EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 29, No 3, 165-170, September 1997

Mutations of DNA Repair Associated Gene, APEX in Human Colorectal Cancer

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Accepted 30 July 1997

Abbreviations: AP, apurinic or apyrimidinic; APE, AP DNA endonuclease

Abstract

Structural and functional changes in the major apurinic/apyrimidinic DNA endonuclease (APEX) gene in human colorectal cancers were investigated. DNAs were prepared from surgically removed 25 human colorectal tissues and direct sequencing of PCR-amplified APEX gene covering the entire protein coding region was performed. Point mutations in 3 and silent mutations in 3 out of 25 colorectal cancer patients were found. Base substitutions in intron II were also found in 2 patients. T ↔ C or some A ↔ G transitions were the most typical pattern of the mutations. AP DNA endonuclease (APE) activities in normal and tumor tissues were 65.7 EU/mg and 21.7 EU/mg, respectively. APEX protein was detected in both normal and tumor tissues and no remarkable difference in the amount of APEX protein between colorectal cancer tissues and their normal counterparts was observed. The incidence of APEX gene mutation in colorectal cancer was 12% which is relatively lower than that of other genes associated with colorectal tumor, but a significant reduction of APE enzyme activities in tumor tissues, especially in those with APEX mutations, was observed. These results indicate that the decreased APE enzyme activity might be closely related to the colorectal tumorigenesis, although no quantitative correlation between APE enzyme activity and APEX content exists.

Keywords: mutations, *APEX* gene, APE activity, colorectal cancer.

Introduction

Cells have evolved a number of mechanisms to ensure the high fidelity transmission of genetic material from one generation to the next generation since muations will lead to genotypes that may be deliterious to the cell. Lesions of DNA can lead to mutations and multiple enzymatic pathways for the repair of these lesions are known (Friedberg, 1990). Recent advances in understanding the genetic basis of malignant diseases have been achieved by researches in colorectal cancer. The cloning and identification of a number of genes involved in colorectal cancer development and susceptibility have been accomplished. *APC*, *hMSH2*, *hMLH1*, *p53* and *ras* genes have been considered to be important involved in colorectal cancer. Mutations in *hMSH2*, *hMLH1* and human homologue of *E. coli mut HL* which are responsible to mismatch repair of DNA are known to be associated with hereditary non polyposis colon cancer (Fishel *et al*., 1993; Han *et al*., 1995). Apurinic or apyrimidinic (AP) lesions in DNA may be generated from spontaneous depurination (Lihdahl and Nyberg, 1972) or from the cleavage of glycosidic bond between various modified bases and deoxyribose unit of DNA (Lindahl, 1979).

AP DNA endonuclease (EC 3. 1. 25. 2; APE) recognizing AP site of DNA and nicking the DNA strand near the AP site, has been shown to be present in most cells, prokaryotes and eukaryotes (Brent, 1976; Thibodeau and Verly, 1980). Five classes of APE in *E. coli* have been reported (Friedberg, 1985) and more than two kinds of the enzyme were shown to be present in human or animal tissues (Ljungquist, 1977; Mosbaugh and Lin, 1980; Kim and Oh, 1990). The major human AP DNA endonuclease (APEX) which has APE, 3'-5'-exonuclease, DNA 3'-diesterase and DNA 3'-phosphatase activities has been purified from human leukocytes and its cDNA has been cloned (Robson *et al*., 1991; Seki *et al*., 1991). Cloning and sequence analysis of genomic DNA of APEX has been reported (Harrison *et al*., 1992; Akiyama *et al*., 1994). Since little is known about APE in human colorectal cancer, we have analyzed the sequence of *APEX* gene in human colorectal cancer tissues in order to find any possible relationship between *APEX* gene and colorectal cancer.

Materials and Methods

Preparation of DNA

High molecular weight chromosomal DNA was prepared from 25 colorectal cancer tissues and some of their non-

cancerous counterparts. Surgically removed fresh colorectal tissues (0.5 g) were homogenized with TES buffer (10 mM Tris, 25 mM EDTA, 0.5 mM NaCl, pH 7.4) by OMNI tissue homogenizer (OMNI internationals, Waterbury, CT, USA), and nuclear fractions were sedimented by centrifugation (600 *g*, 10 min). Proteins in nuclear fraction were digested with proteinase K (1 mg/ml) dissolved in 1% SDS and DNA was isolated by phenol-chloroform extraction according to the method of Davis *et al*. (1994). The purified DNA was dissolved in TE buffer (10 mM Tris-Cl, pH 7.4, 0.1 mM EDTA) and was used as template DNA for PCR amplification of *APEX*.

