in vivo *37*: 2070-2077 (2023)
doi:10.21873/invivo.13304

Expression of Apurinic/Apyrimidinic Endonuclease 1 in Colorectal Cancer and its Relation to Tumor Progression and Prognosis

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Abstract. *Background/Aim: Over-expression of apurinic/apyrimidinic endonuclease 1 (APE1) has been demonstrated to be associated with cancer progression, chemo- and radio-resistance in various cancers. This study examined the expression of APE1 and its relation to tumor progression and prognosis in patients with colorectal cancer (CRC). Materials and Methods: We investigated 193 patients with CRC who received curative surgery for whom formalin-fixed and paraffin-embedded blocks were available, and long-term tumor-specific survival rate analysis was possible. The expression of APE1 was investigated by reverse transcription-polymerase chain reaction, western blotting, and immunohistochemistry in CRC and lymph node tissues. The apoptosis, proliferation, and angiogenesis of CRC cells were determined using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay, and immunohistochemical staining for Ki-67 and CD34 antibodies. Results: APE1 was over-expressed in CRC and metastatic lymph node tissues compared with normal colorectal mucosa and non-metastatic lymph node tissues. Over-expression of APE1 was significantly associated with advanced stage, lymphovascular invasion, perineural invasion, deeper tumor invasion, lymph node metastasis, distant metastasis, and poor survival. Multivariate analysis demonstrated that APE1, perineural invasion, and lymph node metastasis were the independent prognostic factors associated with overall survival. The mean Ki-67 labeling index value of APE1-positive tumors was significantly higher than that of APE1-negative tumors. However, there was no significant association between APE1 expression and the apoptotic index or microvessel density. Conclusion: Over-expression of APE1 is significantly associated with tumor progression and poor survival in patients with CRC. Therefore, APE1 may be a novel biomarker and present a potential prognostic factor for CRC.*

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Key Words: Apurinic/apyrimidinic endonuclease 1, colon neoplasm, prognosis.

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Colorectal cancer (CRC) is one of the most common cancers and the leading causes of cancer-related morbidity and mortality worldwide. Despite the advances of diagnostic and therapeutic modalities, advanced CRC is highly aggressive and associated with poor prognosis (1-3). Therefore, it would be beneficial to identify reliable diagnostic, predictive, and prognostic molecular biomarkers to help the diagnostic and therapeutic processes for CRCs.

Apurinic/apyrimidinic endonuclease 1 (APE1) is a key functional protein involved in the base excision repair pathway that also acts as a redox-dependent modulator, stimulating many transcriptional factors including signal transducer and activator of transcription 3, nuclear factor-kappa B, Fos, activator protein 1, Jun, hypoxia-inducible factor, paired box gene 8, and p53 to maintain cell homeostasis (4-6). This multifunctional APE1 is involved in the pathogenesis of various diseases, including retinal ocular diseases, neurological disorders, inflammatory bowel disease, and cancer (7-9).

APE1 is over-expressed in a variety of cancers and its over-expression correlates with advanced stage and poor prognosis in breast, prostate, lung, liver, and stomach cancers (10-14). Also, over-expression of APE1 has been associated with the resistance against chemotherapeutic agents and

radiotherapy in lung cancer, ovarian cancer, osteosarcoma, pediatric ependymoma, medulloblastoma, and glioblastoma (15-20). Furthermore, inhibition of APE1 impeded tumor progression by inducing tumor cell apoptosis, cell cycle arrest, suppressing epithelial to mesenchymal transition (EMT), and inhibiting angiogenesis in various cancer cells and tumor mouse models (21-24). APE1 was associated with tumor cell growth, radiosensitization, cytotoxicity of chemotherapeutic agents, and metastasis in human CRC cell lines and carcinogen-induced CRC mouse model (25-29). Therefore, APE1 may be a novel biomarker that can serve as a therapeutic target in cancers.

However, until now, there is only few data about the impact of APE1 on tumor progression and prognosis of CRC.

