Restriction Landmark Genomic Scanning (RLGS-M)-based Genome-wide Scanning of Mouse Liver Tumors for Alterations in DNA Methylation Status

Tomoya O. Akama, Yasushi Okazaki, Mitsuteru Ito, Hisato Okuizumi, Hideaki Konno, Masami Muramatsu, Christoph Plass, William A. Held, and Yoshihide Hayashizaki

ABSTRACT

Restriction landmark genomic scanning for methylation (RLGS-M) was used to detect, and subsequently clone, genomic regions with alterations in DNA methylation associated with tumorigenesis. Use of a methylation-sensitive enzyme for the landmark cleavage allows analysis of changes in methylation patterns. In this study, we used RLGS-M to analyze SV40 T antigen-induced mouse liver tumors derived from interspecific F1 hybrids between Mus spretus (S) and C57BL/6 (B6). Because 575 S- and B6-specific RLGS loci/spots have been mapped, tumor-related alterations in the RLGS profile could be immediately localized to specific chromosomal regions. We previously found that the loss of contiguous loci/spots could be attributed primarily to DNA loss, whereas loss of solitary loci/spots could be attributed primarily to DNA methylation. In this study, we examined 30 mouse liver tumor samples for loss of the 507 mapped loci/spots. Fourteen solitary loci/spots found to be absent or reduced in more than 75% of tumor samples were cloned and subjected to DNA sequence analyses. Two loci were identified as α4 integrin and p16/CDKN2, genes reported to be involved in tumorigenesis. Thus, RLGS-M can detect alterations in the methylation status of known tumor suppressor genes and provide a method for detecting and subsequently cloning novel genomic regions that undergo alterations in methylation during tumorigenesis.

INTRODUCTION

Cancer results from a series of epigenetic and genetic changes that may include germ-line and somatic DNA alterations. The identification of cancer-associated genes, such as oncogenes or tumor suppressor genes, has been performed by focusing on the detection of DNA changes in tumor tissues or tumor cell lines. The detection of genomic regions undergoing loss of heterozygosity in tumors has been a powerful strategy for the identification of tumor suppressor genes (1). In the last few years, research has also concentrated on the relation between alterations of DNA methylation status and the onset or the course of tumor progression. DNA methylation is known to be intimately related to the control of gene expression in many normal processes, such as X-chromosome inactivation (2) and genomic imprinting (3). Because methylation of genes, particularly within CpG islands, can silence gene expression and hypomethylation can activate expression, it is reasonable to consider that methylation of tumor suppressor genes or demethylation of oncogenes may be involved in tumor progression (4). The detection of abnormal CpG island methylation in VHL and RB1 genes in a subset of sporadic renal cell carcinomas (5) and retinoblastomas (6—9) is consistent with this hypothesis. Similarly, methylation of the p16/CDKN2 (also known as INK4a and MTS1) tumor suppressor gene has been detected in a variety of common human tumors and tumor-derived cell lines and is associated with the loss of p16/CDKN2 expression (10—15). Until recently, few technologies have been shown to be practical for genome-wide searches for alterations in DNA methylation status. Using RLGS-M (16—18), we previously identified genes that are methylated during development (18) or as a consequence of genomic imprinting (19—20). Thus, the RLGS method has been successfully applied to the genome-wide detection of alterations of DNA methylation status (RLGS-M) as well as to detect deletions or amplification of DNA (17, 21).

Here, we applied RLGS-M for the detection of alterations in DNA methylation status during mouse liver tumorigenesis. We employed transgenic mice containing a mouse MUP enhancer/promoter driving expression of the SV40 T antigen early region (22, 23). Thirty mouse liver tumors from interspecific F1 hybrids between Mus spretus (S) and C57BL/6 (B6) were analyzed. We focused on 507 polymorphic S and B6 loci/spots that were already mapped (24). Although this represents a small subset of CpG islands, 14 loci/spots appeared to have alterations of DNA methylation status. Following cloning and DNA sequence analysis, two of the loci were identified as α4 integrin and p16/CDKN2. Both of these genes have previously been reported to be involved in tumorigenesis. We conclude that RLGS-M can be effectively applied to the genome-wide screening for alterations in DNA methylation status related to tumorigenesis.

