PTEN expression and mutation in colorectal carcinomas

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Abstract. The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) tumor suppressor is a phosphatase that antagonizes the phosphoinositol-3-kinase/AKT signaling pathway and suppresses cell survival as well as cell proliferation. To investigate the molecular role of PTEN expression and mutation in colorectal carcinomas, we performed immunohistochemistry to detect PTEN expression on tissue microarray containing colorectal carcinomas and corresponding adjacent non-cancerous mucosa. PTEN mutation was studied from exon 1 to 9 by PCR, followed by direct sequencing. PTEN expression was then compared with clinicopathological parameters and prognosis of the tumor, including caspase-3 expression. In the present study, PTEN expression was stronger in the adjacent non-cancerous mucosa than carcinoma (P<0.001). Low PTEN expression was positively correlated with tumor size, depth of invasion, lymphatic invasion, lymph node metastasis, higher Dukes staging and reduced caspase-3 expression (P<0.05), but not with venous invasion or differentiation (P>0.05). Univariate analysis suggested that the patients without PTEN expression had shorter survival than the patients with its expression (P=0.003). Multivariate analysis indicated that lymphatic invasion, venous invasion, and PTEN expression were independent prognostic factors for overall colorectal carcinomas (P<0.05). The analysis of mutations revealed only one synonymous mutation in exon 8 (codon 312 Asp: GAC→GAT). These results suggested that down-regulated PTEN expression was involved in the pathogenesis, invasion and metastasis of colorectal carcinomas possibly by regulating the balance between apoptosis and proliferation. PTEN expression may be a good marker for the prognosis of colorectal carcinoma.

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Introduction

Malignant transformation is a biologically complicated process, including the activation of oncogenes and the inactivation of tumor suppressor genes (TSG), which in turn relieves cells from the balance between proliferation and apoptosis to promote tumor development (1). Among genetic changes in malignancies, chromosomal deletion, which could lead to loss of TSG causing uncontrolled proliferation and immortal survival, always provides some new insights into identification of genes critical for initiation, promotion and development of tumor, like PTEN (2).

PTEN gene (phosphatase and tensin homology deleted from human chromosome 10), also referred to as MMAC1 (mutated in multiple advanced cancers) or TEP-1 (TGF-ß-regulated and epithelial cell-enriched phosphatase) is located on human chromosome 10q23.3 and contains nine exons and encodes a 47 kd dual specific protein-phospholipid phosphatase with 403 amino acids (1). Structurally, PTEN is comprised of three parts: i) a 185-amino acid NH_2-teminal phosphatase catalytic domain with high sequence homology to the cytoskeleton protein chicken tensin and bovine auxilin; ii) a COOH-terminal C2 domain; iii) a 50-amino acid COOH-terminal tail with PDZ binding motif and CK2 (formerly casein kinase II) phosphorylation sites. Functionally, it could inhibit shc (src-homology collagen) phosphorylation and therefore blocks the activation of the Ras/MAP-kinase pathway. PTEN also dephosphorylated focal adhesion kinase (FAK), affecting cell adhesion, spreading and recognition. Furthermore, PTEN acts as a phospholipid phosphatase with phosphatidylinosiol 3, 4, 5-triphosphate (PIP_3) as a substrate and one downstream target of PIP_3, protein kinase (Akt/PKB), is continually activated by phosphorylation in cells lacking in functional PTEN (3). Jones *et al* (4) found that activation of PTEN signal pathway could target activation of CPP (cysteine protease protein)-32, resulting in inhibition of cellular apoptosis. These findings suggest

