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BRIEF METHODS

Quantitative Analysis of Promoter Hypermethylation in Laser-Microdissected Archival Specimens

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The methylation of cytosine residues in the promoter region of tumor-suppressor genes is now widely recognized as an alternative mechanism of gene inactivation in cancer (Jones and Laird, 1999). To address the question of whether promoter methylation is an early event in the process of malignant transformation, small and precancerous lesions have to be analyzed. However, these lesions are almost exclusively available only as archival, formalin-fixed, paraffin-embedded pathological specimens. Therefore, highly sensitive methods suitable for the analysis of archival biopsies have to be used.

A very useful approach for the screening of larger series of tumor samples for the methylation status of promoter regions is the methylation-specific PCR (MSP) (Herman et al, 1996). The advantage of this method is its high sensitivity and its ability to analyze many samples in parallel. However, this method provides only qualitative information regarding the methylation status of the sequence analyzed.

During the analysis of various tumor samples, we realized that the qualitative analysis of the methylation status of cytosine-guanine-dinucleotide (CpG) sites in promoters hides, in some cases, huge quantitative differences. These quantitative differences might be more important in the classification of lesions and might have more prognostic significance than pure qualitative information (U Lehmann and H Kreipe, unpublished data).

In this study we first developed a quantitative assay for the measurement of CpG methylation using real-time PCR technology (Lie and Petropoulos, 1998) from Applied Biosystems (Sequence Detection System 7700; Applied Biosystems, Weiterstadt, Germany). We then demonstrated that the methylation status of tumor cells isolated from archival tissue sections by laser-assisted microdissection can be analyzed with this new methodology.

For the efficient detection of methylated cytosine residues after bisulfite conversion in less than 1 ng of genomic DNA, we optimized the protocol for the bisulfite treatment to minimize DNA degradation. Prolonged incubation of DNA with bisulfite at low pH causes serious degradation. This is especially problematic in analyzing DNA from formalin-fixed, paraffin-embedded biopsies because in these cases the DNA is already degraded to a certain extent as a result of the formalin fixation. As a model system, we analyzed the methylation of the promoter region of the tumor-suppressor gene $p16^{INK4A}$ in breast carcinoma using DNA from fresh frozen samples of invasive carcinomas and also from formalin-fixed, paraffin-embedded, laser-microdissected intraductal lesions.

The genomic DNA from fresh frozen tumor biopsies was isolated according to standard protocols. The laser-assisted microdissection of archival histological sections and the subsequent DNA extraction was performed essentially as described (Lehmann et al, 2000).

The extracted DNA was incubated in 200 mM NaOH at 42° C for 30 minutes for complete separation of both strands in a total volume of 50 μl. After the addition of 275 μl of 3.6 M bisulfite containing 1 mM hydroquinone (freshly prepared!), the samples were incubated for 5 hours at 55° C in the dark. Then 375 μl of 6 M sodium iodine containing 1 mM β-mercaptoethanol and 5 μl glass milk suspension (Quiagen, Hilden, Germany) were mixed thoroughly with the reaction mixture. After 10 minutes at room temperature, the samples were centrifuged and the supernatant discarded. The pellet was washed three times with 70% ethanol, once with 20 mM NaOH/90% ethanol, and twice with 90% ethanol. The DNA was eluted from the dried pellet with 25 μl TE-buffer for 15 minutes at 55° C. From this solution, 3 to 6 μl were used for methylation-specific PCR.

