DNA Alterations in Human Aberrant Crypt Foci and Colon Cancers by Random Primed Polymerase Chain Reaction

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Abstract

Colon cancers are the result of the accumulation of multiple genetic alterations. To evaluate the role genomic instability plays during tumor development, we compared DNA fingerprints of 44 aberrant crypt foci (ACF; the earliest identified neoplastic lesion in the colon), 23 cancers, and normal crypts generated by random primers with PCR. The PCR products, separated by PAGE and viewed after silver staining, demonstrate altered fingerprints for 23.3% of the ACF and 95.7% of the cancers. In this first study of human ACF with this approach, the finding of altered DNA fingerprints in these microscopic lesions suggests that genomic instability can occur very early in human colon tumorigenesis.

Introduction

ACF\(^3\) (Fig. 1A) are the earliest neoplastic lesions that can be detected microscopically in whole mounts of human colonic mucosa (1, 2). The prevalence of ACF is increased with familial adenomatous polyposis and colorectal cancer (1, 3, 4). Demonstrations of monoclonality (2) and similar genetic alterations (5, 6) in ACF suggest that ACF are precursors of cancer in human colon. Colorectal tumorigenesis is a stepwise process that involves multiple genetic alterations (7). Mismatch repair deficiency gives rise to microsatellite instability that characterizes hereditary nonpolyposis colorectal cancer. Microsatellite instability is also found in about 15% of sporadic colorectal cancers (8) and a similar proportion of ACF (9, 10). However, most colorectal cancers have multiple chromosomal abnormalities and a high frequency of loss of heterozygosity that are thought to be the result of general chromosomal instability or “CIN” (11). The RAPD method, which amplifies random DNA fragments with single primers of arbitrary nucleotide sequence, provides genomic profiles without prior sequence information and has been used to detect and localize allelic alterations in colon cancer (12–14). By comparing RAPD fingerprints of human ACF and colon cancers with those of normal crypts, genomic alterations were found in 22 of 23 colon cancers and in 10 (23.3%) of 43 ACF analyzed.

Materials and Methods

Samples. Human colon specimens were collected in 4°C saline by the Tissue Procurement Core Facility of the Comprehensive Cancer Center of Case Western Reserve University and University Hospitals of Cleveland. Strips of grossly normal mucosa (located between 4 and 28 cm from the cancer; mean, 14 cm) were separated from the submucosa, snap-frozen flat over liquid nitrogen, and stored at −195°C. ACF and two samples of normal crypts were collected under a dissecting microscope as described by Bird et al. (15) from the same specimen (average area evaluated, 11 cm\(^2\)) of colonic mucosa fixed for 30 min in 70% ethanol and stained with 0.2% methylene blue. From the same patient, cancer samples with >50% malignant cells were obtained from frozen sections adjacent to H&E-stained sections of tumor. In the first experiment, ACF, normal crypts, and tumor from 23 different patients were amplified by PCR with three to eight random primers, depending on how much DNA was available. In the second experiment, 21 ACF and matching normal crypts from 16 additional patients were amplified with 10 random primers. Two samples of normal crypts were used for each patient. One sample contained the same number of crypts as were in the ACF; the second sample contained twice as many crypts to see whether DNA concentration altered the fingerprint pattern. All 39 patients had colorectal cancer; 18 had Dukes’ stage B, 16 had stage C, and 5 had stage D cancer. The patients ranged in age from 34 to 98 (67 ± 13 years) old. All the ACF had 52 ± 36 (range, 13–150) aberrant crypts per focus and covered an area of 1.68 ± 1.16 mm\(^2\) (range, 0.39–4.95 mm\(^2\)); 10 (23%) ACF were from the right colon, and 34 (77%) were from the left colon.