MATERIALS AND METHODS

Transgenic Line and Isolation of Liver Tumors. The MT-D2 transgenic line, which contains the SV40 early region under control of mouse MUP enhancer/promoter, has previously been described (22, 25). In this line, multiple tumors develop which were histopathologically characterized as hepatocellular carcinomas and adenomas. Female MT-D2 (C57BL/6J) mice were mated with M. spretus males. F1 progenies were sacrificed at 10 to 18 months to obtain liver tumors. Tumor samples were dissected and separated from adjacent non-tumor tissue.

DNA Preparation. Normal and tumor DNA were isolated by deproteinization in 10 mM Tris-HCl, pH 8.0; 150 mM EDTA-2Na, pH 8.0; 1% SDS; and 100 μg/ml proteinase K at 55°C for 30 min, followed by phenol-chloroform extraction, dialysis, and ethanol precipitation (26). These samples were dissolved in water and adjusted to 0.5 μg/μl.

RLGS Analysis and Spot Cloning. RLGS, Version 1.8, used in this study was described previously (26, 27). The restriction enzyme combination of NstI-PvuII-PsrI was used in this analysis. Using the new RLGS, Version 1.8, 507 of 575 previously mapped B6- and S-specific spots could be analyzed (24). The RLGS spots were cloned using the restriction trapper-based RLGS spot-cloning methods described previously (28).

Sequence Analysis. Sequence analyses were performed using dye-primer reaction chemistry with the Applied Biosystems model 377 DNA sequence.

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2 To whom requests for reprints should be addressed.
Southern Blot Analysis. DNA (5 μg) was digested with the appropriate restriction endonucleases according to the directions of the supplier, fractionated on 0.8% agarose gels and transferred to Biodine B nylon membranes. The probes used for hybridization were labeled with [α-32P]dCTP by the random primer method (29). Membranes were washed in 0.1 × SSC, 0.5% SDS at 65°C and exposed to FUJI BAS2000 film.

RESULTS

Analysis of Mouse Liver Tumors for Alterations in the DNA Methylation Status Using RLGS-M. The principle of how RLGS-M analysis is used to detect the DNA methylation status is shown in Fig. 1 by a schematic representation of the RLGS patterns of normal tissues from parents, F1 progeny, and F1 tumor tissue. Polymorphic paternal- (S) and maternal- (B) specific spots are transmitted to F1 progeny by Mendelian inheritance, and appear as half-intensity spots. Using a methylation-sensitive enzyme, such as NotI, we can detect alterations of the DNA methylation status at these sites throughout the genome. When comparing tumor DNA relative to control DNA, methylation of the NotI site in the tumor DNA results in spot loss, whereas demethylation of a NotI site results in the appearance of a new spot. An example of the disappearance of the spot due to de novo methylation in a tumor is shown (see Figs. 1 and 2B). To help characterize the alterations of methylation status in tumor DNA, we used interspecific F1 hybrid, strain-specific haploid spots, the chromosomal locations of which are known through genetic analysis (24). The 575 loci/spots (340 B6-specific and 235 S-specific) were mapped by RLGS analysis (NotI-PvuII-PstI) of BSS backcross progeny (24).

Using the new RLGS, Version 1.8 (26), 507 mapped spots can provide useful information for the rapid analysis and localization of genomic alterations such as deletions or methylation changes at landmark sites.

RLGS-M analysis was performed on 30 liver tumor DNA samples from 14 interspecific F1 hybrid mice between M. spretus and a C57BL/6 (B) specific, whereas spots 3 and 4 represent M. spretus (S) specific polymorphic alleles of the same loci. Of nonpolymorphic spots, in the F1 normal tissue of a cross between B and S (BSF1)4, polymorphic spots appear as haploid (half-intensity) spots, and nonpolymorphic spots appear as diploid (full-intensity) spots. In tumor DNA of a BSF1 animal, DNA methylation in the landmark site of a polymorphic spot (B-specific spot 1) leads to the loss of the spot in the RLGS profile. Spot loss can be analyzed in a comparison of normal versus tumor DNA RLGS profiles.