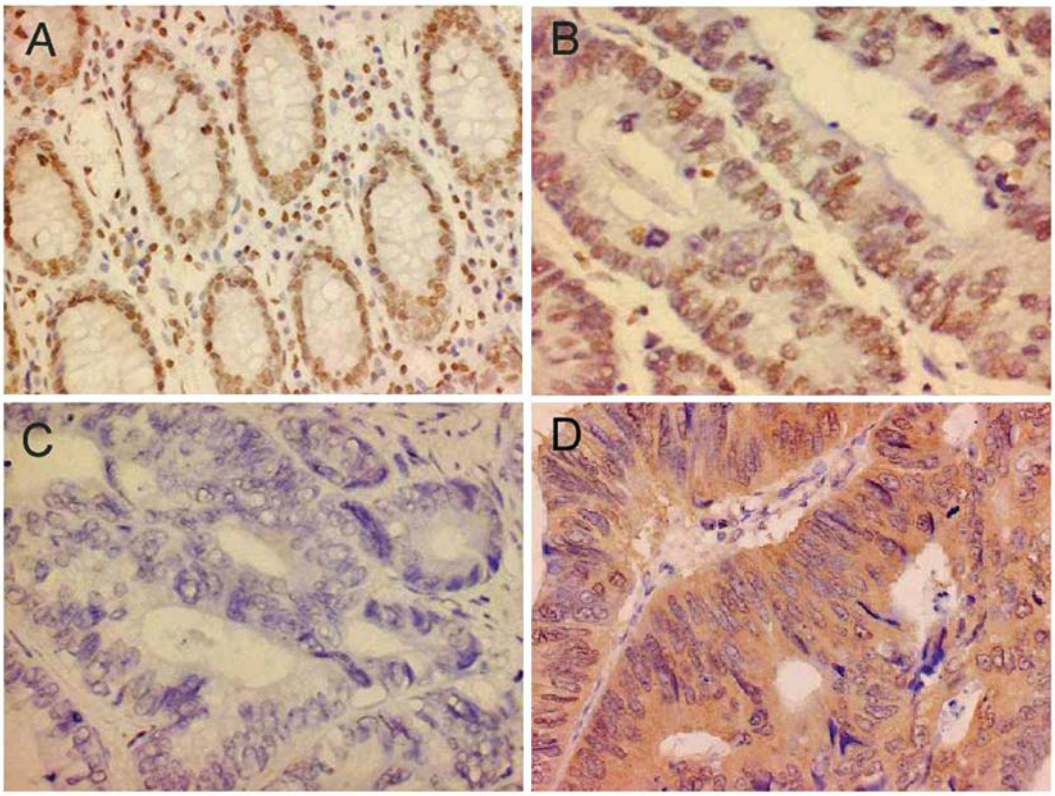

Figure 1. Immunohistochemical staining of colorectal samples. PTEN was positively expressed in the nucleus of the adjacent non-cancerous mucosa (a) and colorectal carcinomas (b). In some cancerous cases, no PTEN expression was detected (c). Caspase-3 was distributed in the cytoplasm of the carcinoma (d).

PTEN plays an essential role in tumorigenesis and progression of maligancies as a TSG.

PTEN gene was found to be one of the most commonly mutated tumor suppressors in human malignancies (5). The hereditary loss of *PTEN* leads to numerous autosomal dominant disorders: Cowden syndrome, Bannayan-Riley-Ruvalcaba syndrome, Lhermitte-Duclos disease, Proteus syndrome and Proteus-like syndrome, which are characterized by the presence of developmental defects, benign hamartomas and an increased risk of cancer (6-9). Genetic alterations of the *PTEN* gene range from point mutations to large chromosomal deletions (10-12). The mutation of PTEN is regularly observed in a considerable fraction of malignancies, such as breast, prostate, colon, endometrial and ovarian carcinomas (13-16).

Colorectal cancer is an important public health problem: there are nearly one million new cases of colorectal cancer diagnosed worldwide each year and half a million deaths. Japan has experienced a marked increase in the incidence of colorectal cancer, and was recently listed in the group of countries with the world's highest incidence rates (17,18). Therefore, we studied the PTEN expression and mutation in Japanese colorectal cancer to clarify the molecular effect of PTEN alteration on the multistep development of colorectal carcinomas.

Materials and methods

Subjects. Three hundred and twenty-seven cases of formalin-fixed and paraffin-embedded colorectal carcinomas and non-cancerous mucosa specimens were collected in Division of Pathology, Takaoka Kouseiren Hospital from 1993 to 2002. The patients with colorectal carcinomas were 184 men and 143 women (18-90 years, mean, 69.16 years). In 128 cases, tumor development was accompanied with lymph node metastasis. None of the patients underwent chemotherapy or radiotherapy before surgery. All provided consent for use of tumor tissue for clinical research and our University Ethics Committee approved the research protocol. Two hundred and seventy-nine cases were followed up by consulting their case documents and telephoning.

