationship between hypomethylation of 5′-regions and levels of transcription of numerous genes (24–26). Densitometric tracings of the bands produced by Hpa II digestion of DNA reveal a ratio of the 2 kilobase band (methylated at CCGG site 5; see Fig. 1) to the 1 kilobase band (unmethylated at CCGG site 5) in 11 MTC tissues from 0 to 0.75 and averaging 0.3 ± 0.07 (SD), as compared to a range in 29 normal adult tissues (lymphocytes, kidney, colon, spleen, lung, and liver) of 1.0 to 7.0, average 2.3 ± 0.5 (P < 0.001). We examined whether the pattern of hypomethylation of site 5 in MTC might be closely related to the high expression of the gene in this neoplasm or to the involvement of this gene in the DNA hypomethylation patterns seen in cancer tissues (1–4). Our results favor the former hypothesis since the average ratio (2.0 ± 0.8) of the 2 kilobase to the 1 kilobase band in 51 DNA samples from other types of human neoplasms (Table 1) was not different from that for the DNA from normal tissues. Moreover, only occasional examples of hypomethylation of CCGG site 5 in the 5′-region of the CT gene were seen in other tumors (Table 1).

Hypermethylation of the CT Gene in Human Cancers. As opposed to the findings of hypomethylation in MTC and the pattern of methylation of CCGG site 5 in normal adult tissues, we found unique patterns of methylation involving other CCGG sites (Table 1) in other human cancers examined and especially in the most aggressive form of lung cancer, small cell lung carcinoma (>80%) and in lymphomas (100%). This finding was extremely surprising, especially for small cell lung carcinoma (SCLC), considering the fact that this cancer is known to have a high degree of endocrine differentiation (27–29), including the presence of low amounts of immunoreactive CT (30–33), and low, but definite, amounts of CT gene mRNA (16, 19). Also, other genes have been shown to be hypomethylated in this tumor (3).

The patterns of hypermethylation were seen in DNA extracted both directly from tumor tissues and from established cell culture lines and were of two main classes. The first involves extensive hypermethylation and was seen most frequently in the classic form of SCLC, including 6 of 9 tissues (examples, Fig. 2B, lanes 1–5) and 2 of 4 lines (OH-3 and NCI H64) established culture lines. Hpa II digestion of genomic DNA from tumors with this pattern reveals high molecular weight bands hybridizing to the CT gene 5′-region probe that were not seen or were seen only in trace amounts in any normal adult tissues (examples, Fig. 2A, lane 2; Fig. 2B, lanes 6–9; Fig. 3, lane 4; Fig. 3B, lanes 6–9) and much less frequently in any other tumor types studied (see last column of Table 2), including other types of lung cancer (2 of 9 adenocarcinomas, 1 of 9 squamous carcinomas, and 0 of 3 large cell carcinomas), lymphomas (1 of 5—an immature T-cell tumor; Fig. 3B, lane 1), and 1 (the HL-60 line of promyelocytic leukemia) of 8 other cancers examined. The dominant DNA fragments in this pattern were >12 kilobases in two SCLC tissues (example, Fig. 2B, lane 1) due to methylation of at least sites 4 through 6 (Fig. 1), a region encompassing exons 1 through 6 of the gene and, most frequently, a 3.1 kilobase fragment [5 of 8 SCLC cultures, 2 of 4 SCLC cultures (examples, Fig. 2B, lanes 2–5)] formed by methylation of sites 3, 4, and 5, thus spanning Msp I sites 2 through 6 (Fig. 1). The sites involved are documented as described in “Materials and Methods,” including derivation of predicted fragments from the 3.1 kilobase region upon double digestion of SCLC DNA with Hpa II plus Bam HI and Hpa II plus Bgl II (sites shown in Fig. 1). An abnormal 2.2 kilobase band was also detected in 3 of the above SCLC DNA's (Fig. 2B, lanes 1–3).