Random alterations due to genomic instability during tumorigenesis, as well as alterations in methylation as described above, are expected to occur. Some alterations, however, may be primary aberrations that promote tumorogenesis. These primary genomic aberrations would be expected to occur at a high frequency in a particular tumor type. Thus, we assume that loci altered in 22 of 30 tumor samples (75%) may have a significant role in tumorogenesis. Twenty-four spots were detected that showed a loss or reduction in spot intensity in 75% of the tumor DNA samples (see Table 1). The loss of these spots could have two primary origins; loss of heterozygosity (i.e., DNA loss) or methylation of the landmark NotI site. To distinguish these two possibilities, we combined the information on spot intensity with that of the chromosomal location. We assume that the chromosomal regions that contain more than two contiguous loci/spot losses are likely to reflect the deletion of DNA as we found previously (23), whereas solitary loci/spot losses preferentially reflect methylation at the landmark sites. Confirmation of this assumption requires the cloning of the spot and the analysis of the tumor-specific alteration by Southern blotting using the spot clone as a probe. By this criterion, we detected loss of 14 solitary spots, which occurred in 22 or more tumors (75%). These 14 spots are shown with their spot number in Fig. 2A. Part of the RLGS gel containing a representative spot, S238, is magnified and shown in Fig. 2B. The intensity of S238 (Fig. 2B, arrow) is clearly reduced in tumor tissue. Characterization of these 14 spots are presented in Table 2. Locus number is designated as D8Rik— or D8Ncvs—. Each of the 14 spots was classified as a solitary loss. Although four of the loci, D5Rik119, D5Ncvs4, D5Rik122, and D5Rik124, are located on chromosome 5, losses of these loci are all solitary. The localization of these four spots, however, is restricted to the centromeric half of the chromosome, indicating that a high frequency of methylation sites associated with tumorgenesis may be localized in this area.

Isolation of Spot Clones and DNA Sequence Analyses. To further characterize the nature of the genomic alteration associated with spot loss at these loci and to potentially identify a gene at the locus, we cloned 13 of the 14 spots using the restriction trapper-based RLGS spot-cloning method (28). Spot S118 was not cloned. DNA sequence analysis and a homology search against DNA databases revealed that two spots, S6 and S238, were identical with mouse α4 integrin and a mouse tumor suppressor gene p16/CDKN2, respectively (Fig. 3). As shown in Fig. 3A, the S6 spot is homologous to the 3' untranslated region of mouse α4 integrin and S238 (Fig. 3B) is homologous to the 3' half of the first exon of p16/CDKN2.

Southern Blot Analyses Using Spot Clone S238 as a Probe. To examine the methylation status of p16/CDKN2 in tumor tissues, Southern blot analysis was performed using the spot clone (S238) as a probe. Representative results are shown in Fig. 4. The S238 probe used for hybridization is identical to the distal half of the first exon of p16/CDKN2. The S238 probe detects a PvuII polymorphism between B6 and S in normal liver DNA (Fig. 4, Lanes 1 and 2). When these PvuII-PvuII fragments were digested with NotI, only the S allele was cleaved into a 3.9-kb band (Lanes 1 and 3). Thus, the NotI site from the spot clone S238 (NotI-PstI fragment) is present in the S allele but not in the B allele.

Fig. 1. Principle of RLGS-M analysis to detect changes in methylation pattern in tumor DNAs. Spots 1 and 2 are C57BL/6 (B) specific, whereas spots 3 and 4 represent M. spretus (S) specific polymorphic alleles of the same loci. C, nonpolymorphic spots. In the F1 normal tissue of a cross between B and S (BSF1), polymorphic spots appear as haploid (half-intensity) spots, and nonpolymorphic spots appear as diploid (full-intensity) spots. In tumor DNA of a BSF1 animal, DNA methylation in the landmark site of a polymorphic spot (B-specific spot 1) leads to the loss of the spot in the RLGS profile. Spot loss can be analyzed in a comparison of normal versus tumor DNA RLGS profiles.

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Table 1. The number of RLGS loci/spots exhibiting different classes of frequency of spot loss or reduction in tumors

<table>
<thead>
<tr>
<th>Tumors with loss or reduction in intensity of spots</th>
<th>Number of loci</th>
</tr>
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<tbody>
<tr>
<td>76–100%</td>
<td>24</td>
</tr>
<tr>
<td>51–75%</td>
<td>53</td>
</tr>
<tr>
<td>26–50%</td>
<td>137</td>
</tr>
<tr>
<td>0–25%</td>
<td>293</td>
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</tbody>
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Approximately 20 tumors were analyzed for each spot.