Pathology. All tissues were fixed in 10% neutralized formalin, embedded in paraffin and cut into 4 μm sections stained with hematoxylin and eosin (HE) to confirm the histological diagnosis and microscopic characteristics. The staging for each colorectal carcinoma was evaluated according to the Dukes staging system indicating the extent of tumor spread (19). Furthermore, tumor size, depth of invasion, lymphatic and venous invasion, and lymph node metastasis of tumors were determined.

Tissue microarray (TMA) and immunohistochemistry. From HE stained sections of the selected tumor cases, representative areas of solid tumor were selected for sampling and 4-mm diameter tissue cores per donor block were punched out and transferred to a recipient block with a maximum 24 cores using a Tissue Microarrayer (Azumaya Kin-1, Japan). Thick sections (4 μm) were consecutively cut from the microarrays and transferred to polylysine-coated glass

Table I. Oligonucleotide primers used in mutation screen of PTEN.

Exon	Primer sequences	Distribution (GI:4240386)	AT (˚C)	Product size (bp)
1	Forward: 5'-CTCCTCCTTTTTCTTCAGCC-3' Reverse: 5'-TATGACCTAGCAACCTGACCA-3'	23305-23324 23527-23547	56	243
3	Forward: 5'-ATAGAAGGGGTATTTGTTGGA-3' Reverse: 5'-ACCTCACTCTAACAAGCAGATA-3'	83319-83339 83590-83611	56	293
4	Forward: 5'-TTCAGGCAATGTTTGTTA-3' Reverse: 5'-CTCGATAATCTGGATGACTCA-3'	88928-88945 89132-89152	48	225
5	Forward: 5'-GCAACATTTCTAAAGTTACCTA-3' Reverse: 5'-TCTGTTTTCCAATAAATTCTC-3'	90904-90925 91269-91289	48	386
6	Forward: 5'-GAGTAACTATTCCCAGTCAGA-3' Reverse: 5'-TAATTTGTTCAAATGCTTCAGA-3'	110087-110107 110351-110372	52	286
7	Forward: 5'-ATCGTTTTTGACAGTTTG-3' Reverse: 5'-CCAATGAAAGTAAAGTACA-3'	115750-115767 15991-116009	48	260
8	Forward: 5'-AGGTGACAGATTTTCTTTTTTA-3' Reverse: 5'-TCAGCTGTACTCCTAGAATTA-3'	118771-118792 119145-119165	52	394
9	Forward: 5'-GTTCATCTGCAAAATGGA-3' Reverse: 5'-TGGTAATCTGACACAATGTCCTA-3'	123153-123170 123527-123549	50	397

AT, annealing temperature.

slides. HE staining was performed for confirmation of tumor tissue (Fig. 1A).

Serial sections of TMA were deparaffinized with xylene, dehydrated with alcohol, and subjected to immunohistochemical staining with intermittent microwave radiation as previously described (20). Mouse anti-human PTEN (1:150, NovoCastra, UK) and rabbit anti-human caspase-3 (1:150, Dako, USA) antibodies were used to detect the respective proteins, with Envision+ system labeled polymer-HRP anti-mouse IgG or anti-rabbit IgG (Dako) as the secondary antibody. Binding was visualized with 3,3'-diaminobenzidine (DAB) and counterstaining with Mayer's hematoxylin was performed to aid orientation. Omission of the primary antibody was used as a negative control.

The immunoreactivity for PTEN was localized in the nucleus, while caspase-3 in the cytoplasm (Fig. 1). One hundred cells were randomly selected and counted from five representative fields of each section blindly by three independent observers (Takano Y., Zheng H.C. and Li X.H.) and the percentage of positive cells in the total counted was graded semi-quantitatively using a four-tier scoring system: negative (-), 0-5%; weakly positive (+), 6-25%; moderately positive (++), 26-50%; and strongly positive (+++), 51-100%.

DNA extraction and checking. Fifty-seven cancer samples were randomly selected for the detection of PTEN mutation. Paraffin-embedded blocks were sectioned at 10 μm and cancer lesions on slides were microdissected with reference to HE staining of consecutive sections, and subject to deparaffinization. DNA was extracted from the deparaffinized samples by standard proteinase K digestion and phenol/chloroform extraction. All the DNA samples were amplified using ß-globin primers, sense: 5'-ACACAACTGTG TTCACTAGC-3' and anti-sense: 5'-GTCTCCTTAAACC TGTCTTG-3' (175 bp) by 30 cycles of denaturation at 95˚C for 30 sec, annealing at 55˚C for 30 sec, and extension at 72˚C for 30 sec to confirm their integrity.