The bisulfite-modified DNA was amplified in a real-time PCR (Fig. 1A) using the following probes and primers: E5: 5'-CRTTATCTACTCTCCCCCTCTCC; E6: 5'-GGTTGGTTATTAGAGGGTGGGG; M-probe: 5'-

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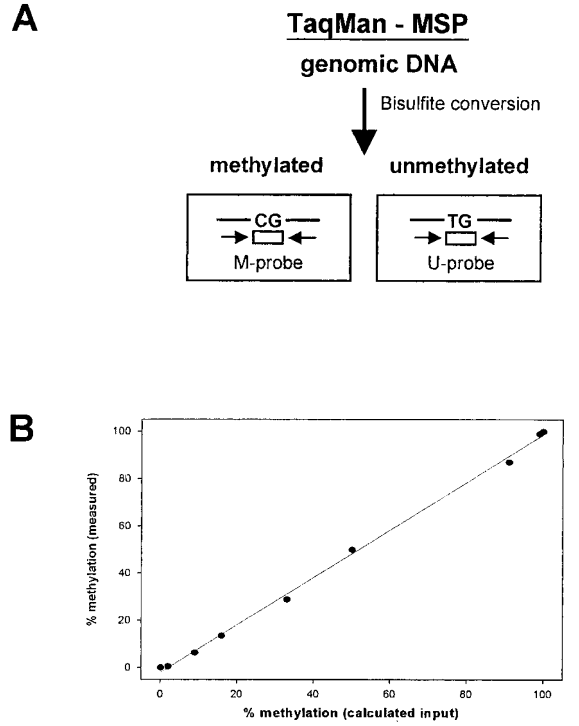

Figure 1.

A, Principle of real-time methylation-specific PCR (MSP): A primer pair containing no potential methylation site in its sequences amplifies a short stretch of DNA containing the cytosine-phosphorothioate-guanine (CpG) sites for which the methylation status is to be determined. The very short length of the amplicon ensures identical amplification efficiencies for methylated and unmethylated alleles. B, Correlation of the measured degree of methylation and the actual input: different mixtures of Raji DNA (completely methylated) and lymphocyte DNA (completely unmethylated) were analyzed. The degree of methylation is calculated as follows: % M = (1/R + 1) × 100, with R = (1 + E)$\Delta^{Ct(M-U)}$ and the measured reaction efficiency E = 0.95. ΔCt(M-U) is the difference of the Ct-values for the amplification of methylated ("M-reaction") and unmethylated ("U-reaction") alleles. The linear correlation ranged from 0.1% methylation to 99.9% methylation (r = 0.998).

FAM-AACCGCCGAACGCACGC-TAMRA; and U-probe: 5'-FAM-CAACCACCAAACACACACAATCCACC-TAMRA. The melting temperatures of the probes and primers were calculated with the PrimerExpress software (Applied Biosystems, Weiterstadt, Germany)

The primer pair E5/E6 amplified both methylated and unmethylated DNA with the very same efficiency (data not shown). The PCR conditions were as follows. After an initial denaturation step at 95° C for 5 minutes, 45 cycles with 15 seconds at 95° C and 60 seconds at 60° C followed. The primer and probe concentrations were 250 nM and 125 nM, respectively. Per reaction, 0.75 units *Taq*-polymerase (Platinum-*Taq*; Life Science Technologies, Karlsruhe, Germany) were used at a magnesium concentration of 2.5 mM in a total volume of 30 μl.

The sensitivity and specificity of the real-time MSP was determined using cloned fragments of bisulfite-modified DNA as a template. These experiments showed that the two hybridization probes were 100% specific for methylated or unmethylated DNA (no cross-reactivity of the M-probe with 10^6 molecules of plasmid containing unmethylated DNA and vice versa, data not shown). Careful mixing experiments showed

