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Detection of CDKN2 Deletions in Tumor Cell Lines and Primary Glioma by Interphase Fluorescence in Situ Hybridization

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Abstract

Deletions of chromosomal band 9p21 have been detected in various tumor types including melanoma, glioma, lung cancer, mesothelioma, and bladder cancer. Recently, the CDKN2 gene (p16INK4a, MTS1, CDK4) has been proposed as a candidate tumor suppressor gene because it is frequently deleted in cell lines derived from multiple tumor types. We performed fluorescence in situ hybridization (FISH) with interphase cells using yeast artificial chromosome clones and a cosmid contig of the CDKN2 region. In 10 cell lines (4 glioma, 2 melanoma, 2 non-small cell lung cancer, 2 bladder cancer) with 9p alterations detected by molecular or cytogenetic analysis, interphase FISH with the CDKN2 cosmid contig detected all 9p deletions previously identified by molecular analysis. Using this probe, FISH analysis of primary glioblastoma tumors revealed homozygous deletions of the CDKN2 region in 6 of 9 tumors (67%) whereas a yeast artificial chromosome probe containing the interferon type I (IFN) gene cluster was deleted in only 4 cases (44%). Thus, it is likely that the CDKN2 region is the target of 9p deletions in gliomas. Interphase FISH will play an important role in defining the clinical significance of 9p deletions in primary tumors because it is especially applicable to clinical samples which may be contaminated by normal cells.

Introduction

The malignant transformation of tumor cells is known to be driven by the accumulation of different genetic events including numerical and structural alterations of distinct chromosomal regions. Among the specific alterations associated with neoplasia, the loss of tumor suppressor genes has been recognized as an important phenomenon. Hemizygous and homozygous deletions of chromosomal band 9p21 have been detected in various tumor types including melanoma, glioma, lung cancer, mesothelioma, and bladder cancer (1). In primary glioma the IFNα gene cluster which is located telomeric to the tumor suppressor region was found to be deleted in up to 50% of the primary tumors (2–4). Recently, the CDKN2 gene (p16INK4a) which encodes an inhibitor of the cyclin-dependent kinase 4 (CDK4), was found to be frequently involved in 9p deletions in tumor cell lines and has been proposed as a candidate tumor suppressor gene (1, 5, 6). In Southern blot analysis, 78% of our glioma cell lines and 45% of primary tumors showed homozygous deletions of CDKN2. However, the frequency of point mutations of CDKN2 in glioblastoma and other primary tumors is rather low (7–9). Therefore, it appears that the preferred mechanism of inactivation of CDKN2 is by homozygous deletions. Interphase FISH is a well established technique for identifying genetic alterations in neoplasias on a single-cell level. This technique is especially suited to analyze clinical tumor samples which are “contaminated” with normal cells. In addition, loss of one allele can be readily determined. We performed interphase FISH analysis on 10 tumor-derived cell lines (4 glioma, 2 melanoma, 2 non-small cell lung cancer, 2 bladder cancer) with rearrangement of the short arm of chromosome 9 detected by molecular or cytogenetic analysis and 9 primary glioblastoma, to determine the accuracy of different probes in detecting 9p deletions in tumor cell lines and primary tumor tissue. To our knowledge, this is the first report showing the utility of interphase FISH to detect deletions of the CDKN2 region in primary tumors.

Materials and Methods

Cell Lines. We used 10 cell lines (4 glioma, 2 melanoma, 2 non-small cell lung carcinoma, and 2 bladder cancer) that had been well characterized by conventional cytogenetic analysis. The cell lines were obtained from the American Type Culture Collection or from the investigators who had established them. Cytogenetic deletions of the short arm of chromosome 9 were detected in 4 of 10 cell lines. The cell lines as well as phytohemagglutinin-stimulated normal peripheral blood cells were harvested using standard cell culture techniques. Metaphase chromosomes were prepared as described previously (11).