PCR. PCR amplification was performed using three individual sets of primers for exons 1-9 of PTEN as described in Table I containing their distribution, annealing temperatures, extension time and amplicon size. Twenty-five microliter of reaction mixtures contained 0.125 μl Takara Ex Taq HS (Takara, Japan) and 0.02 μl PfuUltra High-fidelity DNA polymerase (Stratagene, USA) with 2.0 mM $MgCl_2$, 2.5 μl 10X PCR buffer, 2.5 μl dNTP mixture, 1 μM of each primer set (external primers), and 250 ng of template DNA. PCR conditions were denaturation at 95˚C for 10 min, followed by 30 cycles of denaturation at 95˚C for 15 sec, annealing for 30 sec, and extension at 72˚C. As a termination step, the extension time of the last cycle was increased to 7 min. Sample amplified in the absence of template DNA was employed as negative controls.

DNA direct sequencing. Amplicons were purified with MicroSpin SR-300 columns (Amersham Biosciences, UK). After extraction, the DNA was quantified by Nanodrop ND-100 Spetrophotometer (Laboratory & Medical Supplies, Japan) and then sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) with each

Table II. PTEN expression in colorectal carcinomas and corresponding adjacent non-cancerous mucosa.

Groups	n	PTEN expression				PR (%)	P-value
		-	+	++	+++		
Non-cancerous mucosa	327	2	52	145	127	99.4	<0.001
Carcinomas	327	95	93	87	52	70.9	

PR, positive rate.

Table III. Relationship between PTEN expression and clinicopathological features of colorectal carcinomas.

Clinicopathological features	n	PTEN expression				PR (%)	P-value
		-	+	++	+++		
Age (years)							0.472
<65	123	32	37	33	21	74.0	
≥65	204	63	56	54	31	69.1	
Gender							0.049
Male	184	49	48	52	35	73.4	
Female	143	46	45	35	17	67.8	
Tumor size (cm)							0.022
≤5	211	47	64	60	40	77.7	
>5	116	48	29	27	12	58.6	
Depth of invasion							0.000
T_{is}-T_1	32	4	7	10	11	87.5	
T_2-T_4	295	91	86	77	41	69.2	
Differentiation							0.093
Well-differentiated	165	42	47	49	27	74.5	
Moderately differentiated	148	46	41	38	23	68.9	
Poorly differentiated	14	7	5	0	2	50.0	
Lymphatic invasion							0.033
-	233	62	64	65	42	73.4	
+	94	33	29	22	10	64.9	
Venous invasion							0.091
-	278	75	82	76	45	73.0	
+	49	20	11	11	7	59.2	
Lymph node metastasis							0.012
-	199	48	55	62	34	75.9	
+	128	47	38	25	18	63.3	
Dukes staging							0.011
A + B	199	49	54	61	35	75.4	
C	128	46	39	26	17	64.1	
Caspase-3 expression							<0.001
-	82	37	21	12	12	54.9	
+~+++	245	58	72	75	40	76.3	

PR, positive rate; T_{is}, carcinoma *in situ*; T_1, lamina propria and submucosa; T_2, muscularis propria and subserosa; T_3, exposure to serosa; T_4, invasion into serosa.

Table IV. PTEN mutation screening in colorectal carcinomas.

Exon	n	Amplification no. (%)	DNA sequencing no. (%)	Mutation no. (%)
1	57	52 (91.2)	43 (75.4)	0
3	57	21 (36.8)	14 (24.6)	0
4	57	32 (56.1)	20 (35.1)	0
5	57	16 (28.1)	12 (21.1)	0
6	57	26 (45.6)	14 (24.6)	0
7	57	35 (61.4)	32 (56.1)	0
8	57	10 (17.5)	8 (14)	1 (1.8)
9	57	10 (17.5)	8 (14)	0

