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Advances in Brief

Methylation Patterns in Human Androgen Receptor Gene and Clonality Analysis[1]

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Abstract

Tumor clonality, an important issue in tumor biology, has been analyzed using X-chromosome inactivation studies based on the differential methylation patterns of active and inactive alleles. Recently, a PCR-based androgen receptor gene (*AR*) analysis method was developed that takes advantage of highly polymorphic CAG repeats and nearby *Hpa*II and *Hha*I sites in exon 1 of *AR* at the Xq13 region. However, the data from this assay, which is now widely used, are sometimes uninterpretable and irreproducible for some currently unclear reason. To determine that reason, we analyzed a panel of lung cancer cell lines, using *Hpa*II or *Hha*I restriction enzymes, methylation-specific PCR, and bisulfite genomic sequencing of the polymorphic CAG repeat site of *AR* exon 1, including nearby CpG sites. We found direct evidence of a variable methylation pattern at the restriction sites that prevented proper enzyme cleavage in two lung cancer cell lines (NCI-H292 and NCI-H1944) obtained from female patients who had a polymorphic CAG repeat in *AR* exon 1. Our data suggest that methylation patterns at the CpG sites of *AR* exon 1 are complicated and vary among different individuals. Therefore, the reliability of the PCR-based clonality analysis may require further evaluation.

Introduction

In the somatic cells of female mammals, one of the X chromosomes is inactivated through extensive methylation of cytosine residues in the promoter regions of genes in the chromosome (1, 2). The inactivation occurs randomly between one of the two chromosomes, is somatically heritable, and is believed to be a necessary part of normal mammalian development (3, 4). A fundamental difference between an inactive X chromosome and an active one is the hypermethylation of CpG islands located at the 5′ ends of genes (5–8). On the basis of this feature, assays of RFLP and variable number tandem repeats, using methylation-sensitive restriction enzymes such as *Hpa*II and *Hha*I, which can cleave only specific sequences containing unmethylated cytosine, have been used to determine the clonality of tissues (9–13). However, this approach requires a large amount of high-quality DNA and can only be applied to a limited female population because of the low frequency of polymorphism in the target genomic areas.

As an alternative, a PCR-based method was developed to take advantage of the highly polymorphic CAG repeats and nearby *Hpa*II and *Hha*I sites in exon 1 of *AR*[3] in the Xq13 region (14–16). Because the method requires little DNA and can be applied to most females, it has been widely used to address important clonality issues, using clinical specimens, including paraffin-embedded tissues (17–19). However, the data from the assay are sometimes uninterpretable because of complicated patterns, such as skewing and irreproducibility (20–23). Using the methodology in our laboratory, we have also experienced similar problems in clonality analysis of tumor specimens. Although the mechanisms responsible for these problems currently are unclear, the determination of those underlying mechanisms is crucial for unbiased interpretation of tumor clonality analyses. Consequently, we hypothesized that variable methylation patterns at the restriction sites prevent proper enzyme cleavage and therefore hinder interpretation of data. To prove this, we analyzed the methylation status of *Hpa*II and *Hha*I sites in *AR* exon 1 in a panel of lung cancer cell lines from female patients.

Materials and Methods

Cell Lines and Tissue Samples. We analyzed a panel of lung cancer cell lines from female patients and subjected two of them (NCI-H292 and NCI-H1944) that were found to be polymorphic at the CAG repeat site of *AR* exon 1 to this study. Non-small cell lung cancer samples from female patients were obtained from surgical specimens; of these, three were found to be informative and were subjected to analysis. Tumor and normal tissues were separated. Genomic DNA was extracted by phenol-chloroform extraction and ethanol precipitation in the presence of glycogen.