Arbitrarily Primed-PCR Amplification. Samples of ACF, normal crypts, and tumors were suspended in 1× PCR buffer (10 mm Tris-HCl, pH 9.0, 2% formamide, 50 mm KCl) that contained 200 μg/ml proteinase K (Fisher Scientific, Pittsburgh, PA) and 0.5% Tween 20. After incubation of the sample at 42°C for 24 h, the proteinase K was inactivated at 95°C for 10 min. The extracted DNA was cooled to 4°C in an ice bath and used for PCR without purification.

PCR-based amplification of random DNA segments with single primers of arbitrary nucleotide sequence (12, 16) was used to detect genetic changes. The primers chosen for our studies were 10–24 nucleotides in length, had a G+C content between 46 and 67%, and contained no palindromic sequences (Table 1). Some of the individual primers were combined, as noted below, to generate additional fingerprints as demonstrated previously (16). PCR was carried out in a volume of 50 μl that contained 0.4 μM primer, 20 to 100 ng genomic DNA, 2 mM MgCl\(_2\), 250 μM each dNTP, and 1 unit of Taq polymerase (Fisher Scientific) in 1× PCR buffer. In the first experiment with samples from 23 patients, two-stringency PCR was performed with primers P2, P3, P4, P5, P6, P1+P2, P2+P6, and P4+P6. PCR amplifications were carried out in a thermal cycler (MJ Research, Inc., Watertown, MA) for 10 cycles of low stringency (95°C for 30 s, 36°C for 40 s, and 72°C for 30 s) followed by 30 cycles of high stringency (95°C for 1 min, 50°C for 1 min, and 72°C for 1 min). In the second experiment with 21 samples of ACF and normal crypts, two-stringency PCR was carried out for the primers (P2, P5, P6, and P1+P2) that gave the clearest RAPD fingerprints in the first experiment. An additional six primers (PGKB, P2+P10A, P2+P10B, P2+FO3, P4+FO3, and P5+FO3) were used to amplify these 21 samples for 40 cycles (95°C for 1 min, 36–40°C for 1 min, and 72°C for 2 min). Six μl of PCR products were mixed with loading buffer and separated in 6% polyacrylamide denaturing gel in a sequencing gel electrophoresis apparatus (Model S2, Life Technologies, Inc., Gaithersburg, MD) with 60 W for 3 h. The gels were viewed after silver staining (17).

Semiquantification of PCR Results. The mean absorbance of each PCR band was evaluated with Kodak 1D Image Analysis Software (Scientific Imaging Systems; Eastman Kodak Co., New Haven, CT). A single band (marked “S” in Figs. 1 and 2), that appeared in all of the lanes with near equal intensities, was chosen as a standard band for each patient. The density of each band in a lane was standardized against this S band by dividing the standardized density of the ACF or tumor band by the standardized density of the same band in the normal sample(s), e.g., TA/Ts/Ns. When two normal samples were evaluated for the same patient,
an average of the two standardized values was used. An allelic ratio of 2 or greater was considered a gain of a band; an allelic ratio of 0.5 or less was considered a loss of a band. This is in the same range as used by us and others to determine allelic loss (2, 18).

Results

Reproducible RAPD fingerprints from multiple patients (Fig. 1) were generated by PCR amplification of genomic DNA with each random primer or random primer pair. Samples 4004233 (Fig. 1C) and 4003641 (Fig. 1D) have similar RAPD fingerprints with multiple bands between 100 and 500 bp when amplified with the primer pair P4+P6. For sample 4004233 (Fig. 1C), there are multiple changes that occur in both the ACF and tumor, and additional alterations (gain of bands at a and b) that occur only in the tumor when the RAPD bands are compared with those from normal crypts. For sample 4003641 (Fig. 1D), there are different alterations in both the ACF and the tumor; i.e., there is a loss of a band at d in the ACF that is not seen in the tumor, and there are two alterations in the tumor (a gain of a band at a and a loss of a band at g) that are not seen in the ACF.