The second main class of unusual patterns for Hpa II restriction was seen in DNA from 2 of the other 3 SCLC tissues, the other 2 SCLC culture lines examined (lines OH-1 and NCI H69), 2 of 4 (lines OH-2 and NCI H417) so-called "variant" SCLC tumors (8, 9, 11, 34), 4 of 5 lymphomas (all immature B-cell neoplasms), and much less frequently (Tables 1 and 2) in other tumor types (3 of 9 lung adenocarcinomas, 1 of 9 lung squamous cell, 1 of 3 lung large cell, and 2 of 8 other cancers.

![Image](cancerres.aacrjournals.org)
CALCITONIN GENE IN HUMAN LUNG CANCERS AND LYMPHOMAS

Table 1. Frequency of CCGG sites methylated in tumors and normal tissues studied.

<table>
<thead>
<tr>
<th>DNA source</th>
<th>No.</th>
<th>Methylation of other than CCGG site 5</th>
<th>Decreased methylation of CCGG site 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal adult</td>
<td>29</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>MTC</td>
<td>11</td>
<td>0 (0)</td>
<td>11 (100)</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>5</td>
<td>5 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>SCLC, classic</td>
<td>13</td>
<td>12 (92)</td>
<td>1 (7)</td>
</tr>
<tr>
<td>SCLC, variant</td>
<td>5</td>
<td>4 (80)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>Lung, adenocarcinoma</td>
<td>9</td>
<td>5 (55)</td>
<td>1 (11)</td>
</tr>
<tr>
<td>Lung, squamous</td>
<td>8</td>
<td>2 (25)</td>
<td>1 (12)</td>
</tr>
<tr>
<td>Lung, large cell</td>
<td>3</td>
<td>1 (33)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Othera</td>
<td>8</td>
<td>3 (37)</td>
<td>1 (12)</td>
</tr>
</tbody>
</table>

*a Numbers in parentheses, percentage.
*b Other = 4 colon carcinomas, 3 pheochromocytomas, and 1 promyelocytic leukemia.

studied (1 colon cancer and 1 pheochromocytoma). This restriction profile reveals a less extensive methylation of the CT gene than was seen in the first pattern, but it involves methylation of sites unmodified in normal adult tissues and results in the appearance of novel tumor associated fragments. For example, in one SCLC culture line, a new 1.6 kilobase band (Fig. 3A, lane 1) is formed with the loss of the 0.6 and 1 kilobase bands (due to methylation of site 4 in Fig. 1, with the fragment spanning sites 3 through 5), while in the other SCLC cell culture and 2 SCLC tissues, there is a distinct 1.2 kilobase band (Fig. 3A, lane 2), with retention of all other of the usual bands. Another unusual band, 0.7 kilobase, was seen in combination with the 1.2 kilobase band. This fragment apparently is formed by methylation of site 2, since the 0.5 kilobase band is lost, and it thus spans sites 1–3, as seen in Hpa II digests of DNA from 1 culture of “variant” SCLC (Fig. 3A, lane 3). This 0.7 kilobase band was also seen in one of the SCLC DNAs (Fig. 2B, lane 5) which predominantly contained the first hypermethylation pattern discussed. In the 4 lymphomas, 2 had unique 2.2 and 1.6 kilobase bands (Fig. 3A, lanes 2 and 3), and 2 had the 1.6 kilobase band (Fig. 3A, lanes 4 and 5), with all 4 having a marked reduction of the normal 1.0 and 0.6 kilobase fragments.

Both of the unusual tumor associated Hpa II digestion patterns discussed above are specific for methylation changes within the CT gene and are not due to gene rearrangements. Msp I restrictions of each of the tumor DNAs with abnormal Hpa II patterns resulted in generation of the 3 bands (1.0, 0.6, and 0.5 kilobases) seen in normal genomic DNA (example, Fig. 2B, lane 10). Furthermore, the CT gene methylation changes did not correlate with any overall pattern of DNA methylation in the tumor DNAs studied. Hybridization of the same SCLC DNAs to genes such as crystallin showed partial hypomethylation, as reported in other tumors (2–4), in samples in which the CT gene is extensively hypermethylated (data not shown). Finally, the unusual methylation patterns for the CT gene are specific for tumor DNA in these patients. Hpa II digestion of

Fig. 2. Examples of the analysis of the methylation status of calcitonin gene CCGG sites in DNA from adult normal and various types of tumor tissues. A: lane 1, normal liver DNA digested with Msp I. This Msp I pattern was obtained for all normal and tumor tissues studied; lane 2, normal thyroid DNA from a patient with the hereditary form of MTC, digested with Hpa II; lanes 3 and 4, MTC DNAs digested with Hpa II; lane 5, DNA from normal kidney of patient whose tumor DNA is shown in lane 2, digested with Msp I. This pattern is representative of Msp I digestion of all tumor DNAs which showed abnormal patterns with Hpa II digestion.

