Aberrant Hypermethylation at the bcl-2 Locus at 18q21 in Human Lung Cancers

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ABSTRACT

Accumulating evidence suggests that altered DNA methylation may play a role in the oncogenesis of human neoplasms, including lung cancer. The presence of aberrant hypermethylation at 3p, 9p, 11p, and 17p, which are known to be hot spots for allele loss in lung cancers, is suggested to be a reflection of the existence of tumor suppressor genes in these chromosomal regions. In the present study, we investigated the methylation status of the Rb locus at 13q14 as well as that of the bcl-2 locus at 18q21 in 134 lung cancer specimens, representing all major histological subtypes. As a result, 18q21 was identified to be the fifth chromosomal region affected by frequent tumor-specific aberrant hypermethylation in lung cancers. The occurrence of aberrant hypermethylation at the bcl-2 locus at 18q21 was restricted to non-small cell lung cancers, and among non-small cell lung cancers, such epigenetic aberrations were observed most frequently in adenocarcinomas without any association with bcl-2 expression. Interestingly, allelic loss at the bcl-2 locus was also seen in 40% (7 of 17 informative cases) of adenocarcinomas; this frequency was also the highest among values for the various histological subtypes of lung cancers. These results suggest that aberrant hypermethylation at the bcl-2 locus may reflect a putative tumor suppressor gene residing at 18q21, and aberrant hypermethylation might play a role in its inactivation. In contrast, altered methylation status of the Rb locus appears to be quite rare in lung cancers, if present at all.

INTRODUCTION

Alterations of the pattern of DNA methylation have been recognized as consistent molecular changes in human cancers (1–23). These abnormalities include widespread hypomethylation, regional hypermethylation, and an overall increase in DNA methyltransferase activity that catalyzes DNA methylation. These are thought to have important implications for abnormalities of gene expression, chromosome structure, timing of DNA replication, and chromatin organization (24–26).

Evidence for inactivation of well-defined tumor suppressor genes by aberrant hypermethylation has been reported in a very limited number of human cancers (10, 13, 17, 20–23). Aberrant hypermethylation of the Rb gene was reported to be present in 5–15% of retinoblastomas, whereas silencing of the VHL gene was found in ~20% of clear-cell renal cancers. Silencing of the p16 gene at 9p21 due to aberrant hypermethylation has also been reported in various human cancers, including lung cancers (20–23). In addition, the presence of aberrant hypermethylation in lung cancers has been described at loci on chromosome regions 3p, 11p, and 17p (5, 8, 14), but the possible involvement of such alterations in inactivation of well-defined tumor suppressor genes remains to be studied.

In addition to regional hypermethylation, hypomethylation in growth-promoting genes or dominant oncogenes could also be involved in the oncogenesis of human cancers. Such aberrations at specific sites of the ras and c-myc genes have been reported in human cancers (1, 3, 7, 15), although the potential relevance of these findings remains uncertain because of the lack of evidence for an association between hypomethylation and overexpression of the affected oncogenes.

In the present study, we examined the DNA methylation status of the Rb locus at 13q14 as well as that of the bcl-2 locus at 18q21 in 134 lung cancer specimens representing the four major histological subtypes. These two genes were chosen for the present study for the following reasons: the Rb gene, one of the best studied tumor suppressor genes, is known to be mutated in a considerable fraction of lung cancers (27–31), and evidence for inactivation of this gene by hypermethylation exists in retinoblastomas (10, 13); the bcl-2 gene, which is assumed to contribute to malignancy by preventing apoptosis (32), is often expressed in lung cancers, where unlike the activation seen in follicular lymphomas, its expression does not appear to be due to chromosomal translocations (33–35). We report here that tumorspecific aberrant hypermethylation as well as allelic losses at the bcl-2 locus are frequent events in lung cancers with a clear specificity in the affected histological subtypes, whereas changes in the Rb locus are quite rare, if they exist.

MATERIALS AND METHODS

Tumor Samples and Cell Lines. Tumor samples together with corresponding, uninvolved lung tissue were collected at Aichi Cancer Center and other hospitals in Nagoya, Japan from 134 patients diagnosed histologically as having lung cancer (14 cases of SCLC3 and 120 cases of NSCLC). SCLC samples were obtained either during surgery or at necropsy, whereas NSCLC tumors were collected at surgery. All tissues were quickly frozen in liquid nitrogen and stored at −80°C until analysis. Derivation and culture conditions of lung cancer cell lines, including those with the prefix ACC-LC- which were established at Aichi Cancer Center, have already been reported (36, 37).