The aim of the current study was to examine the expression and prognostic significance of APE1 in human CRCs, including complete long-term follow-up, with special reference to patient survival.

Materials and Methods

Collection of clinical samples. Ten fresh CRC and paired normal colon tissues were collected by colonoscopic biopsy for RNA and protein preparation at Chonnam National University Hwasun Hospital (Jeonnam, Republic of Korea). Tissues were stored in liquid nitrogen until RNA and protein were isolated. Paraffin-embedded tissues (n=193) of CRC used for immunohistochemistry were surgically collected from January 2013 to December 2013 at Chonnam National University Hwasun Hospital. Patients who received radiation therapy or chemotherapy before surgery were excluded. Pathological reports and clinical information at the time of surgery were collected from medical records. Tumors were staged according to the American Joint Committee on Cancer (AJCC) staging system (30). Survival rates were calculated and analyzed from the time of surgery to the follow-up on December 31, 2021. The study was performed with the permission of the independent local ethics committee of the Institutional Review Board of Chonnam National University Hwasun Hospital (IRB No. CNUHH-2023-067). A written informed consent was obtained from each participant prior to tissue acquisition. The participants consented to the collection of histological and clinical data prior to surgery.

Immunohistochemistry. CRC tissues were fixed in 10% formalin for 24 hours, dehydrated in an ethanol series (sequentially from 60% to 100%) and cleared in xylene (100%). We subsequently incubated the tissues in 60˚C paraffin solution and embedded them in paraffin. Paraffin-embedded tissues were cut into sections (4 μm thick) and mounted on slides. Tissue sections were deparaffinized in 100% xylene and rehydrated in a series of ethanol solutions (sequentially from 100% to 70%). For antigen retrieval, tissues were boiled for 10 min in a cooker using retrieval citrate buffer (pH 6.0, Dako, Agilent Technologies, Inc., Carpinteria, CA, USA) and allowed to cool to room temperature. Tissues were treated with Dako REAL peroxidase-blocking solution (Dako, Agilent Technologies, Inc.) at 37˚C for 10 min to inactivate endogenous peroxidase and block non-specific antigens with Dako® Protein Block Serum-Free solution (Dako, Agilent Technologies, Inc.). Sections were incubated with rabbit anti-human APE1 polyclonal antibody, mouse antihuman Ki-67 monoclonal antibody, or mouse anti-human CD34 monoclonal antibody (1:100, Abcam, Cambridge, UK) in at 4˚C overnight. Subsequently, staining of tissues was performed using the Dako REAL Envision HRP/DAB Detection System (Dako, Agilent Technologies, Inc.). Briefly, tissue slides were washed 4 times in TBS-T and then incubated in Envision HRP conjugated secondary antibody (anti-rabbit/mouse) solution for 1 h at room temperature. After washing, tissues were developed with enzyme substrate 3,3-diaminobenzidine (DAB) solution for 10 min at room temperature. Counterstaining was performed with hematoxylin (Sigma, St. Louis, MO, USA) for 5 s.

Determination of APE1 expression. The expression of APE1 was assessed using semiquantitative scoring system of immunohistochemical staining. The final score was calculated by multiplying the score of staining intensity by the distribution of positive cells. Staining intensity was scored as 0, negative; 1, weak; 2, moderate; 3, strong. Distribution of positive cells was graded as 1, 0-25%; 2, 26-50%; 3, 51-75%; 4, 76-100%. The mean of the final score was 6, samples with a final score of less than 6 were considered negative for APE1 expression and 7-12 were considered positive. Assessment of staining was performed by two independent observers without knowledge of clinical outcome data such as tumor stage and survival.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from frozen tumor tissue of patients with CRC using the Trizol reagent (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The concentration of total RNA was quantified using NanoDropTM2000/2000c Spectrophotometers (Thermo Fisher Scientific, Inc.). Total RNA (500 ng) was reverse-transcribed to cDNA using Oligo-dT and MMLV transcription reagents (Promega, Madison, WI, USA). PCR amplification of specific genes in cDNA was performed using gene-specific primers and Go Taq® DNA polymerase (Promega). The following specific primers were used; APE1 forward 5'-GCAGCAGGAGAGGGCCCAGC-3'/reverse 5'-GGTGTGTTGGG GTAGAGGTGCC-3'; GAPDH forward 5'-ACCACAGTCCATG CCATCAC-3'/reverse 5'-TCCACCACCCTGTTGCTGTA-3'. The synthesized PCR products were separated on a 1% agarose gel and visualized using HiQ BlueMango stain solution (bioD, Gyeoggi, Republic of Korea). The PCR bands were quantified using the Multi Gauge V3.2 software (Fujifilm, Tokyo, Japan).