***Hpa*II and *Hha*I Enzyme Digestion and PCR-based AR Analysis.** DNA samples (0.2–2 μg of each) were incubated overnight at 37°C with 20 units of *Hpa*II or *Hha*I (Life Technologies, Inc.) in a 20-μl reaction volume. Simultaneously, the same amount of each DNA was incubated without enzyme in a mock reaction. Three pairs of PCR primers were used to amplify *AR* exon 1: AR1 forward (5′-GTGCGCGAAGTGATCCAG AA-3′) and AR1 reverse (5′-TCTGGGACGCAACCTCTCTC-3′); AR2 forward (5′-AGA GGCCGC-GAGCGCAGCACCTC-3′) and AR2 reverse (5′-GCTGTGAAGGTTGCTG-TTCCTCAT-3′); AR3 forward (5′-AGCACCTCCCGGCGCCAGTTTGC-3′) and AR3 reverse (5′-TTCCTCATCCAGGACCAGGTAGCC-3′). One of the primers was end labeled with [γ-^{32}P]dATP (4500 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) and T4 DNA polynucleotide kinase (New England Biolabs, Beverly, MA). Each PCR amplification was performed in an 8-μl volume containing 200 μM dNTP, 1.5 mM $MgCl_2$, 0.4 μM of PCR primers including 0.01 μM [γ-^{32}P]-labeled primer, and 0.5 units of Hotstart Taq DNA polymerase (Qiagen). Reactions were performed under the following conditions: one initial cycle of 95°C for 15 min, annealing temperature for 1 min, and 72°C for 1 min; 39 cycles of 94°C for 30 s, annealing temperature for 1 min, and 72°C for 1 min; and a final extension for 5 min at 72°C. The annealing temperatures for each primer were 56–62°C. The PCR products were separated on 6% denaturing acrylamide-formamide gel for 2–4 h and autoradiographed to X-ray film.

Bisulfite Modification of Genomic DNA. Genomic DNA (1 μg) was treated with sodium bisulfite in a 50-μl reaction volume as described previously (24). Chemical conversion of cytosine to uracil is known to occur at rate of nearly 100%; however, methylated cytosine cannot be converted to uracil by the reaction (25). After modification, the two strands of DNA are no longer complementary. Therefore, strand-specific primers were designed for differential amplification of the methylated fragments of bisulfite-modified *AR* genes. To amplify an allele with unmethylated *Hpa*II sites, the following primer pair was used: ARU1 forward (5′-TGTGTGTGAAGTGATTTA-GAATTTGG-3′) and ARU1 reverse (5′-AATAACCTATAAAACCTCTA-CAATAAA-3′). To amplify both alleles regardless of methylation status at the *Hpa*II and *Hha*I sites in *AR* exon 1, the following primers were designed and used: ARS forward (5′-AAGATTTATTGAGGAGTTTTTT AGAAT-3′) and ARS reverse (5′-AATAACCTATAAAACCTCTACAATAAA-3′). PCR was performed under the same conditions as described above. Annealing temperatures ranged from 56°C to 62°C. The PCR products were separated on 6%

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[3] The abbreviation used is: *AR*, androgen receptor gene.

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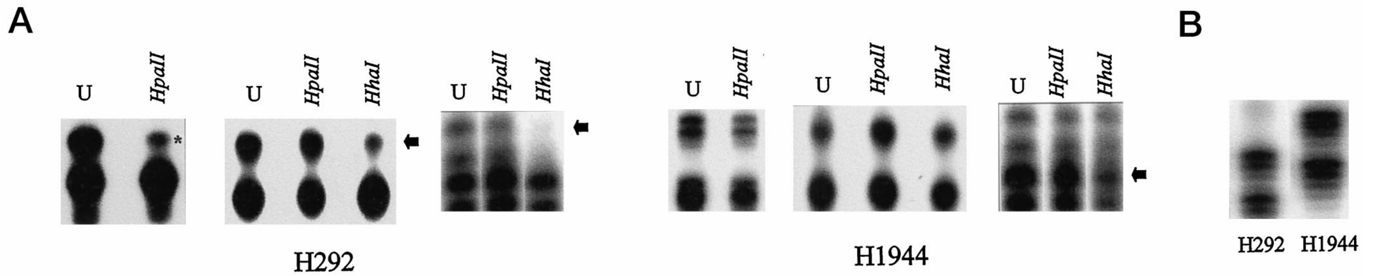