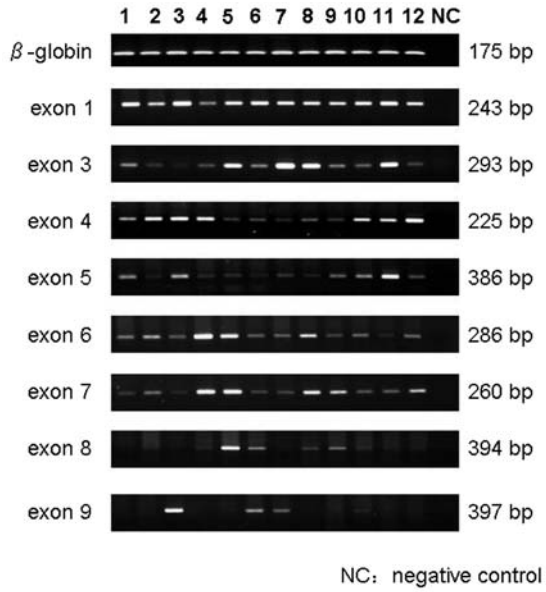

Figure 2. Detection of *ß-globin* and PTEN in colorectal carcinomas. ß-globin was positive in all cases of colorectal carcinomas. Exons 1, 3-9 of *PTEN* were positive in some carcinoma cases by PCR. NC, negative control.

pair of forward and reverse primers. The sequence data were compared with PTEN sequence (Genbank GI: 4240386) using BLAST.

Statistical analysis. Statistical evaluation was performed using the Spearman correlation test to analyze rank data. Kaplan-Meier survival plots were generated and comparisons between survival curves were made with the log-rank statistic. The Cox's proportional hazards model was employed for multivariate analysis. P<0.05 was considered as statistically significant. SPSS 10.0 software was employed to analyze the data.

Results

Expression of PTEN in corresponding non-cancerous mucosa and colorectal carcinomas. In the present study, PTEN was positively stained in the nucleus of corresponding non-cancerous mucosa (Fig. 1A) or carcinoma cells (Fig. 1B) and infiltrating inflammatory cells. Whereas caspase-3 immunoreactivity distributed to the cytoplasm of carcinoma cells (Fig. 1D). PTEN was positively detected in 325 of 327 (99.4%) cases in corresponding non-cancerous mucosa. However, in some cancerous cases, no PTEN expression was detected (Fig. 1C). In total, 232 of 327 (70.9%) cases positively expressed PTEN protein in colorectal carcinomas. PTEN expression in colorectal carcinoma was statistically lower than the adjacent non-cancerous mucosa (P<0.001) (Table II).

The relationship between the expression of PTEN and clinicopathological features of colorectal carcinoma. As Table III shows, PTEN expression was negatively correlated with tumor size, depth of invasion, lymphatic invasion, lymph node metastasis, lower Dukes staging, and lower caspase-3 expression (P<0.05), but not with patient age, differentiation and venous invasion (P>0.05). The male carcinoma patients exhibited stronger PTEN expression than females (P<0.05).

The amplification, sequencing and mutation analysis to exon 1,3-9 of PTEN in colorectal carcinoma. After DNA extraction, ß-globin was evaluated as a control of DNA integrity and 57 samples displayed clear bands for the following PTEN amplification (Fig. 2). We did not detect exon 2 of PTEN despite changing primers twice. As summarized in Table IV, the amplification rate of PTEN exons 1 and 4 are 91.2% and 61.4%, higher than the others. However, exons 8 and 9 displayed the lowest detection rate (17.5%). Due to the weaker amplification of PCR, some samples were not sequenced although we repeated the experiments several times. Among these amplicon, only one synonymous mutation was found in exon 8 (codon 312 Asp: GAC→GAT) (Fig. 3).

Univariate and multivariate survival analysis. Follow-up information was available on 279 colorectal carcinoma patients for periods ranging from 0.9 months to 12.1 years (mean, 66.8 months). Univariate analysis using the Kaplan-Meier method suggested that the patients without PTEN expression had shorter survival than the patients with its expression (P=0.003) (Fig. 4). Multivariate analysis using Cox's proportional hazards model indicated that lymphatic invasion, venous invasion and PTEN expression were independent prognostic factors for overall colorectal carcinomas