Protein isolation and western blotting. CRC tissue was homogenized by adding M-PER® mammalian protein extraction reagent (Thermo, Rockford, IL, USA) with Halt™ Phosphatase inhibitor and Protease inhibitor cocktail (Thermo) and centrifuged. The separated supernatant (total protein) was quantified using a BCA™ protein assay (Thermo Fisher Scientific, Inc.). Proteins (20 μg) were electrophoresed using 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane (PVDF, Millipore, Billerica, MA, USA). Membranes were treated with 5% BSA solution to block non-specific binding and incubated with the specific antibody at 1:1,000 dilution. The following antibodies were used; Antibodies against APE1 (Abcam) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoblotted proteins were revealed using the enhanced chemiluminescence detection system HRP substrate (Millipore) and the luminescent image analyzer LAS-4000

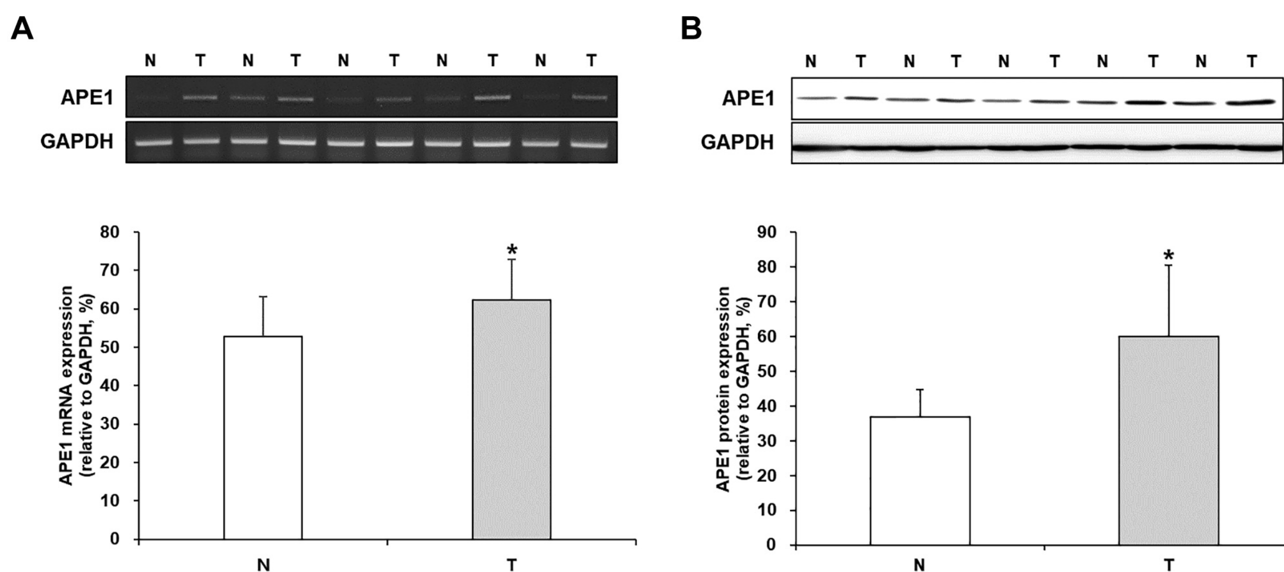

Figure 1. *Apurinic/apyrimidinic endonuclease 1 (APE1) expression in colorectal cancer and normal colorectal tissues using reverse transcription-polymerase chain reaction and western blotting. APE1 is significantly overexpressed in cancer tissues compared to paired normal tissue at the mRNA (A) and protein levels (B) in colonoscopic biopsy specimens. Each bar represents the mean±SD of 20 cases. *p<0.05 vs. normal colorectal mucosa tissue. SD: Standard deviation; N: normal colorectal mucosa tissue; T: colorectal cancer tissue.*