Fig. 1. *A*, results of three independent experiments using PCR-based analysis. The *left panel* represents cell line H292, and the *right panel* represents cell line H1944. The cleavage enzymes are indicated on the *top* of each *lane*. *U* indicates undigested DNA. In cell line H292, cleavage of the upper allele by *Hpa*II (*) was clearly observed in the first experiment but not in the other two independent experiments. The clear cleavage of the upper allele by *Hha*I was consistently found in the second and third experiments (as indicated by *arrows*). In cell line H1944, a random inactivation pattern was observed in all experiments except those with *Hha*I cleavage in the third experiment, in which the lower allele was skewed (as indicated by an *arrow*). *B*, PCR products using the methylation-specific primer sets for the unmethylated sequence of a *Hpa*II site in *AR* exon 1.

denaturing acrylamide-formamide gels for 2–4 h and autoradiographed on X-ray film.

Cloning of PCR Products and Bisulfite Genomic Sequencing. A TA cloning kit (Invitrogen) was used for cloning *AR* gene segments. Fresh PCR products using ARS primers that contained both methylated and unmethylated sequences in a 1-μl volume were cloned into TA vectors as specified by the manufacturer. For sequencing, 500 ng of plasmid DNA containing inserts was used as template for a sequencing reaction with AmpliCycle sequencing kit (Perkin-Elmer). T7 primer for vector sequence was end labeled. Cycling condition were followed as specified by the manufacturer.

Results and Discussion

In the last few years, a PCR-based assay of clonality that uses the methylation-sensitive restriction enzymes *Hpa*II and *Hha*I has become widely used to analyze clonality in clinical specimens, including paraffin-embedded tissues. However, the data from the assay are sometimes uninterpretable for reasons currently unclear. Using the methodology in our laboratory, we have experienced similar problems in analyzing tumor specimens (Fig. 1). We analyzed a panel of lung cancer cell lines from females and found that two of them (NCI-H292 and NCI-H1944) were polymorphic at the CAG repeat site of *AR* exon 1. Using *Hpa*II or *Hha*I as restriction enzymes, we found that different experiments could produce inconsistent results in both these cell lines (Fig. 1*A*), suggesting that even in a clonal cell population the assay might produce results that are difficult to interpret. We hypothesized that variable methylation patterns at the restriction sites prevented proper enzyme cleavage. To test this hypothesis, we modified genomic DNA from the two cell lines, using sodium bisulfite to convert unmethylated cytosine to uracil. We then designed PCR primer sets specific for an unmethylated sequence at a *Hpa*II site and used them to amplify the modified DNA. We found that the primer sets could not be used to amplify unmodified DNA but could amplify both alleles of the *AR* exon 1 fragment, suggesting that the unmethylated sequence was present in both parental alleles (Fig. 1*B*).