Patient Materials. Tumor specimens obtained from 9 patients undergoing biopsy or resection of brain tumors were frozen in liquid nitrogen and stored at −70°C. The tumors were graded as glioblastoma multiforme according to the WHO classification system. Touch preparations were made by touching a freshly cut and thawed tumor surface on a slide. The slides were fixed in methanol:glacial acetic acid (3:1), treated with 5 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany), and postfixed in 0.5% paraformaldehyde (Sigma Chemical Co., St. Louis, MO).

FISH Probes. YAC A88E10 (330 kilobases), later referred to as YAC 11, and YAC 802B11 (1450 kilobases), later referred to as YAC 23, were obtained by screening the St. Louis and the CEPH YAC libraries with IFNα1 primers (12). YAC 883G5 (1100 kilobases), later referred to as YAC 17, were obtained from the CEPH MegaYAC library by screening with D9S966 primers (13). YAC 284D6 (320 kilobases), later referred to as YAC 10/2, from chromosomal band 8q22 was used as a control probe (14).

Eight cosmids encompassing a 250-kilobase region around CDKN2 were used. The cosmids contig was assembled by screening a flow-sorted human chromosome 9 library (Lawrence Livermore Laboratories) with probes from a YAC contig of the region. The exact localization of the FISH probes is shown in Fig. 1. FISH probes were prepared as described previously (10). YACs were purified on a pulsed-field gel. The DNA of the excised YAC bands as well as the cosmid DNA (20–100 pg) was amplified using a SIA (15). The amplification products were PCR labeled with biotin-11-dUTP (Enzo Diagnostics) and finally treated with DNsase (DNase I, 200 pg/ml for 10–20 min: Boehringer Mannheim) to reduce the average fragment size to 150–450 base pairs. pHR98, a variant satellite 3 sequence, which hybridizes specifically to the heterochromatinic region of chromosome 9 (9q9), was used to determine the copy number of chromosome 9 (16). The plasmid with a 158-base pair insert was amplified by SIA, PCR labeled with Spectrum Orange-11-dUTP (Imgenetics, Framingham, MA), and treated with DNsase as described. The copy

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3 The abbreviations used are: IFN, interferon type I; FISH, fluorescence in situ hybridization; YAC, yeast artificial chromosome; CDK4, cyclin-dependent kinase; SIA, sequence independent amplification.

4 O. I. Olopade et al., unpublished data.

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Table 1 Interphase analysis of normal peripheral blood

<table>
<thead>
<tr>
<th>FISH probe</th>
<th>Region/ marker</th>
<th>No. of hybridization signals/cell (%)</th>
</tr>
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<tbody>
<tr>
<td>pHuR</td>
<td>9qh</td>
<td>0.02 ± 0.06*</td>
</tr>
<tr>
<td>YAC 17</td>
<td>D9S966</td>
<td>0.30 ± 0.27</td>
</tr>
<tr>
<td>COS p16</td>
<td>CDKN2</td>
<td>0.10 ± 0.19</td>
</tr>
<tr>
<td>YAC 11</td>
<td>IFN A</td>
<td>0.06 ± 0.10</td>
</tr>
<tr>
<td>YAC 23</td>
<td>IFN A</td>
<td>0.04 ± 0.08</td>
</tr>
<tr>
<td>YAC 10/2</td>
<td>8q22</td>
<td>0.16 ± 0.18</td>
</tr>
</tbody>
</table>

* Mean ± SD of hybridization signals/cell.

number of chromosome 8 was determined by a centromeric FISH probe CEP 8 Spectrum Orange (Imagensics).