(Fujifilm). The protein bands were quantified using the Multi Gauge V3.2 software (Fujifilm).

Assessment of tumor cell apoptosis. Apoptotic tumor cells were detected using the DeadEnd™ Colorimetric terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) System (Promega). Briefly, the tissue sections were deparaffinized and rehydrated through xylene and a graded alcohol series. The tissue sections were incubated for 15 min in proteinase K solution to increase permeability and the enzyme terminal deoxynucleotide transferase (TdT) reaction and biotinylated nucleotide mix were added for 60 min in a 37˚C humidified chamber. End-labeling cells were developed using the streptavidin horseradish peroxidase (HRP) and the enzyme substrate 3,3-diaminobenzidine (DAB). Counterstaining was performed with hematoxylin for 5 s. The whole tissue was observed in a high magnification field (magnification 40×10) and the highest labeled area was photographed. The apoptotic indices (AI) are presented as number of positive nuclei containing apoptotic body among 1,000 tumor cell nuclei.

Assessment of tumor cell proliferation. Proliferative tumor cells were identified by immunohistochemical staining using an anti-Ki-67 antibody. Ki-67-positive cells showed distinct immunoreactive staining of cell nuclei. The Ki-67 labeling index (KI) was calculated as the number of Ki-67-positive nuclei per 1,000 tumor cell nuclei.

Determination of microvessel density (MVD). Quantification of microvessel (MVD) was performed using immunohistochemical staining with an anti-CD34 antibody. Brown-stained cells or clusters of endothelial cells clearly separated from adjacent tumor cells, and other connective tissue were considered as vessels. The immunostained tissue was screened at low magnification to identify three areas with the highest density of vessels (hot spots). Then, hot spots for each case were photographed at a higher magnification (×400). Vessels were counted in the five areas of hot spots at 400× magnification and expressed as the mean number of vessels in these areas.

Statistical analysis. Data analysis was performed using IBM Statistical Package for the Social Sciences (SPSS/PC+ 20.0, IBM, Armonk, NY, USA). The association between APE1 expression and clinicopathological parameters of colorectal cancer was assessed using Partitioning Chi-square and Fisher's exact tests. Overall survival (OS) was plotted using the Kaplan-Meier method, and statistical differences between groups were analyzed using the log-rank test. The prognostic significance of APE1 expression was determined by multivariate analysis using a Cox regression model. Group comparisons were performed using *t*-test. *p*-Values <0.05 were considered statistically significant.

Results

APE1 is over-expressed in CRC and metastatic lymph node tissues compared to normal colorectal mucosa and non-metastatic lymph node. We measured the expression of APE1 at mRNA and protein levels using RT-PCR, western blotting, and immunohistochemistry in CRC in paired normal colorectal mucosa, metastatic or non-metastatic lymph node tissues of same patients taken by colonoscopic biopsy and surgery. In colonoscopic biopsy specimens, APE1 was over-expressed in CRC tissues compared to paired normal colorectal mucosa at mRNA and protein levels (Figure 1A and B). Densitometric analyses showed that the mRNA and protein expression of APE1 were significantly higher in CRC