To directly analyze methylation status at these restriction sites,

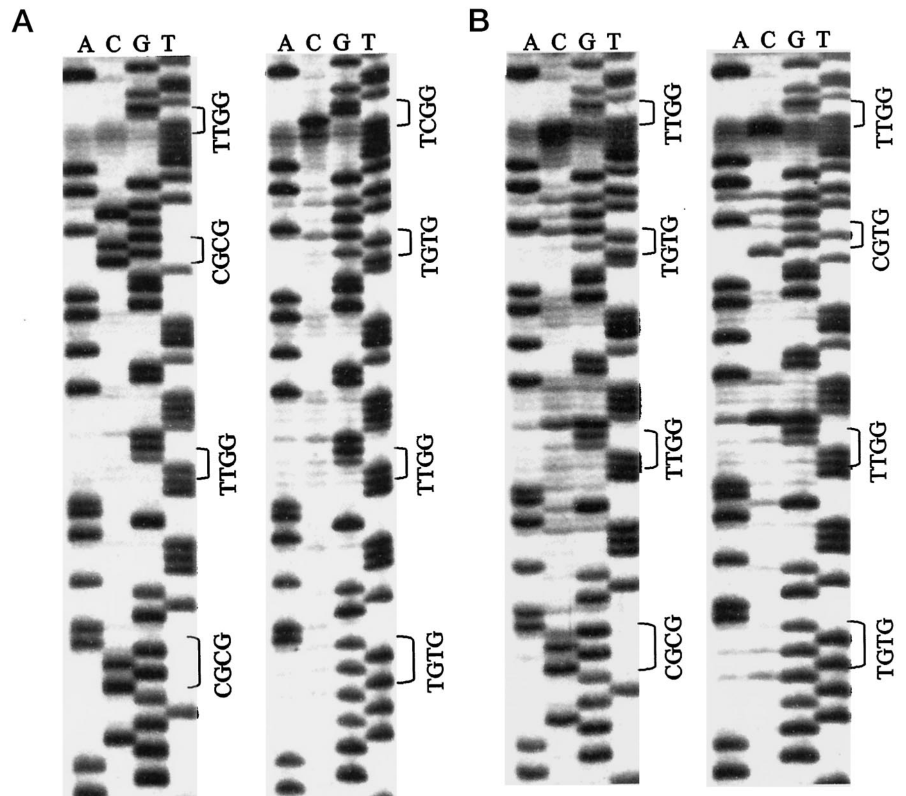

Fig. 2. Sequencing analysis of bisulfite-modified genomic DNA. *A*, clones from cell line H292; *B*, clones from cell line H1944. The original sequence of *AR* exon 1 should be 5′-AGAGCGTG***CGCG***AAGTGATCCAGAAC-***CCGG***GCCCCAGGCACCCAGAGGC***CGCG***AGCGCAG-CACCTC***CCGG***CGCCA-AG-3′. Both *Hha*I and *Hpa*II sites are indicated in bold italics. In *panel A*, the shorter allele of line H292 (*left lane*) was methylated at both *Hha*I sites but not at the two *Hpa*II sites. The longer allele (*right lane*) was methylated at only one cytosine residue of a *Hpa*II site. In *panel B*, the shorter allele (*left lane*) was methylated at two cytosine residues of one *Hha*I site. The longer allele (*right lane*) was methylated at only one cytosine residue in the other *Hha*I site.

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specific primer sets were designed to amplify fragments that included two *Hpa*II and two *Hha*I sites and the CAG repeats in *AR* exon 1 regardless of its methylation status. Amplified PCR fragments were cloned, and sequences in individual clones were analyzed. Clones from line NCI-H292 contained either 16 or 19 CAG repeats, and clones from line NCI-H1944 contained 20 or 24 CAG repeats, representing alleles from either the maternal or paternal parent, respectively.

In line NCI-H292, the shorter allele was methylated at 12 cytosine residues, including those at the *Hha*I sites, but not at the *Hpa*II sites (Fig. 2*A*. The longer allele was methylated at three cytosine residues, including one at a *Hpa*II site (Fig. 2*A*). This result theoretically suggests that when *Hpa*II is used for cleavage, no PCR product should be obtained from the cell line. Thus, the inconsistent patterns of PCR amplification after the different enzyme digestions observed in Fig. 1 were likely due to the incomplete cleavage and preferential amplification of one of the remaining undigested alleles in some experiments. In fact, the longer allele contains unmethylated *Hha*I sites and therefore could have been digested, which is consistent with our results (Fig. 1*A*).

Line NCI-H1944 exhibited different methylation patterns from line NCI-H292. The shorter allele was methylated at three cytosine residues, including one at a *Hha*I site, whereas the longer allele was methylated at only one cytosine (*i.e.,* at the same *Hha*I site as in the shorter allele; Fig. 2*B*). Because the *Hpa*II sites were not methylated in both alleles in the cell line, digestion using *Hpa*II could not generate a desirable result. Similarly, because one of the two *Hha*I sites was not methylated in both alleles, the use of *Hha*I could cleave both alleles as well. Therefore, the clonality of line NCI-H1944 could not be assessed reliably by either *Hpa*II or *Hha*I. Together, our data suggest that methylation patterns at the CpG sites of *AR* exon 1 are complicated and vary among different individuals. Thus, the reliability of the PCR-based clonality analysis may require further evaluation.

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