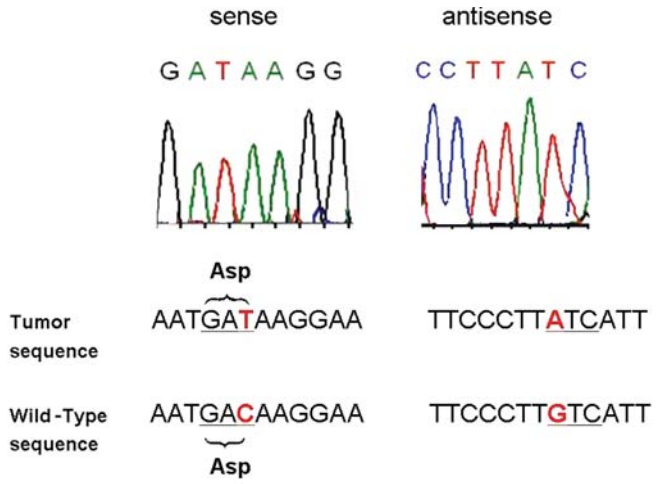

Figure 3. PTEN mutation in colorectal carcinomas. The DNA sequencing analysis of case 39. Only one synonymous mutation (codon 312 GAT→GAC) of exon 8 was noted.

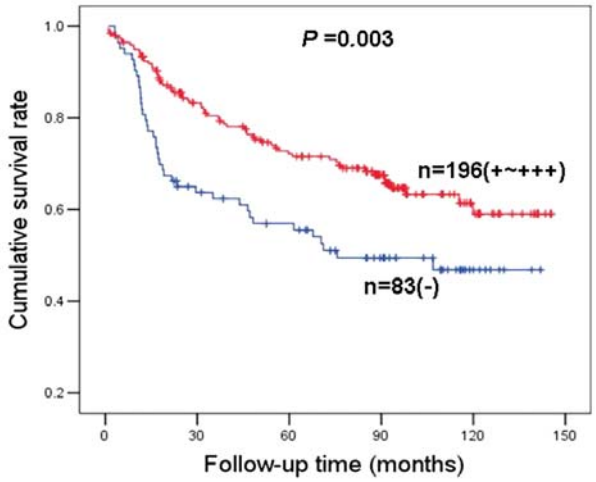

Figure 4. Correlation between the status of PTEN expression and prognosis of the patients with colorectal carcinomas. Kaplan-Meier curves for cumulative survival rate of patients according to the PTEN expression in all the colorectal carcinomas.

(P<0.05), but not age, gender, tumor size, depth of invasion, differentiation, lymph node metastasis or Dukes staging (Table V).

Discussion

PTEN, a tumor-suppressing gene, is involved in cellular differentiation, reproduction and apoptosis, as well as cellular adhesion and mobility and its loss or down-regulation plays an important role in the multiple steps of tumorigenesis and progression of malignancies (21). Enhanced tumorigenesis has also been observed in PTEN-deficient mice, which eventually develop loss of heterozygosity (LOH) of the remaining PTEN allele, leading to the appearance of tumors in the endometrium, liver, prostate, gastrointestinal tract, thyroid and thymus (22,23). In the present study, we used the anti-PTEN antibody recognizing a 200 amino acid C-terminal region of PTEN molecule and found that it was localized in the nucleus of colonic epithelial and carcinoma cells as used previously (1,2,24) although other investigators described cytoplasmic localization of PTEN protein using different antibodies (25-27). It was considered that the function of nuclear PTEN is not the same as cytoplasmic PTEN. Nuclear PTEN plays a role in chromosome stability, DNA repair, cell cycle arrest and cellular stability. It was postulated that simple diffusion, export dependent on a putative cytoplasmic localization signal, active shuttling by the RAN GTPase or major vault protein (MVP), phosphorylation-dependent shuttling and monoubiquitylation-dependent import may be possible mechanisms for the nucleo-cytoplasmic shuttling of PTEN (28). Levels of nuclear PTEN may influence the cell cycle or the antiapoptotic activity of nucleophosmin (29-31). In our study, colorectal carcinomas displayed statistically lower PTEN expression than corresponding non-cancerous mucosa. The down-regulated PTEN might be involved in the pathogenesis of colorectal carcinomas and be attributable to genetic or epigenetic changes, such as mutation, LOH, hypermethylation and microsatellite instability in various malignancies as described previously (1,3).

Table V. Multivariate analysis of clinicopathological variables for survival with colorectal carcinomas.

