ABSTRACT

Almost all tumors from patients with hereditary non-polyposis colon carcinoma and approximately 10%–15% of sporadic colon and gastric carcinomas contain widespread deletions within mono- and dinucleotide repeat sequences in their DNA. This is referred to as the replication error (RER+) phenotype and, in the case of colon carcinoma, is often associated with an improved tumor prognosis and possibly also with response to chemotherapy. The RER+ status of tumors is usually determined by examining several dinucleotide and mononucleotide repeats for size variations when compared with the matching normal DNA. This is referred to as the replication error (RER+) phenotype and, in the case of colon carcinoma, is often associated with an improved tumor prognosis and possibly also with response to chemotherapy. The RER+ status of tumors is usually determined by examining several dinucleotide and mononucleotide repeats for size variations when compared with the matching normal DNA.

INTRODUCTION

Microsatellite instability, or the replication error (RER+) phenotype, was first observed in the DNA of tumors from patients with the familial cancer syndrome, hereditary non-polyposis colon carcinoma (HNPCC) (9). RER+ tumors are characterized by frequent alterations in the size of mono- and dinucleotide repeat sequences. In HNPCC cases, this is due to constitutional mutations in one of the DNA mismatch repair genes, the most common being hMSH2 and hMLH1. RER+ is also observed in a small proportion of sporadic tumors from the HNPCC spectrum of malignancies, particularly colon, gastric and endometrial carcinomas (5). RER+ colon carcinomas have distinctive clinicopathological features that include an almost exclusive location in the proximal colon, mucinous or poorly differentiated histology, the presence of tumor infiltrating lymphocytes and a better prognosis. In a retrospective series of 185 proximal colon carcinomas, we recently found that RER+ tumors respond well to adjuvant chemotherapy as observed by significantly improved patient survival (4). It has been established that analysis of the BAT-26 poly(A) tract located within intron 5 of the hMSH2 gene is sufficient to determine the RER+ status of tumors with more than 99% accuracy (23). This mononucleotide repeat sequence is particularly susceptible to deletions in RER+ tumors, thereby avoiding the need to screen several different mononucleotide and dinucleotide repeats for size variations. Although BAT-26 is quasi-monomorphic (99.92%) in pure Caucasian populations, some 10% of Africans have shortened alleles (17,18). Therefore, to avoid false positives, germ-line DNA should be tested in parallel to the tumor DNA in populations with significant African genetic admixture. The properties of BAT-26 allow for an 80% reduction in the number of PCR and electrophoretic analyses previously used to determine RER+ status.

We recently described a rapid and non-isotopic method for the identification of RER+ based on SSCP analysis of deletions within BAT-26 (7). Although relatively simple and inexpensive, this method is difficult to standardize in routine pathology laboratories because of the nature of the gel electrophoresis and silver staining systems used. The sensitivity of the SSCP technique depends on the gel composition and running temperature, while the reproducibility of silver staining depends on the quality of the reagents used, in particular the ultrapure water.

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By running precast quality-controlled gels in a temperature-regulated DNA analyzer that uses fluorescent signal detection, these variables are removed from the assay. The capacity for high throughput of samples and the ability to analyze formalin-fixed, paraffin-embedded tumor material should allow the widespread introduction of this method for routine screening of RER+ status.

**MATERIALS AND METHODS**

**Tumor DNA**

Tumor DNA was obtained from 188 colon carcinomas that had been surgically resected between 1991 and 1996 at the Sir Charles Gairdner Hospital (Nedlands, Australia). All tumor DNA originated in the proximal colon and were of Dukes’ stage C. Ten-micron thick sections were cut from formalin-fixed, paraffin-embedded blocks of each tumor. One of these was incubated fixed, paraffin-embedded blocks of colon carcinomas that had been surgically resected between 1991 and 1996 at the Sir Charles Gairdner Hospital (Nedlands, Australia). All tumor DNA originated in the proximal colon and were of Dukes’ stage C. Ten-micron thick sections were cut from formalin-fixed, paraffin-embedded blocks of each tumor. One of these was incubated

**PCR of the BAT-26 poly(A) Tract**

Primers used for amplification of the BAT-26 poly(A) repeat (23) were as follows: forward 5’TGACTACTTTTGACCTTGACCC-3’ and reverse 5’AACCATACACGTTTTAACC-3’. These were fluorescently labeled at the 5’ end with HEX™ dye (GeneWorks, Adelaide, Australia). PCR conditions were the same as previously described (23) and comprised 5 min denaturation at 94°C, 2 min annealing at 45°C, 2 min extension at 70°C, followed by 32 cycles of 1 min annealing at 45°C, 1 min extension at 70°C and 30 s denaturation at 94°C. The program was terminated by 5 min extension at 70°C. A PCR product of 121 bp was obtained.

**Fluorescence SSCP**

One microliter of fluorescently labeled PCR product was mixed with 2 µL of deionized formamide loading buffer containing 0.05% dextran blue and heated to 94°C for 3 min. One microliter of this mixture was then loaded onto a precast, non-denaturing polyacrylamide gel (80 µm thickness, 18 cm length; Corbett Research, Sydney, Australia) mounted in a DNA fragment analyzer capable of detecting the HEX fluorochrome (Corbett Research). The sample was pulse-loaded for 20 s at 1200 V before rinsing the wells and running the gel for 90 min at 1200 V. Gel temperature was maintained at a constant 22°C throughout the run by the use of an inbuilt cooling unit. The electrophoretogram was analyzed using ONE-Dscan® 1.3 software (Fairfax, VA, USA). Tumor samples displaying abnormal, shortened bands were reamplified and reanalyzed for confirmation using the same method.

