Analysis of Loss of Heterozygosity in Microdissected Tumor Cells from Cervical Carcinoma Using Fluorescent dUTP Labeling of PCR Products

ABSTRACT

The use of fluorescently labeled nucleotides (F-dUTP) in the PCR for genotyping of microsatellite polymorphisms was investigated. Microdissected tumor cells from cervical squamous cancer biopsies were compared with cells from surrounding normal tissue for loss of heterozygosity (LOH) at the mismatch repair gene hMLH1. Removal of unincorporated F-dUTP before analysis is necessary to reduce background fluorescence; ethanol-precipitation was found to be as efficient as the use of a spin column for this purpose. The gel resolution was sufficient to distinguish alleles differing by about four nucleotides. Alleles differing by only one dinucleotide repeat were possible to identify, but the ratio of alleles was difficult to assert with any reliability due to the wide peaks obtained. Single primer-pair and nested amplification systems were shown in reconstruction experiments to reliably quantitate the ratio of alleles. Of 36 cervical cancer biopsies, amplification and typing of the hMLH1 microsatellite marker was successful in 20 cases. Among 9 informative (heterozygous) biopsies, 2 (22%) were found to show LOH.

INTRODUCTION

Cervical squamous cancer is the most frequent form of genital cancer (10) and one of the leading causes of female death from cancer (12). A number of chromosomal regions have been reported to be altered in cervical cancer biopsies (2,6,9,14). With the possible exception of the tumor suppressor gene p53 located on 17p, no susceptibility genes for cervical cancer have been identified. Mutations in the hMLH1 gene, on 3p21.3-23 (3), have been found in hereditary non-polyposis colorectal cancer (5) and proposed to affect DNA repair and lower the fidelity of replication (11). Based on the observation that loss of heterozygosity (LOH) on 3p has been observed in cervical cancer patients (14), we decided to determine if the hMLH1 gene is structurally altered in cervical carcinoma biopsies.

Rapid and inexpensive methods for analyses of genetic markers, such as microsatellite polymorphisms, are essential to studies of genetic disease and for mapping of cancer genes. There is strong need to evaluate methods for inexpensive, nonradioactive labeling methods of polymerase chain reaction (PCR) fragments (1). The high cost of attaching fluorescent molecules to the PCR primers makes alternative techniques, such as incorporation labeling, potentially interesting. We have therefore evaluated a novel method for fluorescent labeling of the PCR products using incorporation of fluorescently labeled nucleotides (F-dUTP) during PCR (13).

MATERIALS AND METHODS

Isolation of Tissue and Template DNA Extraction

Fixed and paraffin-embedded biopsies from 36 cervical cancer patients (23 cervical squamous cancer in situ and 13 cervical carcinoma) were sliced in thin sections, placed on microscope slides and stained with haematoxylin/eosin. Normal and squamous tumor tissue were marked out on each slide and retrieved by microdissection. The material was subjected to proteinase K treatment (0.25 mg/mL) in 1% sodium dodecyl sulfate (SDS) at 60°C for 2–3 h before the enzyme was inactivated by incubation at 90°C for 10 min. DNA was isolated by two phenol-extractions, followed by one chloroform-extraction and precipitated by adding 1/10 the volume of 3 M sodium acetate and 3 times the volume of 99.9% ethanol. The DNA pellet was washed once with 70% ethanol, dried and resuspended in 50 µL TE-low buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA).

PCR Amplification

For the LOH analysis, the PCR primers for the D3S1611 marker (5),
located in an intron of the hMLH1 gene, were used. For samples from which very limited amounts of DNA were obtained, a new 5′ primer was used in a nested PCR (Figure 1). The PCR was performed in 50 µL containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 10% glycerol, 1% Nonidet® P-40 (NP40)/Tween® 20, 0.8 mM dNTPs (0.2 mM of each of them), 1.0 µM F-dUTPs, labeled with the rhodamine dyes R110 or R6G (Perkin-Elmer/Applied Biosystems Division [PE/ABI], Foster City, USA) (none in the first round of PCR if nested system was used), 2 U of AmpliTaq® DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA), 0.3 µM of each primer and 5 µL DNA from the biopsy. In the nested PCR experiments, 2 µL of PCR product from the first round were used as template for the second round. Amplification was carried out in a GeneAmp® PCR System 2400 instrument (Perkin-Elmer). The amplification was performed for 30 cycles, each with 94°C for 30 s, 55°C for 30 s and 72°C for 15 s. In the nested PCR experiments, the same PCR cycle was used.

Purification of PCR Product

The volume of reactions was brought up to 100 µL by dH₂O, and they were extracted twice with 100 µL of a phenol:H₂O:chloroform-mixture (proportions 34:9:7). The PCR products were precipitated by adding 15 µL of 2 M sodium acetate, pH 4.5, and 300 µL of 99.9% ethanol. As an alternative, purification by QIAquick™ Spin columns (Qiagen, Hilden, Germany) was used. We used an elution volume of 30 µL.