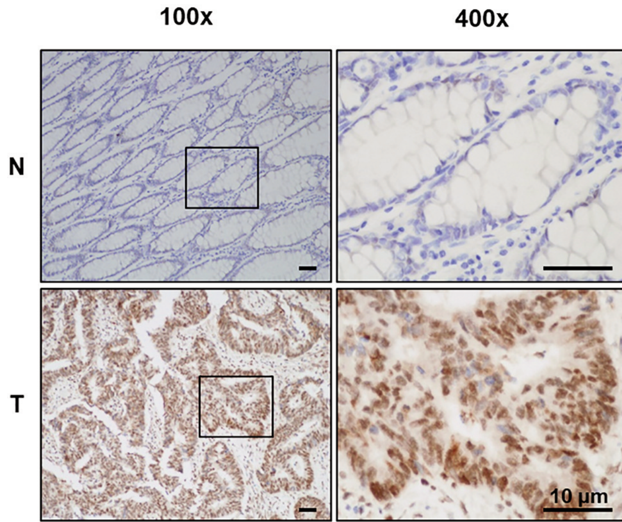

Figure 2. *Apurinic/apyrimidinic endonuclease 1 (APE1) expression in colorectal cancer and normal colorectal tissues using immunohistochemistry. The immunostaining for the APE1 protein was absent or weak in the normal colorectal mucosa. The immunostaining for the APE1 protein was predominantly identified in the cytoplasm of tumor cells and was not detectable in the tumor stroma (100×, 400×). N: Normal colorectal mucosa tissue; T: colorectal cancer tissue; Scale bar=10 μm.*

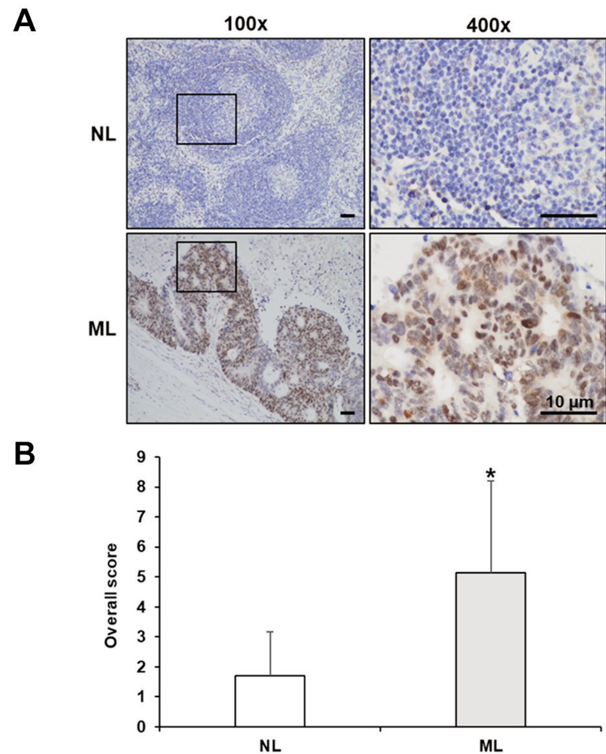

Figure 3. *Apurinic/apyrimidinic endonuclease 1 (APE1) expression in non-metastatic lymph node and metastatic lymph node tissues using immunohistochemistry. The immunostaining for APE1 in metastatic lymph node tissues was significantly stronger than that in non-metastatic lymph node tissues (100×, 400×). Each bar represents the mean±SD of 20 cases. *p<0.05 vs. non-metastatic lymph node tissue. SD: Standard deviation; NL: non-metastatic lymph node tissue; ML: metastatic lymph node tissue; Scale bar=10 μm.*

tissues than that in normal colorectal mucosa (p=0.030 and p=0.045, respectively) (Figure 1A and B). Immunostaining indicated that APE1 protein was not expressed or weakly expressed in the normal colorectal mucosa. In contrast, immunostaining showed that APE1 protein in CRC tissues was predominantly present in the cytoplasm of the tumor cells, but not in the surrounding stroma (Figure 2). The immunostaining of APE1 in metastatic lymph node tissues was significantly stronger than that in non-metastatic lymph node tissues (Figure 3A). The overall score for immunostaining of APE1 in metastatic lymph node tissues was significantly higher than that in non-metastatic lymph node (p<0.001) (Figure 3B).