Southern Blot Analysis. Five µg of genomic DNA were digested with appropriate enzymes and subjected to electrophoresis on 0.8–1.3% agarose gels. DNA was then transferred to Hybond N+ nylon membranes (Amersham) in 0.1 N NaOH/0.1 M NaCl. Hybridization, washing, autoradiography, and probe stripping were performed under standard conditions. The following restriction enzymes were used together with either SacI (8 units µg/µl) or HindIII (10 units µg/µl) to analyze methylation status: methylation-sensitive SacII (20 units µg/µl) and HpaII (12 units µg/µl), and a methylation-insensitive isochizomer of HpaII, MspI (10 units µg/µl). EcoRI (8 units µg/µl) was also used to study loss of heterozygosity at the bcl-2 locus. Allelic loss was scored when the decrease in signal intensity by densitometric tracings was greater than 50% in tumor specimens.

Probes used in the present study were a 1.8-kb EcoRI–HindIII fragment of the 5’ end of the Rb gene (p123M1.8; a generous gift from Dr. T. Dryja, Harvard Medical School, Boston, MA) (13) as well as a 1.1-kb SacI–SacII fragment, a 1.7-kb BamHI fragment, and a 1.9-kb SacII fragment of the bcl-2 gene (38). A 1.4-kb mitochondrial DNA probe (L3200-H4552; a generous gift from Dr. M. Tanaka, Nagoya University School of Medicine, Nagoya, Japan) was used to confirm complete digestion of DNA with methylation-sensitive enzymes because mitochondrial DNA is not subject to methylation (39).

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1 To whom requests for reprints should be addressed.

3 The abbreviations used are: SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer.

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RESULTS

Absence of Aberrant Hypermethylation of the Rb Locus in Lung Cancers. We examined 134 lung cancer specimens for methylation abnormalities in the 5' end of the Rb gene. Double digestion with SacI and SacII invariably yielded 3.8- and 1.6-kb bands in both tumors and normal lung tissues, indicating the absence of aberrant methylation at the site recognized with SacII within the 5' end of the Rb gene (Fig. 1). HpaII sites surrounding exon 1 were also found to be unmethylated in both lung cancers and normal lungs, indicating the absence of aberrant methylation (data not shown).

Tumor-specific Aberrant Hypermethylation of the bcl-2 Locus in Lung Cancers. We also examined methylation status of the bcl-2 locus in the 134 lung cancer specimens by Southern blot analysis using a 1.1-kb SacI-SacII fragment as a probe (Fig. 2). An SacII site examined here was found to be consistently unmethylated in the corresponding normal lung specimens of 44 cases (data shown only for case 1). In contrast, the appearance of a novel 1.3-kb band indicated the presence of aberrant hypermethylation in 28 (21%) of the 134 cases.

Lung cancers are categorized into small cell and non-small cell categories. In the present study, a significant difference in the occurrence of the aberrant hypermethylation was found to exist between the two histological types, i.e., aberrant hypermethylation was observed in 28 (23%) of 120 NSCLCs, whereas none (0%) of 14 SCLCs showed such abnormalities (P = 0.031 by Fisher’s exact probability test; Table 1). Furthermore, among NSCLCs, aberrant hypermethylation appeared to be significantly more frequent in lesions with non-squamous histologies (19 of 48; 40%) when compared with squamous cell carcinomas (9 of 72; 13%), and the difference was statistically highly significant (P = 0.0007 by Fisher’s exact probability test).

Using an additional methylation-sensitive enzyme, HpaII, the extent of aberrant hypermethylation was also assessed. Twenty-two (79%) of 28 tumor samples that showed aberrant hypermethylation at the SacII site exhibited the presence of additional 0.9-kb and/or 1.3-kb fragments to various degrees, suggesting that this abnormality was not confined to the specific SacII site (Fig. 3). We noted that degrees of hypermethylation at the SacII site in each case were generally correlated with those at the HpaII sites, whereas global methylation status

Northern Blot Analysis. Ten µg of total RNA were electrophoresed on a 1% agarose gel containing formaldehyde, transferred to a Gene Screen (Dupont), and hybridized with the bcl-2 probe. The bcl-2 probe was a 1.3-kb BamHI-EcoRI fragment of a bcl-2 cDNA clone, #58 (40).
did not show such a relationship in our preliminary analysis using a centromeric minor satellite probe (data not shown).

We also examined whether or not lung cancer specimens showing aberrant hypermethylation in the 5′ exons of the bcl-2 gene also exhibit such epigenetic changes in the promoter region. Unlike the case with hypermethylation of the p16 gene (20–23), the SacII and HpaII sites in the bcl-2 promoter region were found to be unmethylated, regardless of the presence or absence of the above described aberrant hypermethylation (Fig. 4; data not shown for HpaII).