**FISH Procedure.** Two color FISH with YAC or cosmid probes and a centromeric probe was performed as described previously (17). The hybridization solution contained approximately 0.1 µg of each probe, 1 µg of human Cot1-DNA (BRL), 0.6 µg of human placental DNA, and 3 µg of salmon sperm DNA/slide in a 10-µl volume. The biotinylated probes were detected with FITC-conjugated avidin. The slides were counterstained with 4',6'-diamidino-2-phenylindole dihydrochloride and were analyzed using epifluorescence and a single-pass filter (Chroma Technology) to avoid superimposition of the centromeric and the YAC signals. For interphase analysis of the cell lines, the FISH signals of a total of 500 single, intact cells were counted by 2 independent observers. In addition, 25 metaphase cells of each cell line were analyzed. In the tumor samples 100 single intact cells were analyzed. For Fig. 2, separate autoradiographic signal comparable to the control; —, no signal; p, partial deletion of the IFNA gene cluster (only some of the multiple bands are present); ND, not done; NSCLC, non-small cell lung carcinoma.

The number of chromosome 9 copies were determined by FISH analysis of the centromere 9 probe (pHuR 98). The presence of the interferon A cluster (IFNA) and of the molecular markers REY24, CDKN2, D9S966, and D9S171 were determined by Southern blots.

**Molecular Analysis.** Cell line DNA was extracted and treated with restriction enzyme (HindIII), electrophoresed on a 1% agarose gel, and transferred to a nylon-based nitrocellulose membrane (Gene Screen Plus; NEN, Boston). DNA filters were hybridized with 32P-labeled probes from 9p2i and exposed to X-ray film. The probes used were REY24, CDKN2 cDNA, D9S966, and D9S171 (13).

**Results**

**Interphase Analysis in Normal Peripheral Blood.** To determine the reliability of the FISH probes in nonmalignant cells, ten test hybridizations of peripheral blood cells from normal individuals were performed with each probe (Table 1). Both centromeric and YAC probes showed an almost identical distribution of signals/cell comparable to previously published results for centromeric probes. In 500 nuclei scored, 2 signals were detected in 94–97% of the cells.

**Interphase Analysis in Tumor Cell Lines.** 9p deletions were determined by molecular analysis in 9 of 10 cell lines (Table 2). All deletions were detected as well by Interphase FISH with the COSp16 probe. The results of the FISH analysis are summarized as a deletion map (Fig. 1). YAC 23 was homozygously deleted in one cell line (H322). YAC 11 which covers the proximal IFN gene cluster was absent in 2 cell lines (U410, H322); only one copy was retained in one cell line (H4). Moreover, the intensity of the hybridization signals for YAC 11 was significantly reduced in 3 cell lines (H290, H4, A172). Previous detailed molecular analysis revealed that the distal deletion breakpoints of these cell lines lie within the YAC 11 region (3, 18). Therefore the intensity of the signal is reduced. However, we were still able to detect signals for YAC 11 in one cell line [H4 (Fig. 2a)] even though 90% of the YAC region was deleted. YAC 17 was homozygously deleted in 2 cell lines (H290, RT4), the number of copies was reduced in 2 other cell lines (A172, H4), and 1 cell line showed a partial deletion of one allele (U410). The cosmid probe which covers the region of CDKN2 was homozygously deleted in 8 of 10 cell lines. In one cell line (T98) the signal was significantly reduced indicating a partial deletion of the region. Southern blot analysis showed a homozygous deletion of CDKN2 in this cell line, whereas another molecular marker of the region was retained (data not shown). In 5 cell lines (H4, U410, HS294T, RT4, UM-UC3), both control probes, the chromosome 8 centromere probe and the YAC 10/2 probe, showed a similar distribution of signals/cell indicating the comparable hybridization efficiency of centromeric and YAC probes. However, in the 5 remaining cell lines (all with 4 or more copies of...
latter group the number of signals/cell was highly comparable to the centromere 9 data. In contrast, in the cell lines with homozygous deletions 99.5 ± 0.4% (SD) of the cells showed no hybridization signal (Fig. 3A). Nonhomozygous deletions could be detected with a similar accuracy. Thus, there was good concordance between the molecular results and the FISH data. All the homozygous deletions determined by molecular analysis were detected by Interphase FISH (Table 2). In addition, cell lines with a partial loss of the IFN gene cluster had a reduced intensity of the hybridization signal of YAC 11.