AP DNA (substrate for APE enzyme assay) was prepared from ^{3}H-labeled *E. coli* chromosomal DNA by the method described by Kim and Oh (1993). The number average size (molecula weight, M_r) of AP DNA prepared in this preparation was 4.6 kb (3.036 x 10^6 Da) and contained 12 AP sites per molecule of DNA when calculated from equation of Zubroff and Sarma (1976); number of AP site/molecule=[M_r of control AP DNA/M_r of AP site excised AP DNA]-1; where Mn=ΣWi (ΣWi/Mi); Wi=Weight of DNA in each fraction and Mi=Molecular weight of DNA in each fraction migrated upon agarose gel electrophoresis.

Amplification and sequencing of APEX gene

For the amplification of entire protein coding regions of *APEX* oligodeoxyribonucleotide primers (Figure 1) were designed and synthesized by ordering to Bioneer Co., Seoul, Korea. DNA segments corresponding to exon II through exon V of *APEX* were amplified by PCR according to the method of Saiki (1988) using each primer set and template DNA, and the amplified DNAs were purified by agarose gel (1%) electrophoresis followed by the isolation of them using Gene Clean II kit (BIO 101, La Jolla, CA, USA). DNA sequencing reaction was performed by direct thermal cyclic sequencing method using amplicycle sequencing kit (Perkin Elmer, Branchburg, NJ, USA). The sequencing reaction products were subjected to SDS polyacrylamide sequencing gel (8%) electrophoresis, and autoradiography of the dried gel was carried out to identify the nucleotide sequence of *APEX* gene.

Enzyme assay

APE enzyme activity in colorectal cancer tissues and their counterparts were determined as described by Thibodeau *et al*. (1980). Fresh tissues dissolved in TES buffer (1 g/ml) were homogenized with glass homogenizer and nuclear fraction was sedimented by centrifugation (600 *g*, 10 min). APE in the nuclear fraction was extracted with 1 M KCl in TES buffer for 2 hours at 4˚C. The extracted APE was centrifuged at 12,000 *g* for 10 min, and the supernatant was dialyzed against TES buffer overnight at 4˚C. Protein contents in the dialyzed samples were determined by Bradford method (Bradford, 1976) and they were used for enzyme assay. Enzyme assay was performed according to the method described by Kim and Oh (1993). In brief, the reaction mixture of 100 μl of 0.1 M Tris-HCl buffer (pH 8.0) containing [^{3}H]AP DNA and 100 μl of enzyme solution was incubated for 15 min at 37˚C. The reaction was terminated by addition of 100 μl of ice-cold 2 mg/ml bovine serum albumin solution followed by addition of 60 μl of 30% perchloric acid. The mixture was stand for 15 min at 0˚C and centrifuged for 15 min at 12,000 *g*. The supernatant (100 μl) was removed carefully and radioactivity in it was counted by liquid scintillation counter (Packard, Downers Grove, IL, USA). The radioactivity in the supernatant after alkaline treatment of [^{3}H]AP DNA was assumed to be 100% AP site excised, and the relative radioactivity in the supernatant obtained from the enzyme reaction was used for the calculation of enzyme activity. Enzyme unit was defined as pmol of substrate (AP site) excised per min.

Western blot immunodetection

APEX protein expressed in the colorectal tissues was detected by Western blot immunodetection by the method described by Burnette (1981). A portion of APE protein prepared for the enzyme assay was subjected to SDS-polyacrylamide gel (12%) according to the method of Laemmli (1970), and proteins in the gel were electrotransfered onto the nitrocellulose membrane. APEX protein on the membrane was immunodetected using polyclonal anti-APEX rabbit antiserum (kindly supplied by Dr. S. Mitra, University of Texas, Galveston, TX, USA) as primary antibody and peroxidase-conjugated anti-rabbit IgG antibody as secondary antibody. Peroxidase positive