Fig. 3. Further examples of calcitonin gene CCGG site methylation in tumors and normal tissues. A: lane 1, 1.6 kilobase band and loss of 0.6 kilobase band in SCLC DNA (Hpa II digestion); lane 2, 1.2 kilobase band in SCLC DNA (Hpa II digestion); lane 3, 1.2 and 0.7 kilobase bands with loss of 0.5 kilobase band in "variant (1)" form SCLC DNA (Hpa II digestion); lane 4, DNA from normal liver of patient whose tumor DNA is shown in lane 2 (Hpa II digestion); lane 5, DNA from normal lung of patient whose tumor DNA is shown in lane 2, digested with Msp I. This pattern is representative of Msp I digestion of all tumor DNAs which showed abnormal patterns with Hpa II digestion.

Downloaded from cancerres.aacrjournals.org on January 6, 2018. © 1986 American Association for Cancer Research.
DNA from normal liver, lung and kidney from patients with either pattern of hypermethylation of the CT gene in their tumor DNA gave a normal pattern (examples, Fig. 2B, lanes 6–8; Fig. 3A, lanes 4 and 5), as did representative samples of control tissues from other patients (example, for normal lung DNA to compare to lung cancers, Fig. 2B, lane 9: peripheral white cells to compare to lymphomas, Fig. 3B, lanes 6–9).

Correlation of the Methylation Findings with Past Data for L-DOPA Decarboxylase Activity. At present it is not known whether our findings have functional significance for the development and/or the phenotypic expression of any human cancer. We also cannot say whether the methylation patterns seen are a specific consequence of abnormal methylation in tumor cell DNA or may reflect methylation patterns specific to the compartment of normal cells from which the tumors arose in adult renewing tissue. However, among the forms of lung cancer, we have found a striking correlation between the distribution of neuroendocrine features and the methylation status of the CT gene. We have compared (Table 2) the present methylation results with our previous data for activity of L-DOPA decarboxylase (29, 35), a biogenic amine synthesizing enzyme which closely parallels neuroendocrine differentiation of small peptide hormone secreting cells in general (27) and the presence of the neuroendocrine phenotype among the spectrum of lung carcinomas in particular (29, 35). The frequency for finding high activities of L-DOPA decarboxylase among the different types of lung cancers correlates directly ($r = 0.997$) with the percentage of the incidence for finding one or more of the hypermethylation patterns.

Moreover, as shown in the last column of Table 2, the pattern for extensive methylation of the 5'-region of the CT gene, in which the 3.1 kilobase or a larger band is observed, has particular relationships to the phenotypes within the spectrum of human lung cancers. In tissues or cell cultures demonstrating the classic SCLC phenotype and having high L-DOPA decarboxylase activity, we find the highest percentage of the tumor DNAs (61%) with this extensive methylation pattern. In contrast, all of the other types of lung cancer have an incidence of 22% or less. Second, examination of DNA from a subtype of SCLC, the so-called “variant” SCLC tumors and/or cell culture lines (8, 9, 11, 34), bears special mention. These tumors of SCLC lineage are characterized by a loss of the usual neuroendocrine biochemistry profile which characterizes classic SCLC tissues and/or cell lines (8, 9, 29, 34), have a more vigorous growth pattern in cell culture (8, 9), have amplification of c-myc (36) or other myc- family related genes, and are relatively resistant to radiation in vitro as compared to culture lines of classic SCLC (37, 38). While all of these variant SCLC tissues or cultures gave abnormal patterns of methylation, only 1 (line NCI H60) of 5 had the extensive methylation pattern (Table 2). Three had the presence of the unusual low molecular weight bands, seen most frequently in lymphomas, and one culture line was fully hypomethylated (line NCI H82).