Clinicopathological parameters	Relative risk (95% CI)	P-value
Age (≥65 years)	1.280 (0.816-2.008)	0.283
Gender	1.076 (0.706-1.639)	0.733
Tumor size (≥5 cm)	0.812 (0.523 -1.261)	0.354
Depth of invasion (into muscularis propria)	3.020 (0.378-24.152)	0.468
Differentiation (poor)	1.144 (0.795-1.647)	0.297
Lymphatic invasion (+)	1.613 (1.024-2.540)	0.039
Venous invasion (+)	2.183 (1.296-3.677)	0.003
Lymph node metastasis (+)	1.769 (0.731-4.283)	0.206
Dukes staging (C)	1.672 (0.799-3.498)	0.172
PTEN expression (+~+++)	0.653 (0.432-0.986)	0.042

CI, confidence interval.

Low expression of PTEN gene product was implicated in the clinicopathological stage and metastasis of various malignancies (1,3). We found that decreased PTEN expression was closely linked to tumor size, invasive depth, lymph node metastasis, and higher Dukes staging of colorectal carcinomas, similar to a previous report (32). Furthermore, univariate analysis showed that the patients without PTEN expression showed poorer prognosis than the patiens with its expression. Multivariate analysis indicated that PTEN expression and lymphatic invasion, venous invasion were independent prognostic factors for overall colorectal carcinomas. It was suggested that down-regulated expression of the PTEN protein probably contributed to growth, invasion, and metastasis of colorectal carcinoma and could be considered as a good marker to indicate the aggressive behaviors and poor prognosis of colorectal carcinomas. Several putative mechanisms relating to tumor suppression of PTEN have been reported such as inhibiting cell mobility and facilitating cell adhesion by dephosphorating FAK, inhibiting cell apoptosis and increasing cell growth by dephosphorating PIP3 and restraining cell differentiation by inhibiting MAPK signal pathway. Abnormal expression of the PTEN protein was involved in progression of colorectal carcinoma by its effects on cell mobility, adhesion, apoptosis, growth and differentiation (1,3,21).

As a negative regulator of the conserved insulin/IGF-PI3K signaling pathway, conditional deletion of PTEN caused increased proliferation, decreased apoptosis and tumorigenesis. PTEN's promotion of the apoptotic response may cooperate with its function in cell cycle arrest, and may be mediated by more than one mechanism (28,29). Caspase-3 is an apoptosis related enzyme and ultimately execute apoptotic cell death. Active caspase-3 consists of large and small subunits, which are released from the procaspase through proteolytic processing, plays a key role in apoptosis (32,33). Schwartzbauer *et al* (34) found increased PTEN expression by recombinant adenovirus in cultured neonatal rat primary cardiomyocytes caused cardiomyocyte apoptosis as evidenced by increased caspase-3 activity and cleaved poly(A) DP-ribose polymerase. Here, the PTEN expression was positively correlated with caspase-3 expression in colorectal carcinomas. Consequently, we could infer that low expression of PTEN decreased expression of caspase-3 to cause tumor cells apoptosis dysfunction, which is the theoretic basis of contribution of PTEN to tumorigenesis and progression of colorectal carcinomas.

Since germline mutations of the PTEN gene are found in two hamartoma disorders called Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome, different types of mutations in the PTEN locus such as frameshifts, missense mutations, and splicing variants were found to be associated with a broad range of human cancers (35-37). These mutations sometimes induce early termination of the translation and subsequently cause the production of unstable messages and/or immature gene products, which are no longer able to form functional phosphatase domain and completely lose the phosphatase activity, but most of missense mutations results in robust decrease in the phosphatase activity (38-40). Although some reports showed 17%-19.5% mutation of PTEN (15, 25), in our study, only one synonymous mutation was detected in PTEN exon 8 (codon 312 Asp: GAC→GAT). To improve the accuracy of the PCR amplification and sequence, we employed the high-fidelity PfuUltra™ polymerase in PCR reaction mixture and sequenced each amplicon from both ends although some samples were not yet available for the direct sequencing. Additionally, we carefully analyzed the starting and ending data of DNA sequence because of its instability. It was also reported that PTEN expression was frequently diminished at the transcriptional/translational level. DNA methylation, transcriptional repression and microRNA-directed mRNA degradation and translational disruption appear to reduce PTEN expression in some cancers (41,42). Therefore, it might be concluded that mutation of PTEN was not common in Japanese colorectal carcinomas.

In summary, the study suggested that down-regulated PTEN expression was involved in pathogenesis, invasion and metastasis of colorectal carcinomas possibly by regulating the balance between apoptosis and proliferation. PTEN expression may be a good marker for prognosis of colorectal carcinoma.

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