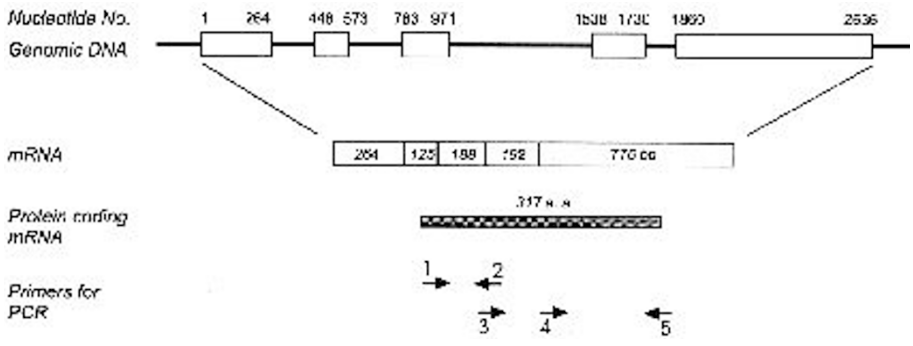

Figure 1. Schematic representation of *APEX* gene and the design of primers for the PCR-amplification of exon sequences. The corresponding primer sequences are as follows. 1, Sense (exon II), 5'-CGTTCGTAACGGGAATG-3'; 2, antisense (exon III), 5'-TCAAATTC CACTC-3'; 3, sense (exon IV), 5'-TACA GTGGGTAAAGGAAGAA-3'; 4, sense (exon V), 5'-GTG GATCCGAGGAGGAG CATGAT-3'; 5, antisense (exon V), 5'-GACTCGAGTCACAGTGCTAGGT-3'.

signals were detected by ECL Western blotting protocol (Amersham, Buckinghamshire, England).

Results

Mutations of APEX gene in colorectal cancer tissues

Point mutations in the exon of *APEX* gene were found in 3 out of 25 colorectal cancer patients (Table 1, Figure 2). Leucine (CTT) at codon 108 of *APEX* gene was mutated to arginine (CGT) in one patient (No. 1), and serine (TCG) at codon 164 of *APEX* gene was also mutated to proline (CCG) in another patient (No. 3). Point mutations at 2 codons, GGC (No. 41, glycine) to AGC (serine) and CCT (No. 59, proline) to TCT (serine) were found in one patients (No. 11). Silent mutations at codon 293 (CCT→CCC), at 58 (AAT→AAG) and at 206 (GTG→GTA) in three patients were found (Table 1, Figure 3). Base substitutions at nucleotide +587 and at +680 in intron II of *APEX* gene were found in two patients (Table 1, Figure 3).

APE enzyme activities in colorectal cancer tissues

APE enzyme activities in colorectal tumor tissues were significantly lower than that of the normal counterparts (Table 2, Figure 4). The mean specific activities of APE in normal and tumor tissues were 65.7 ± 26.7 and 21.7 ± 12.8 EU/mg protein, respectively. APE enzyme activities in tumor tissues which had point mutations in *APEX* gene (17.7, 10.3, 6.3 EU/mg protein) were far lower than the average APE enzyme activity in all tumor tissues (Figure 4).

APEX proteins in colorectal cancer tissues

APEX protein was detected in colorectal cancer tissues as well as in their normal counterparts, and there was no remarkable difference in the intensity of APEX between tumor and normal tissues (Figure 5). APEX protein was also detected in the colorectal tumor tissues that have point mutations in APEX gene with a similar intensity.

Discussion

In recent years the cloning and identification of a number

Table 1. Mutations of the major human AP DNA endonuclease (APEX) gene in colorectal cancer patients

Patient	Codon	Point mutation	Codon	Silent Mutation	Intron II
1	108	CTT (Leu)→CGT (Arg)	293	CCT (Pro)→CCC (Pro)	
3	164	TCG (Ser)→CCG (Pro)			
5			58	AAT (Lys)→AAG (Lys)	A→G[a]
6					T→C[b]
8			206	GTG (Val)→GTA (Val)	
11	41	GGC (Gly)→AGC (Ser)			
	59	CCT (Pro)→TCT (Ser)			

[a] Nucleotide No. +587

[b] Nucleotide No. +680

Nucleotide No. +1 represents the first nucleotide at the transcription initiation site.