Interphase Analysis in Tumor Specimens. To determine 9p deletions in primary tumors, we analyzed 9 brain tumors, pathologically classified as glioblastoma multiforme, using the FISH probes YAC 11, COSp16, and YAC 10/2 for detection of the deletion of the proximal IFN gene cluster (YAC 11), the CDKN2 region (COSp16), and a control probe (YAC 10/2). Of 9 tumors, 4 tumors (44%) had a deletion of the proximal IFN gene cluster [YAC 11 (Fig. 3B)]. No cosmid signal for CDKN2 was detectable in 5 tumors. In one additional tumor sample [sample 1 (Fig. 3B)] the intensity of the hybridization signals of the cosmid contig was significantly reduced in comparison to the control YACs, indicating a partial deletion of the cosmid contig.

In one tumor sample [sample 7 (Fig. 3B)] 7% of the cells did not show any YAC 11 signal. This tumor had only one copy of chromosome 8. Therefore, the number of cells without hybridization signal probably results from an incomplete hybridization efficiency. In aneuploid tumors (6 cases, determined by interphase FISH) a subpopulation of cells (13.4 ± 4.8%) had 2 copies of chromosome 9. This cell population was not identified in cell lines and probably represents the contamination with normal cells (stromal cells, lymphocytes, etc.).

Discussion

Interphase FISH analysis is a well established method to determine chromosomal aberrations in hematological malignancies and solid tumors. Using the appropriate probes, interphase analysis is able to detect chromosomal aberrations in clinical tumor specimens contaminated with normal cells and is also able to detect these changes in small subpopulations of cells. In this study, we describe the analysis of 10 cell lines derived from gliomas, melanomas, non-small lung cancer, and bladder cancer and 9 primary gliomas using interphase FISH analysis. For our experiments, we generated FISH probes from YACs and a cosmid contig by a SIA technique developed in our laboratory (10). This procedure yields consistent and strong FISH signals for interphase analysis. In contrast, single cosmids of the 9p region had a hybridization signal of only moderate intensity due to the small insert size. At present, FISH probes of YACs or similar vectors have been generated previously by Alu-PCR (19). This amplification technique is limited by the number of Alu sequences per clone which varies considerably. Hybridization of YAC probes generated by Alu-PCR to extended chromatin preparations showed incomplete repre-
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Fig. 3. A, FISH analysis in 10 tumor cell lines. The percentage of cells without hybridization signal (YAC 10/2, YAC 11, COSp16) is shown. B, FISH analysis in 9 glioblastomas. The percentage of cells without hybridization signal (YAC 10/2, YAC 11, COSp16) is shown.

Our data suggest that the 250-kilobase region covered by the cosmide contig includes the target gene of the 9p deletions in primary gliomas. However, the frequency of point mutations detected in primary tumors is rather low (8, 9). Therefore, the simultaneous deletion of the neighboring genes may be responsible for the selective growth advantage for the malignant cells. Hannon and Beach (24) proposed that p15 (MT52, CDKN2α), a transforming growth factor β-regulated member of the p16 family, also plays an important role in carcinogenesis. p15 maps approximately 20 kilobases centromeric to CDKN2 and is included in our cosmide contig (1). It may well be that the predominant mechanism of 9p rearrangements in primary tumors is the deletion of a large genomic region which would inactivate both genes in one step. In fact, in cell lines as well as in primary glioblastoma, the vast majority of deletions includes both genes (1, 5, 9).

Therefore, we believe that homozygous deletions are the predominant mechanism for inactivating this region. Because further mapping data are crucial to determine the clinical significance of these rather large deletions in primary tumors, FISH will play an important role in characterizing the deletions.

Recently, the overexpression of CDK4, the target molecule of p16, was proposed as an additional mechanism of functional p16 inactivation (22, 23). Both events would result in a disorganization of the cell cycle. However, in a number of cell lines and primary gliomas the homozygous deletion of CDKN2 was the much more frequent event (22, 23).

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