Fig. 2. RLGS-M profile of normal BSF, liver DNA produced by the enzyme combination of NotI-PvuII-PstI. A. 14 spots showing loss (methylation) in more than 75% of 30 hepatoma samples are indicated with arrows. B, part of the RLGS profile containing spot S238 (arrow) is magnified and shown from normal (1) and tumor (2) DNA.

absent from the B6 allele. Lanes 5–12 (Fig. 4) show DNA from tumor samples digested with both NotI and PvuII. In tumor XX-2 and K3-1 (Fig. 4, Lanes 5 and 6), the NotI-PvuII 3.9-kb S allele is reduced in intensity while the 6.0-kb PvuII-PvuII S band increased in intensity, indicating that the NotI site of S allele has become methylated. Tumors L3-3, F3-1B, and F3-5 show both methylated and unmethylated S bands of almost the same intensity (Lanes 7, 9, and 10), suggesting that 50% of the S allele of the p16 gene has become methylated. Tumors B3-1 and N3-2C have no methylated 6.0-kb S band (Lanes 8 and 12) but the intensity of the unmethylated 3.9-kb band is decreased, implying that the S allele of p16/CDKN2 gene was partially lost in this tumor sample. In tumor G3-2, both the methylated and unmethylated S bands are decreased and the B6 band is completely absent (Lane 11), indicating that the S allele of the p16/CDKN2 gene is partially methylated and the B6 allele is deleted.

Discussion

Alterations in DNA methylation are consistently found associated with tumorigenesis and may play a variety of roles in tumor progression (4). Several studies indicate that the methylation of CpG islands can be correlated with transcriptional silencing of tumor suppressor
genes, suggesting that the epigenetic alteration of DNA could represent a common mechanism for gene inactivation during tumor progression (4, 10, 12–15). Evidence supporting the inactivation of tumor suppressor genes through hypermethylation has been primarily derived for known tumor suppressor genes. An exception is HIC-1, a new candidate tumor suppressor gene that was identified through its aberrant hypermethylation in multiple types of known cancers (30). Although regional hypermethylation is frequently found in cancer, most of the genes associated with these sites are unknown.

Most methods that have been developed in recent years to rapidly scan the genome for genetic alterations related to cancer depend on nucleic acid hybridization to detect gain or loss of DNA sequences. These methods, which include comparative genomic hybridization (31), RDA (32), and PCR analysis of simple sequence length polymorphisms (33), can be performed without using PCR (28). In addition, because approximately 90% of the NotI sites are estimated to reside in CpG islands (37), RLGS, using NotI as the restriction landmark, preferentially scans these gene-rich regions. Thus, RLGS may identify differentially methylated genomic regions that would be missed by PCR-based methods.

Spot loss can be due to either loss of specific DNA sequences or methylation of the NotI site, resulting in the absence of NotI cleavage and subsequent labeling by the fill-in reaction at that site. Our studies indicate that most spot loss due to methylation occurs at solitary locations in the genome, whereas DNA loss appears to involve two or more contiguous loci (23). In the work presented here, we used the extensive genetic mapping data available for BSF1 mice to distinguish solitary and contiguous RLGS loci/spots.

We identified 14 RLGS loci/spots, the loss of which in tumors was likely to be due to methylation because the loss was solitary and did not involve contiguous loci. These alterations appeared to be related to the tumorigenic process because losses occurred in more than 75% of the tumors analyzed. To further characterize the loci and the nature of the amplification of GC-rich sequences may bias these methods toward the amplification of regions with moderate or low GC content. RLGS can be performed with as little as 1.5 μg of DNA, and cloning can be performed without using PCR (28). In addition, because approximately 90% of the NotI sites are estimated to reside in CpG islands (37), RLGS, using NotI as the restriction landmark, preferentially scans these gene-rich regions. Thus, RLGS may identify differentially methylated genomic regions that would be missed by PCR-based methods.

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of the genetic alteration involved, spot clones were isolated that corresponded to 13 of the 14 loci. Two of the spot clones were found to be identical to known sequences (Fig. 3) and correspond to genes with known tumor suppressor function.