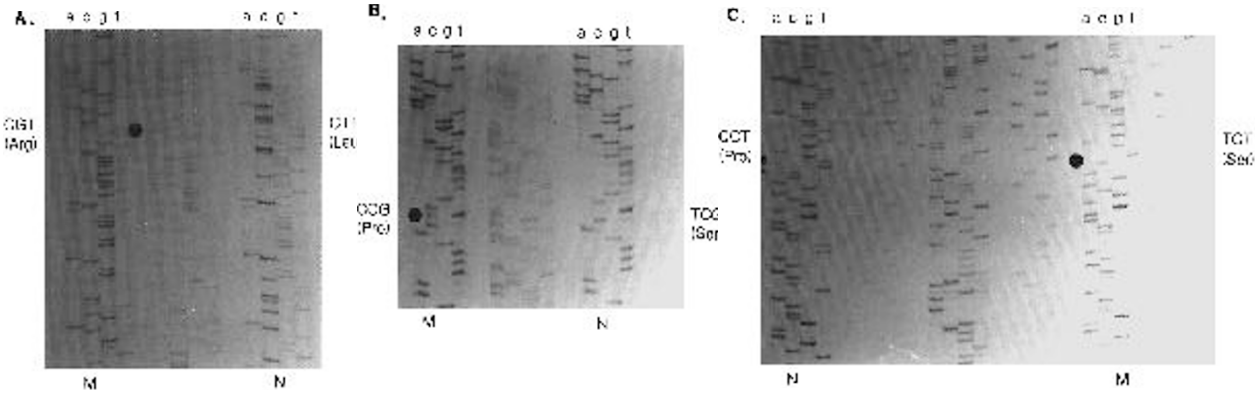

Figure 2. Point mutations in *APEX* gene in colorectal cancer patients. **A** (patient No. 1): Point mutation at codon 108 (T→G, ●); **B** (patient No.3), Point mutation at codon 164 (T→C, ●); **C** (patient No. 11), Point mutation at codon 59 (C→T, ●); N (normal) and M (mutant) are indicated.

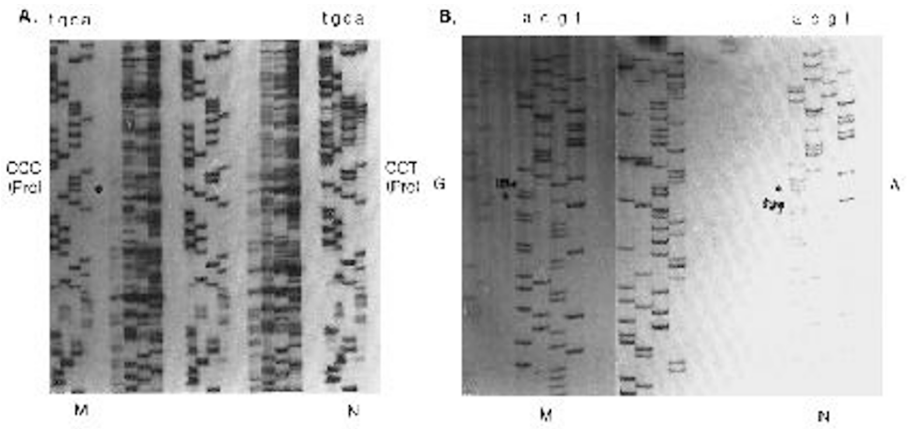

Figure 3. Mutations without amino acid changes in *APEX* gene in colorectal cancer patients. **A**, Silent mutation (patient No. 1) at codon 293 (T→C, ●) is indicated; **B**, A base substitution (patient No. 5) at nucleotide +587 (A→G, ●) is indicated. N (normal) and M (mutant) are indicated.

Table 2. AP DNA endonuclease activities in human colorectal tumor tissues. AP DNA endonuclease was prepared from surgically removed colorectal tissues by 1 M KCl extraction followed by dialysis overnight against a buffer (10 mM Tris-HCl, pH 7.0) according to the method described in the text. EU is defined as the amount of AP site (pmol) hydrolyzed for 15 min.

Group	No. subjects	APE activity (EU/mg)
Normal tissue	4	65.7 ± 26.7
Tumor tissue	25	21.7 ± 12.8

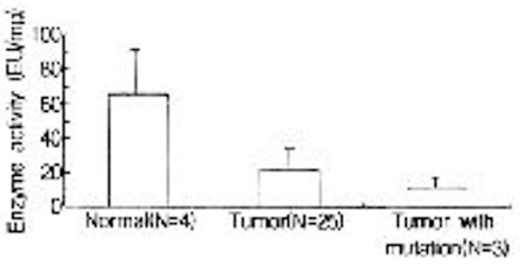

Figure 4. AP DNA endonuclease activities in human colorectal cancer tissues and their normal counterparts. N represents the number of patients and bars indicate standard deviations.

of genes involved in colorectal cancer development, and mutations in *adenomatous polyposis coli* (*APC*) gene, human homologue to the bacterial *Mut LHS* mismatch-repair gene, and human homologue to yeast genes involved in mismatch repair, *PMS1*, *MLH1* or *MSH2* have been reported (Miyoshi *et al*., 1992; Powell *et al*., 1992; Fishel *et al*., 1993; Cunningham and Dunlop, 1994).