Over-expression of APE1 is significantly associated with tumor progression and poor prognosis in CRC. To study the prognostic role of APE1 in CRC progression, we investigated the association between the expression of the APE1 protein immunohistochemically in formalin-fixed and paraffin-embedded tissue blocks obtained from 193 patients with CRC and clinicopathological data, including long-term survival. Based on our criteria, expression of APE1 was detected in 76 of the 193 (39.4%) CRCs analyzed (Table I). Positive expression of APE1 was significantly associated with advanced stage, lymphovascular invasion, perineural invasion, deeper tumor invasion, lymph node metastasis, and distant metastasis (p<0.001, p=0.009, p=0.004, p=0.042, p<0.001, and p<0.001, respectively) (Table I). Moreover, the overall survival for patients with positive expression of APE1 was significantly lower than those with negative expression (p<0.001) (Figure 4). After performing a Cox multivariate regression analysis, APE1, perineural invasion, and lymph node metastasis were the independent prognostic factors associated with overall survival when adjusted for several covariates, including perineural invasion, lymph node metastasis, and depth of invasion with a hazard ratio of 1.626 (95%CI=1.003-2.636; p=0.049), 2.355 (95%CI=1.426-3.891; p=0.001), and 2.418 (95%CI=1.410-4.417; p=0.001) (Table II).

Correlation between APE1 expression and tumor cell apoptosis, proliferation, and angiogenesis of CRCs. All tumor samples were assessed by performing TUNEL assay, immunostaining of Ki-67 and CD34 to determine the apoptosis, proliferation, and angiogenesis of CRC cells. Representative photomicrographs showing positive TUNEL

Table I. *Correlation between Apurinic/apyrimidinic endonuclease 1 (APE1) expression and clinicopathological parameters of colorectal cancer.*

Parameters	Total (n=193)	APE1 Negative (n=117)	APE1 Positive (n=76)	*p*-Value
Age (years)				0.595
<70.5	97	57	40	
≥70.5	96	60	36	
Sex				0.101
Male	108	71	37	
Female	85	46	39	
Stage				<0.001
I	43	33	10	
II	58	46	12	
III	49	27	22	
IV	43	11	32	
Tumor size (cm)				0.126
<4.9	97	64	33	
≥4.9	96	53	43	
Histological type				0.631
Differentiated	170	102	68	
Undifferentiated	23	15	8	
Lymphovascular invasion				0.009
Negative	144	95	49	
Positive	49	22	27	
Perineural invasion				0.004
Negative	137	92	45	
Positive	56	25	31	
Depth of invasion (T)				0.042
T1/T2	51	37	14	
T3/T4	142	80	62	
Lymph node metastasis (N)				<0.001
N0	110	81	29	
N1-3	83	36	47	
Distant metastasis (M)				<0.001
M0	149	104	45	
M1	44	13	31	

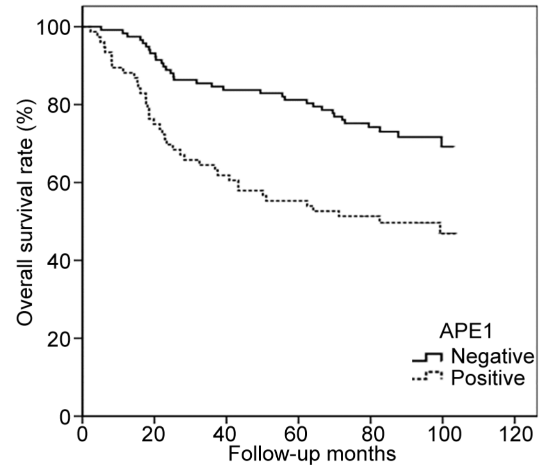

Figure 4. *Kaplan-Meier survival curve for the correlation of overall survival with positive (dotted line) and negative (solid line) apurinic/apyrimidinic endonuclease 1 (APE1) expression. The overall survival of patients with APE1-positive tumors was significantly lower than that of patients with APE1-negative tumors (p<0.001).*