A comparison of a chemically induced mouse liver tumor with normal liver using methylation-sensitive RDA identified several alterations in methylation in the tumor DNA (35). These included hypomethylation of LINE1 repetitive sequences, reduction of mitochondrial DNA gene dosage, and hypermethylation of an unknown sequence. In the RLGS studies presented here, we concentrated on the 507 B6- and S-specific loci/spots that had been mapped previously (24). The appearance of new RLGS spots, possibly due to hypomethylation, has been observed but these spots have not been cloned or characterized.4 The known sequences identified by RLGS (α4 integrin and p16/CDKN2) were not identified using the MS-RDA. However, it would be interesting to determine whether or not these regions are methylated in chemically induced liver tumors. It is not known whether there is any correspondence between unknown sequences identified by RLGS and by methylation-sensitive RDA.

Spot clone S6 was found to be derived from the α4 integrin gene. Previous work has shown that expression of α4β1 integrin is inversely correlated with the invasive potential of B16 melanoma cell lines (38). Overexpression of α4β1 was found to suppress the invasive potential of the B16 melanoma cells, whereas treatment with anti-α4 antibodies increased invasiveness. Although we have not yet performed the appropriate studies, the silencing of α4 integrin expression through methylation could therefore contribute to the malignant phenotype. This would be consistent with our previous observation that some well separated MT-D2/spretus liver tumor nodules derived from a single liver had identical genetic alterations, suggesting invasion or metastasis within the liver (39).

Spot clone S238 was found to contain sequences identical to exon 1 of the p16/CDKN2 (INK4a, MTS1) gene. Homozygous and heterozygous deletion and point mutations of this gene on 9p21 are commonly observed in human cancers (40–42). Several reports also indicate that methylation of a 5′ CpG island that includes exon 1 is associated with transcriptional silencing of p16 in human tumors (10, 12–15). The product of the p16/CDKN2 gene, p16, binds to CDK4 and CDK6, preventing their interaction with cyclin D and subsequent cell cycle progression. The CDKN2 gene also encodes an unrelated protein, p19, which arises from an alternative reading frame. This protein (p19) is also capable of inducing growth arrest, although the mechanism is not understood (43).

Southern blot analysis of DNAs from the liver tumors using the S238 spot clone indicated that the spreitus NotI site within exon 1 was completely or almost completely methylated in 19 of 24 samples. Five samples showed partial methylation (Fig. 4, Lanes 8 and 12) or a combination of partial methylation and partial gene loss (Fig. 4, Lane 11). It is not known whether the tumors are heterogenous with respect to alterations at this locus. This region (exon 1) corresponds to the region of the p16/CDKN2 gene that is hypermethylated in human tumors and results in transcriptional silencing (15). Currently, it is not clear whether methylation silences p16 expression in the liver tumors analyzed in this study.

Because the liver tumors are induced as a result of SV40 T antigen expression, the Rb gene is presumably “inactive.” p16 is considered to be upstream of pRb because it normally complexes with CDK4 and/or CDK6 inhibiting their interaction with cyclin D and the subsequent phosphorylation of pRb that is necessary for cell cycle progression from G1 to S (44). Thus, it is somewhat difficult to perceive what the consequence of loss of p16 expression would be in a Rb-negative cell. Indeed, ectopic expression of p16 in Rb-negative cells does not result in G1 arrest (44). Recent experiments indicate that p16 mRNA levels are elevated in Rb-negative cells or in SV40 T antigen-transformed cells, suggesting that Rb is normally a transcriptional repressor of p16 (45).

Then what is the significance of p16 methylation and its presumed transcriptional silencing in the SV40 T antigen-induced liver tumors? Clearly, more work is necessary to elucidate the consequence of p16 methylation in this system. Recent studies indicate that D type cyclins interact with a novel myb-like transcription factor, DMP1 (46). This suggests that cyclin D-dependent kinases may regulate gene expression through a Rb-dependent mechanism. This pathway may function to link regulation of some genes with the cell cycle but may still be susceptible to regulation via p16-CDK4/CDK6 interaction. The methylation of p16 in the SV40 T antigen-induced liver tumors may perturb this pathway.

We conclude that RLGS-M can be used for a genome-wide search for tumor-related alterations in the DNA methylation status. RLGS-M can detect alterations in known tumor suppressor genes and provides a means for detecting and cloning novel genomic regions that have alterations in methylation status related to tumor progression.

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