In addition, each random primer or random primer pair generated a unique RAPD fingerprint for each patient (Fig. 2). Amplifications were successful for 43 of 44 ACF samples; 10 (23.3%) of 43 ACF showed a gain and/or loss of RAPD bands compared with the fingerprints of their corresponding normal crypts (Table 2; Figs. 1 and 2). One of the ACF (from patient 4002483) showed RAPD alterations with three primers (Table 2). For three ACF, somewhat similar alterations of RAPD fingerprints were observed both in the ACF and cancer samples, compared with fingerprints of corresponding normal crypts from these same patients (Fig. 1C, discussed above; Fig. 2, A and C). For the ACF in Fig. 2C, there was an additional loss of a band at allele a that was not seen in the tumor. For most ACF, the genomic changes detected in the ACF differed from those seen in the corresponding cancer samples (Fig. 1, C and D and Fig. 2, B, E, and F). In the second experiment, one ACF shows both the gain and loss of DNA bands (Fig. 2D), whereas two ACF (Fig. 2, G and H) show only a loss of DNA bands when their RAPD fingerprints are compared with the fingerprints from normal crypts (tumor samples not done). It is interesting that the sizes of the ACF with altered bands vary widely, from 13 crypts to 109 crypts, i.e., the smallest ACF with 13 crypts is included but not the largest ACF with 150 crypts. In fact, 7 of the 10 ACF with altered bands have less than 52 crypts, the average size of the 44 ACF analyzed. The age, sex, location in the colon, and stage of colon cancer did not influence the occurrence of altered bands in the ACF that were analyzed.

As illustrated (Figs. 1 and 2), cancer samples generally showed more altered bands per RAPD fingerprint than did the ACF with the same primer. All 23 cancer samples were successfully amplified, and 22 (95.7%) of 23 cancers showed altered fingerprints when compared with the fingerprints of their corresponding normal crypts. The cancer samples also had more altered RAPD fingerprints per primer than did the ACF (Fig. 3); 17 tumors exhibited genomic alterations with two or more random primers. The total number of altered RAPD fingerprints per successful amplification was low for ACF (12 of 309 or 3.9%) compared with cancer samples (77 of 134 or 57.5%; P < 0.01). The main genetic alterations are gain and/or loss of RAPD bands that are observed both in ACF and cancer samples compared with the fingerprints of normal crypts. In addition to these quantitative alterations, we also observed qualitative changes in the relative intensity of DNA bands between those from ACF or cancer samples and those from normal crypts (Fig. 1D, allele d in tumor).

Discussion

To our knowledge, this is the first report of altered DNA fingerprints in human ACF, the earliest identified neoplastic lesions in the colon (2). Genome-wide alterations identified with RAPD in 23.3% of