staining, immunohistochemical staining for Ki-67 and CD34 in CRC tissues are shown in Figure 5. The correlation between APE1 expression and AI, KI or MVD is summarized in Table III. The KI of the 193 tumor samples ranged from 21.9 to 86.0, with a mean KI of 59.7±16.6. The mean KI value of APE1-positive tumors was 66.4±13.5, which was significantly higher than that of APE1-negative tumors (*p*=0.019). The AI of the 193 tumor samples ranged from 0.9 to 30.0, with a mean AI of 9.0±6.7. No significant difference was observed between APE1 expression and AI (*p*=0.704). The MVD of the 193 tumor samples ranged from 3.5 to 429.0, with a mean MVD of 106.2±82.4. No significant difference was observed between APE1 expression and MVD (*p*=0.762).

Discussion

APE1 functions in the base excision repair pathways of the DNA damage and acts as a redox-dependent modulator that stimulates many transcriptional factors (4-6). The APE1 protein plays a critical role in embryonic development, tissue regeneration, and tumor development and progression (7-9).

Previously, APE1 was shown to be over-expressed in a variety of cancers and its over-expression associated with advanced stage, poor prognosis, and chemo-, and radio-therapy resistance (10-20). Also, the activity of APE1 is associated with apoptosis, cell cycle, proliferation, colony formation, and angiogenesis of cancer cells. Inhibition of APE1 slows growth and progression in a variety of cancer types (21-29). Therefore, APE1 inhibitors have therapeutic potential.

Until now, there was little data on the effect of APE1 on tumor progression and prognosis of human CRC. Therefore, we measured the expression of APE1 in CRC tissues and paired normal colorectal mucosa tissues using RT-PCR and western blotting. Its expression was found to be significantly higher in CRC tissues than in normal tissues at mRNA and protein levels in colonoscopic biopsy specimens. Also, we compared the expression of APE1 in non-metastatic lymph node and metastatic lymph node tissues using immuno-histochemistry. The overall score for immunostaining of APE1 in metastatic lymph node tissues was significantly higher than that in nonmetastatic lymph node tissues in surgical specimens. These observations suggest that APE1 may play a critical role in human CRC development and progression.

Table II. *Cox multivariate regression of the association between Apurinic/apyrimidinic endonuclease 1 (APE1) immunoreactivity and survival in colorectal cancer adjusted for clinicopathological parameters.*

Covariate	HR	95%CI	*p*-Value
APE1 expression			
Negative	1.000	1.003-2.636	0.049
Positive	1.626		
Perineural invasion			
Negative	1.000	1.426-3.891	0.001
Positive	2.355		
Lymph node metastasis (N)			
N0	1.000	1.410-4.147	0.001
N1-3	2.418		
Depth of invasion (T)			
T1-2	1.000	0.688-2.902	0.347
T3-4	1.413		

HR: Hazard ratio; CI: confidence interval.

Table III. *Association of Apurinic/apyrimidinic endonuclease 1 (APE1) expression with tumor cell apoptosis, proliferation, and angiogenesis in colorectal cancers.*

		APE1 expression		
Indices (mean±SD)	Total (n=193)	Negative (n=117)	Positive (n=76)	*p*-Value
AI	9.0±6.7	9.5±8.0	8.6±5.7	0.704
KI	59.7±16.6	54.1±17.1	66.4±13.5	0.019
MVD	106.2±82.4	102.8±69.7	108.5±90.4	0.762

KI: Ki-67 labeling index; AI: apoptotic index; MVD: microvessel density; SD: standard deviation.