### DISCUSSION

In the present study, we have described an unusual pattern of hypermethylation in human tumors, involving the 5'-region of a human small polypeptide hormone gene. To our knowledge, this represents the first observation of hypermethylation of an endogenous gene in human tumor cell DNA and the first association of a gene specific methylation pattern with tumors of a given histological type. This observation may be contrasted with the previously reported finding of DNA hypomethylation in tumors (1–4). As several investigators have pointed out (24–26, 39, 40), the mechanism underlying hypomethylation need not involve a demethylating enzyme but rather may be an abrogation of maintenance methylation during DNA replication. Development of hypermethylation, on the other hand, requires de novo methylase activity. De novo methylation has been reported previously to take place in viral infection (41, 42) and has also been inferred to occur in some cellular DNA sequences in early mammalian development (43–45; also reviewed in Refs. 24, 40, and 42). Our finding of hypermethylation in lung tumors and lymphomas suggests either that de novo methylation occurred during the development of the tumors or that these tumors derived from a normal progenitor cell population, the DNA of which was methylated in the 5'-region of the CT gene and possibly also in other genes.

With regard to specific expression of the CT gene, we commented previously that the hypomethylation found in MTC is consistent with the very high degree of CT gene expression in this tumor and in the normal cells from which it arises. Interestingly, multiple studies have suggested that there is a high incidence of small amounts of CT production in all types of human lung cancer (30–33) and in normal pulmonary endocrine cells, which may be the parent cell for SCLC (46). In a previous report (16), we determined that 5 of the lung cancer culture lines used in the present study and now shown to contain hypermethylation patterns for the CT gene contain significant amounts of mRNA from the calcitonin gene. However, the amounts of CT gene mRNA in lung cancers are orders of magnitude lower than in MTC (16, 19). The methylation of site 5 in most tissues may correlate with substantially decreasing expression of the CT gene, but it does not appear to fully block transcription. The methylation of other 5'-region CGGG sites in the CT gene, such as we now find in some non-MTC tumors, may not play a role in further modulating expression of this gene. Further studies will be required to pursue these questions.

The major significance of our findings may be to provide a new molecular marker for events in development and/or tumor progression within the spectrum of human lung cancers and in lymphomas. For example, a mounting body of evidence suggests that each of the four major types of human lung cancer (small cell, adenoc, squamous, and large cell carcinoma) arises as a consequence of multiple directions of differentiation ongoing within a single endodermal cell lineage in the bronchial mucosa (for review, see Refs. 34 and 47). We and others (8, 9, 11, 37) have presented evidence that the SCLC neuroendocrine phenotype can change toward that of the less treatment responsive non-SCLC lung cancers with time. This shift may involve formation of a variant SCLC cell type which has lost neuroen-
doctrine features (8, 9) and which has been one focus of the present study. This cell type is now shown to generally have a less extensive CT gene methylation pattern than does classic SCLC.

A significant fraction of non-SCLC lung cancers, especially adenocarcinomas, has been shown to have biochemical features of neuroendocrine differentiation (29, 35) and now CT gene hypermethylation. It is not known whether these tumors are more sensitive to treatment than are most of the traditionally resistant non-SCLC lung cancers. The present finding of a unique pattern of DNA methylation in SCLC and in some non-SCLC lung cancers suggests further study to determine whether this molecular event may be useful as an additional marker for more precise phenotyping to predict the therapeutic responsiveness of lung tumors in individual patients.

Finally, from the standpoint of tumor aggressiveness and clinical behavior, recent findings of Babiss et al. (48) may have which is partially supported by NIH Grant 13148.

REFERENCES


36. Carney, D. N., Mitchell, J. B., and Kinsella, T. J. In vitro radiation and

Downloaded from cancerres.aacrjournals.org on January 6, 2018. © 1986 American Association for Cancer Research.
CALCITONIN GENE IN HUMAN LUNG CANCERS AND LYMPHOMAS


DNA Methylation Patterns of the Calcitonin Gene in Human Lung Cancers and Lymphomas

Stephen B. Baylin, Jo W. M. Höppener, André de Bustros, et al.