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Fig. 1. DNA fingerprints from two different patients (4004233 shown in C and 4003641 shown in D) amplified with the same random primer (P4+P6). A, an ACF with 48 crypts from 4004233 that was used for the DNA fingerprint in C (Lane A). B, frozen section of cancer stained with H&E from 4004233 that was used for the DNA fingerprint in C (Lane T). C and D, DNA fingerprints of normal crypts (Lane N), ACF (Lane A), and cancers (Lane T) from patients 4004233 and 4003641, respectively. Arrows show the altered DNA bands, i.e., ACF and/or tumor bands with allelic ratios of 0.5 or less or with allelic ratios of 2.0 or greater; numbers at left, length in bp. In C (patient 4004233), losses of DNA bands for alleles c, d, e, f, and g (allelic ratios of 0.2–0.5) are observed in both ACF and tumor; gains of DNA bands for alleles a and b (allelic ratios 2.4 and 3.0, respectively) are seen only in tumor. In D (patient 4003641), there is a loss of allele d (allelic ratio, 0.3) only in the ACF, whereas the tumor shows a gain of a band at a (allelic ratio, 2.6) and a loss of a band at g (allelic ratio, 0.5).
ACF suggest that chromosomal instability is a very early event and might play a crucial role during the development of some colorectal cancers. There are previous reports of chromosomal instability as early as the polyp stage (19, 20), and some have suggested that genetic instability is required for the development of tumors (discussed in Refs. 20 and 21). Although RAPD was not used to identify genomic alterations in those polyp studies, Peinado et al. (13) found that the ACF and tumor bands with allelic ratios of 0.5 or less or with allelic ratios of 2.0 or greater. Similar gain of a DNA band is seen in both ACF and cancer (allelic ratios 2.6 and 2.3, respectively) from the same patient in column A. In column B, only the tumor shows a gain of a DNA band at a (allelic ratios 2.4 for Lane T and 1.5 for Lane A) and only the ACF shows a gain of a DNA band at b (allelic ratios 2.0 for Lane A and 1.6 for Lane T). Both the ACF and tumor show the gain of multiple bands in C, but only the ACF shows the loss of a band at a (allelic ratio 0.4). The ACF in D shows the gain of DNA bands at alleles a, b, c, d (allelic ratios, 2.1 and 2.9) and the loss of bands at alleles c and d (allelic ratios, 0.5). In E, the gain of DNA bands are seen in alleles a, b, c, d, f, g, i, and j (allelic ratios, 2.1–3.6) in the cancer, whereas the loss of DNA bands in alleles e and h (allelic ratios, 0.5) are seen in the ACF. In F, the loss of a DNA band at allele a (allelic ratio, 0.3) is seen in the ACF with the gain of bands at alleles b, c, and d (allelic ratios, 3.1–8) in the cancer. Multiple losses of DNA bands in ACF were observed in G (allelic ratios, 0.2–0.4) and H (allelic ratios, 0.3–0.5).

The advantages of RAPD for our studies are that it requires only small amounts of DNA to generate genome-wide fingerprints that can show multiple alterations and it does not require prior knowledge of DNA sequences. Two-stringency PCR was used in our first study as used previously in the study of human colon tumors (13). The initial, low-stringency condition allows a large number of hybridizations to take place throughout the genome; the second, high-stringency condition allows only the segments that closely match the primer to be amplified further. In addition we added primers in pair-wise combinations, which produce distinct genomic fingerprints different from those generated with either single primer alone (16). Whereas these conditions produced fingerprints that demonstrate multiple differences between normal mucosa and 95.7% of our cancer samples, they did not reveal as high a proportion (7 of 22, or 32%) of altered human ACF as had been reported in the rat study (14). In hopes of improving our results in the second experiment with 21 additional ACF, we used only four primers from our first experiment and added six new primers with a single stringency (12). This time only 3 of 21 or 14.3% of the ACF showed alterations. From these very limited experiments, it appears that the two-stringency PCR with the original primers, especially P4+P6 and P5, was more effective with our very small sample sizes.
samples of human DNA. Consequently, our results likely underestimate the amount of chromosomal instability in human ACF.

The wide range of sizes of ACF, from 13 to 109 crypts, with altered fingerprints, also argues that chromosomal instability occurs very early in colon tumorigenesis. Some ACF (Fig. 2, A, C, E, and B, C, E, and F) have gains of DNA bands that are similar to those seen in the tumor samples from the same patient. These results suggest that these alterations observed in the tumors occurred early in the ACF and persisted in the final cancer. One ACF and tumor (Fig. 1C) show multiple similar losses of DNA bands, but the tumor shows additional alterations. This supports the hypothesis that ACF are early precursors that gain additional alterations to become cancer. However, several ACF (Figs. 1D and 2, B, C, E, and F) show losses or gains of DNA bands that are not seen in the cancers from the same patients. One possible explanation is that these changes observed in the ACF do not contribute to tumorigenesis, i.e., these ACF are not likely to persist. A second equally plausible explanation is that each tumor develops independently along its own unique pathway, and not every change observed in each ACF will be observed in all tumors.

In summary, the observations of altered fingerprints in microscopic lesions known as ACF suggest that chromosomal instability can occur very early in colon tumorigenesis and may be a driving factor of this process. Future studies of larger numbers of ACF and cancers with RAPD might aid in finding the earliest molecular changes that occur in colon tumorigenesis.

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References

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