Table 1: Aberrant hypermethylation and allelic loss at the bcl-2 locus in human lung cancers

<table>
<thead>
<tr>
<th>Histological type</th>
<th>No. examined</th>
<th>Hypermethylation(^a)</th>
<th>Allelic loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCLC</td>
<td>14</td>
<td>0%(^b)</td>
<td>0% (3)(^c)</td>
</tr>
<tr>
<td>NSCLC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>72</td>
<td>13%</td>
<td>17% (29)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>40</td>
<td>40%</td>
<td>40% (17)</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>8</td>
<td>38%</td>
<td>0% (3)</td>
</tr>
</tbody>
</table>

\(^a\) Frequency of aberrant hypermethylation at the studied SacII site.

\(^b\) Numbers in parentheses, the numbers of informative cases for the EcoRI polymorphism.

Allelic Loss at the bcl-2 Locus in Lung Cancers. Identification of aberrant hypermethylation of the bcl-2 locus led us to examine allelic loss at this locus using its EcoRI polymorphism (Table 1; Ref. 41). Allelic loss at the bcl-2 locus was observed in 12 (24%) of 49 informative NSCLC cases, whereas none (0%) of 3 informative SCLCs carried allelic loss at this locus. It is interesting to note that adenocarcinomas, which exhibited aberrant hypermethylation most frequently (40%) among NSCLCs, showed the highest frequency (7 of 17 informative cases; 40%) of allelic loss at the bcl-2 locus, suggesting a possible relationship between aberrant hypermethylation and allelic loss at the bcl-2 locus.

Allele Specificity of Aberrant Hypermethylation of the bcl-2 Locus. The existence of an EcoRI polymorphism in the bcl-2 gene (41) also allowed us to examine aberrant hypermethylation in lung cancers in relation to their allele specificities by Southern blot analysis using DNAs double-digested with EcoRI and SacII (Fig. 5). Among 52 cases heterozygous for the EcoRI polymorphism, 13 exhibited aberrant hypermethylation, as indicated by the appearance of additional 13- and 7-kb bands. Five cases, which carried loss of an allele corresponding to either 5- or 11-kb bands, exhibited aberrant hypermethylation in the other remaining allele (data shown for cases 32 and 28). The almost complete absence of the unmethylated 1.1-kb band observed in case 32, which appeared to have negligible normal cell contamination as judged from clear allelic loss, indicated the presence of aberrant hypermethylation in virtually every tumor cell. Four lung cancer specimens (data shown for cases 24, 35, and 115) appeared to have aberrant hypermethylation in both alleles, resulting in the appearance of additional 13- and 7-kb bands, whereas the remaining four cases exhibited allele-specific hypermethylation in which only a single allele was selectively methylated, resulting in the generation of either 13- or 7-kb bands (data shown for cases 21 and 77). We note that similar allele-specific methylation was reported in the Rb gene in a retinoblastoma case in which inactivation of Rb was suspected to be due to a mutation and a hypermethylation in the two alleles (13).

Among 39 heterozygous cases without aberrant hypermethylation, 7 demonstrated allelic loss, and the remaining 32 cases retained both alleles of the bcl-2 gene.

Aberrant Hypermethylation and Expression of the bcl-2 Gene in Lung Cancer Cell Lines. To avoid the normal tissue contamination inevitable with primary lung tumors, we also performed Southern blot analysis using lung cancer cell lines cultured in vitro. Among 11 NSCLC cell lines examined here, 4 (PC-12, NCI-H460, SK-MES-1, and Calu1) showed complete hypermethylation, leading to the disappearance of the 1.1-kb unmethylated band, while both unmethylated (1.1 kb) and methylated (1.3 kb) bands were observed in RERF-LC-A1 and Calu6 (Fig. 6a). These results suggest the possibility that contamination of normal stromal cells and lymphocytes might have contributed significantly to the persistence of the unmethylated band in most primary lung tumors, although cancer cells with complete hypermethylation might have been selected out in vitro from the mixtures of cells with varying degrees of hypermethylation.

We next investigated whether there is any relationship between methylation status and expression of the bcl-2 gene by Northern blot analysis using the same panel of cell lines. Expression of the bcl-2 gene was observed in cell lines both with and without aberrant hypermethylation, indicating that the presence of aberrant hypermethylation does not correlate with the relative levels of bcl-2 expression (Fig. 6b).
DISCUSSION

The presence of aberrant hypermethylation in lung cancers has been described at 3p, 9p (p16), 11p, and 17p, chromosomal regions known to undergo frequent allelic loss in this tumor type (5, 8, 14, 20–23). These observations lend support to the hypothesis that aberrant hypermethylation may nonrandomly mark chromosomal regions that harbor tumor suppressor genes. Indeed, a candidate tumor suppressor gene, termed HIC-1 (hypermethylated in cancer), was isolated recently using the presence of hypermethylation at 17p13.3 as a useful molecular signpost (42).