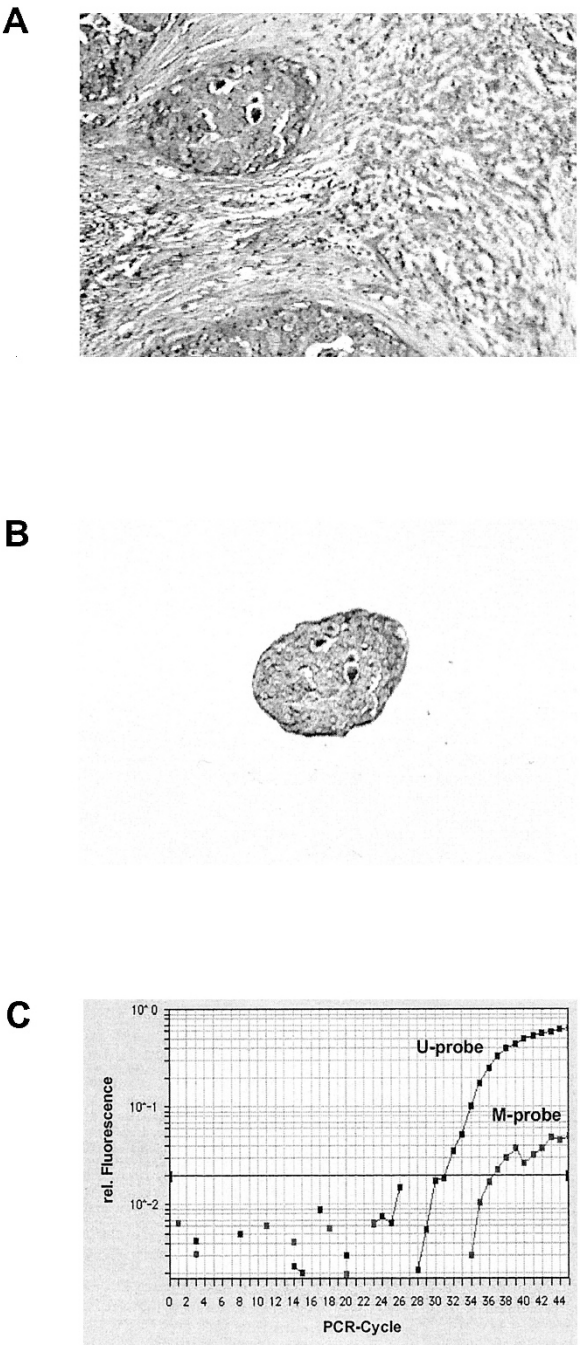

Figure 2.

Isolation of pure intraductal tumor cells using laser-assisted microdissection. A, Methylene blue-stained section of a ductal carcinoma of the breast. The reduced optical quality is due to the fact that the slides are not coverslipped for microdissection (×100). B, Isolated intraductal tumor cells in the lid of a reaction tube (×100). C, Amplification plot of the real-time MSP for intraductal breast tumor cells.

that less than 1% methylated alleles could be detected in a quantitative manner in an unmethylated background. The linear correlation between the calculated and the observed ratio ranged from 0.1% methylation to 99.9% methylation (Fig. 1B).

Because both methylated and unmethylated alleles are amplified by the same primer pair with equal efficiency (data not shown), the ratio of both species

can be directly calculated from a shift in the amplification plot. Therefore, in contrast to the method described by Dennis et al (1999), the preparation of standard solutions of bisulfite-modified DNA required for an absolute quantification is not necessary. Because the yield of the bisulfite conversion is slightly varying and the stability of bisulfite-treated DNA is limited, the preparation and storage of standard solutions is a huge practical problem.

In a series of invasive breast carcinomas, we found a level of methylation in the promoter region of the *p16^{INK4A}* gene of up to 60% whereas, in intraductal lesions isolated from archival tissue sections by laser-assisted microdissection, no methylation or only a very low level of methylation (1% and less, Fig. 2) could be detected. These results clearly indicate a dynamic change in the methylation pattern during breast cancer progression, at least for the *p16^{INK4A} gene.*

In addition to the quantitative information provided by this new assay, further advantages are the speed and high throughput of the 96-well–based, real-time PCR system and the omission of all postamplification steps, which greatly reduces the work load and the risk of contamination. Now, retrospective studies analyzing quantitatively the methylation status of tumor-suppressor genes in morphologically defined tumor cells or cells from precursor lesions are possible.

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