Previously, expression of APE1 was found to be a prognostic value in a variety of cancers. Therefore, to document the prognostic significance of APE1 expression, we examined the correlations between APE1 immunostaining and various clinicopathological parameters including survival of patients with CRC. Our study showed that positive expression of APE1 was significantly associated with advanced stage, lymphovascular invasion, perineural invasion, deeper tumor invasion, lymph node metastasis, distant metastasis, and poor survival. After performing a Cox multivariate regression analysis, APE1, perineural invasion, and lymph node metastasis were found to be independent prognostic factors associated with overall survival when adjusted for several covariates, including perineural invasion, lymph node metastasis, and depth of invasion. Previously, over-expression of APE1 was related to a poor prognosis in patients with liver metastases from CRC (31). Also, over-expression of APE1 was associated with poor prognosis and resistance to oxaliplatin-based adjuvant chemotherapy in small sized stage III patients with CRC (32). Therefore, APE1 may be a prognostic and predictive biomarker of oncologic outcomes in patients with CRC.

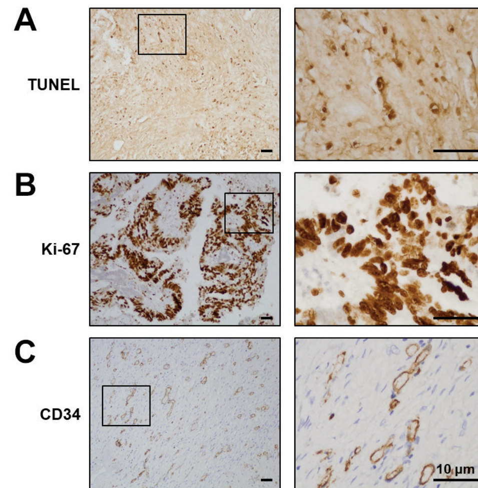

Figure 5. *Representative photomicrographs showing positive TUNEL staining, and immunostaining for Ki-67 and CD34 in colorectal cancer tissues. (A) Detection of apoptotic cells using TUNEL staining. Apoptotic cells with classic features of DNA condensation are shown to have a halo consisting of pyknotic nucleus and shrunken cytoplasm in colorectal cancer tissues; no TUNEL staining is observed in normal colorectal mucosa (100×, 400×). (B) Immunostaining for Ki-67 shows strong nuclear positivity in the colorectal cancer cells but is rarely positive in the normal colorectal mucosa (100×, 400×). (C) Immunostaining for CD34 is rarely observed in the normal colorectal mucosa and is intense in small blood vessels and capillaries (100×, 400×). Scale bar=10 μm.*

Cancer progression and metastasis are complex processes arising from multiple alterations of proto-oncogenes and tumor suppressor genes, resulting in aggressive phenotype of cancer cells including cell growth, migration, invasion, survival, epithelial-mesenchymal transition (EMT), drug resistance, and angiogenesis (33-35). Previously, APE1 over-expression was shown to inhibit apoptosis, and cause cell cycle arrest, enhanced EMT and angiogenesis in a variety of cancer cells. In contrast, APE1 knockdown was shown to induce apoptosis and cell cycle arrest and inhibit EMT and angiogenesis (21-24). Furthermore, inhibition of APE1 was found to impede tumor progression in tumor mouse models (25-29).

Finally, we evaluated the correlation of APE1 expression with apoptosis, proliferation, and angiogenesis in human CRC tissues. We observed that the mean KI value of APE1-positive tumors was significantly higher than that of APE1-negative tumors. However, no significant association was observed between APE1 expression and AI or MVD value. Previously, APE1 was found to be associated with enhanced tumor cell proliferation index, evaluated by Ki-67 immunostaining in an azoxymethane-induced CRC mouse model (29). These results suggest that APE1 is associated with tumor progression by influencing tumor cell proliferation in CRC.

Taken together, over-expression of APE1 is significantly associated with tumor progression and poor survival of patients with CRC. Therefore, APE1 may be a potential prognostic and predictive biomarker for CRC.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization: Ji-Yun Hong, Sung-Bum Cho, and Young-Eun Joo. Execution of most of the experiments: Hyung-Hoon Oh, Young-Lan Park, and Sun-Young Park. Design of experiments and drafting the article: Ji-Yun Hong and Sung-Bum Cho. Supervision: Young-Eun Joo. Approval of final article: all Authors.

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Received May 17, 2023
Revised June 6, 2023
Accepted June 7, 2023