The present study demonstrates 18q21 to be the fifth described chromosomal region with frequent tumor-specific aberrant hypermethylation in lung cancers. We found aberrant hypermethylation of the bcl-2 locus as opposed to the hypomethylation expected from bcl-2 expression in lung cancers. Since the present study shows that the presence of aberrant hypermethylation does not correlate with the relative levels of bcl-2 expression, the bcl-2 gene itself might consequently be an innocent bystander of epigenetic changes, since it has been suggested that DNA methylation can form a methylation domain starting from certain sequence elements, termed "centers of methylation" (43).

Shiseki et al. (44) reported previously that lung cancers frequently carry 18q deletions. In the present study, both hypermethylation and allelic loss were found to be frequent in a specific histological subtype of NSCLC, i.e., the adenocarcinoma, in contrast to the absence of such alterations in the biologically and clinicopathologically distinct lung cancer subtype, SCLC. Together with the growing body of experimental evidence implying a role for altered DNA methylation in oncogenesis (45–49), the present study suggests that this chromosomal region may harbor a putative tumor suppressor gene, especially for adenocarcinomas, and that aberrant hypermethylation might be involved in inactivation processes, perhaps by silencing gene expression and/or causing a high rate of mutation of methylated cytosine residues. It is interesting to note that 18q21 has been shown to carry at least two putative tumor suppressor genes, DCC and Maspin, whose inactivation in colon and breast cancers, respectively, is thought to be achieved mostly by repression of their expression (50, 51).

The present study also demonstrates that aberrant hypermethylation at the Rb locus is very rare, if it exists at all, in lung cancers, in contrast to the previous reports of a 5–15% occurrence in spontaneous retinoblastomas (10, 13). In this regard, Szyf et al. (52) demonstrated that de novo methylation activities may vary depending on cell types. It is also possible that the Rb locus may be less susceptible to DNA methylation than the other loci described at 3p, 9p (pl6), 1ip, and lip, chromosomal regions known to harbor tumor suppressor genes.

Fig. 4. Representative results of Southern blot analysis showing the absence of aberrant hypermethylation at the SacII sites in the promoter region, as indicated by the disappearance of the 8-kb band, observed by single digestion with HindIII. The appearance of a 4.5-kb band depends on the presence (cases 5, 8, 24, 33, 42, and 115) or absence (cases 121, 45, and 46) of aberrant hypermethylation at the SacII site in exon 2. A restriction map of the first and second exon of the bcl-2 gene (shaded boxes) and flanking regions is shown in relation to the location of the probe of the 1.9-kb SacII fragment. Below are origins of the restriction fragments observed in the present study. Hi, HindIII; II, SacII.

Fig. 5. Representative results of Southern blot analysis to examine allele specificity of aberrant hypermethylation of the bcl-2 gene in lung cancers. The appearance of 13- and 7-kb bands reflects aberrant hypermethylation of alleles corresponding to the 11- and 5-kb unmethylated bands. Aberrant hypermethylation of both alleles is evident for cases 24, 35, and 115, whereas allele-specific hypermethylation is present in cases 21 and 77. Cases 32 and 28 demonstrate loss of alleles corresponding to the 5- and 11-kb bands, respectively, and aberrant hypermethylation is observed for the remaining alleles. A restriction map of the first and second exon of the bcl-2 gene (shaded boxes) and flanking regions is shown in relation to the location of the probe of the 1.7-kb BamHI fragment. Below are origins of the restriction fragments observed in the present study. R, EcoR I; II, SacII. *, a polymorphic EcoRI site (41).
methylated than the \( bcl-2 \) locus, even in the same cell type (i.e., bronchial and pulmonary epithelial cells), since differential occurrence of aberrant hypermethylation among several loci in different chromosomal regions was reported during immortalization and transformation of human bronchial epithelial cells (46).

A final point that requires investigation is whether aberrant hypermethylation at the \( bcl-2 \) locus in primary tumors, especially of adenocarcinomas, is associated with more aggressive clinical phenotypes and shorter survival, since a significantly higher frequency of 18q deletions has been reported in brain metastases of NSCLCs than in the primary tumors, suggesting a potential relation to tumor progression (44).

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