Figure 1. Sequence of the marker D3S1611. The length of the fragment varies from 128–138 bases (single PCR) or 89–98 bases (nested PCR).

Figure 2. Example of purification of the PCR products. The fragment with an estimated size of 158 bp represents an unspecific amplification product. Electropherogram from a run of (A) a non-purified PCR product and (B) a PCR product purified by phenol:chloroform:dH₂O extraction. The fragments with sizes below 120 bp that are removed by the purification represents unincorporated F-dUMP, F-dUDP and F-dUTP, while the fragment around 220 bp represent a fluorescent by-product generated during the PCR.

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Detection of the Products

To detect amplified fragments, 1.5 µL of the purified PCR products were analyzed on a Model 373 DNA Analysis System using the GENESCAN™ 672 software (both from PE/ABI) (8).

Calculation of Allele Ratios

To estimate the degree of LOH, i.e., the allelic loss in the heterozygous paired normal and tumor samples, the peak area values were used in the following way (4). A ratio was calculated as \((T_1/T_2)/(N_1/N_2)\), where \(T_1\) and \(N_1\) are the area values of the shorter allele for the tumor and normal sample, respectively, and \(T_2\) and \(N_2\) are the area values of the longer allele for the tumor and normal sample, respectively. In cases where the allele ratio calculated by this equation was above 1.0, the ratio was converted by using \(1/[(T_1/T_2)/(N_1/N_2)]\) to give a result in the range from 0.0–1.0. A value at or below 0.50 was taken to indicate LOH.

RESULTS

Purification of PCR Products

Two alternative protocols for the purification of PCR products were used. An example of the results of the precipitation protocol is shown in Figure 2, A and B. The results of the spin-column method were indistinguishable from those of the precipitation method.

Allelic Resolution Using F-dUTP-Labeled PCR Products

We investigated resolution of the system for alleles of the dinucleotide polymorphism in the \(hMLH1\) gene. Alleles differing by 4, 6, 8 and 10 bases, i.e., alleles differing by 2, 3 and 4 repeat units, were easily separated and their proportion possible to determine (see below). However, for alleles differing by only 2 bases, broad peaks made it difficult to determine relative proportions of the alleles. For accurate determination of allele ratios for such samples, labeled primers should be used.

Accuracy of the Quantitation of Allele Proportion

A reconstitution experiment using a mixture of two homozygous DNA samples in a spectrum of proportions showed that there is a linear relationship between input ratio and measured values in the entire spectrum of allele proportions (Figure 3A). Similar results were obtained both for single and nested PCR (Figure 3, A and B).

Loss of Heterozygosity

In total, 36 patient biopsies were examined. In samples from 20 of these, it was possible to amplify the marker from both normal and tumor DNA. Of these 20 biopsies, 9 (45%) were found to be heterozygous for the D3S1611 marker. In 2 (22%) of the 9 informative samples, LOH was detected using the cut-off value of 0.50 (Table 1).

DISCUSSION

Labeling by incorporation of F-dUTP during PCR represents a simple and cost-effective alternative to using fluorescently labeled primers that can be generally applied. We observed no change in sensitivity of the PCR when the F-dUTP was present. The main disadvantages of the incorporation method are the necessity for purification before the gel analysis and the tendency to yield broader peaks, possibly due to nonquantitative incorporation of label, incomplete denaturation of the secondary structures or unequal distribution of fluorophores between the two samples.
strands. The broad peaks, resulting from the incorporation of dUTP, may occasionally result in split peaks, which leads to difficulties in accurate quantitation of the proportion of two alleles. Our results show that accurate quantitation is possible when alleles differ by more than 4 bases, but for alleles differing by fewer bases, the method has to be used with caution. If the labeling method is used solely for genotyping, correct typing results may be possible to obtain even for alleles differing only by 2 bases. The reconstitution experiment showed that the system was able to accurately determine the proportion of the two alleles, even when a nested PCR system was used.

In our study, 2 out of 9 patients (22%) showed loss of the hMLH1 marker in their tumor cells. Thus, our results do not suggest that large deletions of the hMLH1 gene, or that of nearby locus, is a common feature of cervical carcinomas. Previously, chromosomal loss of regions on 3p, near the hMLH1 gene, have been found in between 43%–100% of tumors (7,14). The discordance between studies may be due to technical differences. Also, the chromosomal location of the markers used in these studies was not identical, and deletions in the 3p14-21 region may have gone undetected using the D3S1611 marker. Further studies, using larger patient cohorts, are needed to determine the involvement of LOH in this region in cervical cancer development.

REFERENCES


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