An increased sensitivity of APE to genotoxic compounds such as H_2O_2 or alkylating agents in rat glioma cells has been suggested (Ono *et al*., 1994), but no evidences that the APE gene is related to colorectal cancer have been documented yet.

In the present study, the incidence of point mutation in *APEX* was about 12% (3 out of 25) which is somehow very low as compared to the incidence of point mutations in *K-ras* (40-60 %) and *p53* (75-80 %) genes in colorectal cancer (Wiggers *et al*., 1988; Ponz *et al*., 1992; Levin *et al*., 1991). This result indicates that mutations in *APEX* gene may not have a significant role in colorectal carcinogenesis. However the reduced APE enzyme activity in colorectal cancer tissue of *APEX* mutant implicates that mutation of *APEX* gene may cause the defect in AP DNA repair function which may eventually lead to instability of many genes including oncogenes and antioncogenes.

APE enzyme activity in colorectal cancer tissues was significantly lower than that of their normal counterparts in this study, indicating that the enzyme activity is essential

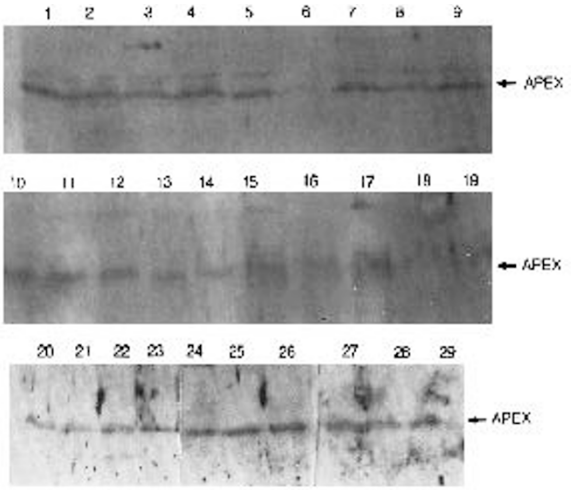

Figure 5. Western blot immunodetection of APEX protein in human colorectal tumor tissues. Tissues (300 mg each) were homogenized in 300 μl of solubilizing solution (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl, pH 7.5) with Polytron tissue homogenizer and centrifuged for 10 min at 12,000 rpm. Portions of supernatants were subjected to SDS polyacrylamide gel electrophoresis. Proteins were electrotransfered onto nitrocellulose membrane and immunodetected using anti-APEX antiserum according to ECL detection protocol. Lane 1-4, normal counterpart of colorectal tumor tissues. Lane 5-29, Tumor tissues.

for the maintenance of normal cellular functions which may be generated from healthy genes. APE enzyme activity measured in the present study represents the sum of all types of APE enzyme and the proportions of *APEX* gene product in the colorectal tissues is not known. So other types of APE gene products in addition to APEX should be encountered in the evaluation of the role of APE in colorectal cancer generation. Mutations in other types of *APE* gene have not been analyzed in the present study and the incidence of mutations in oncogenes or antioncogenes in the mutants of *APEX* have not been determined.

In a colorectal tumorigenesis model by Vogelstein (Vogelstein, 1988; Fearon and Vogelstein, 1990), multiple genes were thought to be involved in various steps of tumorigenesis; mutations in *APC* or *MCC* in the early step, *K-ras* at the intermediate step and *p53* or *DCC* genes in the late step. Therefore the study on the change of APE activity in various steps of tumorigenesis will help the understanding how the reduced APE activity affect on the colorectal tumorigenesis.

A similar quantities of APEX protein expressed in the colorectal cancer tissues and their normal counterparts in spite of a significant difference in the APE enzyme activity between them indicate that APEX protein might not contribute much to the total APE activity present. But the reduced APE enzyme activity in the mutants of *APEX* gene implicates that biological activity of the mutant *APEX* gene products is extremely low.

Acknowledgements

This study was supported by Research Fund (1995-1996) of Yonsei Cancer Research Institute, Yonsei University-College of Medicine. We thank to Dr. Jin-Sup Choi for his kind supply of tissue samples.

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