**RESULTS AND DISCUSSION**

In most RER+ tumors, the 26 deoxyadenosines in the BAT-26 poly(A) tract are typically shortened by 4–16 bp in length (6). These mutations are usually clonal, although several smaller bands are occasionally seen. We recently showed that silver-stained SSCP gels only 8 cm in length can readily detect deletions of as little as 3 bp in BAT-26 (7). Together with the observation that screening for BAT-26 deletion is more than 99% accurate for the identification of RER+ status (23), we tested the possibility that F-SSCP could be used as a routine assay for RER+.

Figure 1 shows an example of the screening of colon tumor DNA for deletions in the BAT-26 poly(A) tract using F-SSCP. Tumors with the RER+ phenotype (Figure 1, lanes 3, 7, 11 and 12) are clearly observed to have one or more smaller sized bands in addition to the normal banding pattern. Previous studies in our laboratory using denaturing isotopic gels run on manual sequencing apparatus identified several RER+ tumors with 2 or 3 bp deletions (7). As seen by the migration pattern of one of these samples with a 3 bp deletion (Figure 1, lane 7), the sensitivity of F-SSCP is such that it will detect most shortened BAT-26 alleles described in RER+ tumors (6). DNA derived from formalin-fixed and paraffin-embedded archival tumor material up to 8 years old gave results similar to those obtained from frozen tumor specimens. The incidence

![Figure 1](image-url)

**Figure 1. Identification of the RER+ phenotype in colon cancers by F-SSCP screening for deletions in the BAT-26 mononucleotide repeat.** Additional aberrantly migrating bands (arrows) are clearly visible in lanes 3, 7, 11 and 12, indicating the presence of the RER+ phenotype in these tumors. All other tumor samples contain the normal BAT-26 banding profile.
of RER+ observed using F-SSCP in a series of Dukes’ stage C, proximal colon carcinomas was 19% (35/188). This frequency is almost identical to that obtained previously in our laboratory in a separate tumor series and using several microsatellite markers (8).

The reported frequency of BAT-26 polymorphisms in pure Caucasian populations (0.08%) is sufficiently low (18) for false positives not to be a concern in the routine analysis of MSI. However the relatively high frequency (approximately 8%–12%) of shortened BAT-26 alleles in Africans (17,18) requires that germ-line DNA also be tested in all individuals from populations with significant African genetic admixture to avoid false positives. This can be carried out by simultaneous BAT-26 analysis of DNA obtained from sections of non-tumor tissue blocks prepared routinely during the histological processing of all surgical specimens.

F-SSCP screening of BAT-26 has several advantages that make it attractive for the routine analysis of RER+ status in clinical tumor specimens. First, the use of fluorescent PCR product avoids the need for hazardous radioisotopes or for time-consuming silver stain methods. Second, F-SSCP can be standardized by the use of calibrated and temperature-controlled electrophoresis systems that are considerably less expensive and easier to operate than automated DNA sequencing apparatus. Full standardization can be achieved by the use of disposable, pre-cast, quality-controlled, non-denaturing gels, which also obviates the need to pour gels and to clean glass plates at the beginning and end of each run, respectively. Third, the F-SSCP system we describe has the capacity to screen up to 120 tumor samples per day for RER+ status (4 gel runs of 90 min each, 30 samples per run), providing significant economies of scale for routine analysis. This throughput is threefold higher than the silver stain method and results in significant savings in the time required to pour, polymerize and then stain gels with the latter system.

Since the initial descriptions of F-SSCP (3,11,15), this method has been described for analysis of p53 gene mutations (13,16,19,21), K-ras mutations (10) and clonality (14) in tumors, β-globin mutations in β-thalassemia (20) and for genotyping of viruses (2) and bacteria (22). Our study is the first to use F-SSCP for the routine analysis of RER+ status in clinical tumor specimens. Positive identification of this phenotype can help to direct cancer patients who may belong to the HNPCC syndrome for further genetic testing (1). However, not all tumors from HNPCC cases show deletions in mononucleotide repeats (5). For the identification of suspected HNPCC cases, the
F-SSCP molecular assay should be used in conjunction with clinical information on the family history of cancer and with the distinctive histopathological criteria described recently by Jass et al. (12) for RER+ tumors.

If all results are positive, testing for germline mutations in the DNA repair genes hMLH1 and hMSH2 would appear warranted. Confirmation of our recent observations on the predictive value of RER+ in colon cancer (4) would also demand the availability of a sensitive, reproducible and inexpensive assay system for this genetic alteration. Although the distinctive histopathological features of RER+ tumors have been shown to allow the identification of this phenotype with 93% accuracy (12), we believe that molecular screening of BAT-26 using F-SSCP is the method of choice for routine analysis because of the reasons given above and because it does not rely on subjective assessment of morphological criteria.

REFERENCES

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