• COLORECTAL CANCER •

# Expression and significance of PTEN, hypoxia-inducible factor-1 alpha in colorectal adenoma and adenocarcinoma

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## Abstract

**AIM:** To investigate the expression and significance of PTEN, hypoxia-inducible factor-1 alpha (HIF-1$\alpha$), and targeting gene VEGF during colorectal carciogenesis.

**METHODS:** Total 71 cases colorectal neoplasms (9 cases of colorectal adenoma and 62 colorectal adenocarcinoma) were formalin fixed and paraffin-embedded, and all specimens were evaluated for PTEN  mRNA, HIF-1$\alpha$ mRNA and VEGF protein expression. PTEN mRNA, HIF-1$\alpha$ mRNA were detected by in situ hybridization. VEGF protein was identified by citrate-microwave SP immunohistochemical method.

**RESULTS:** There were significant differences in PTEN, HIF-1$\alpha$ and VEGF expression between colorectal adenomas and colorectal adenocarcinoma ($P<0.05$). The level of PTEN expression decreased as the pathologic stage increased. Conversely, HIF-1$\alpha$ and VEGF expression increased with the Dukes stage as follows: stage A (0.1029±0.0457: 0.1207± 0.0436), stage B (0.1656±0.0329: 0.1572±0.0514), and stage C+D (0.2335±0.0748: 0.2219±0.0803). For PTEN expression, there was a significant difference among Dukes stage A, B, and C+D, and the level of PTEN expression was found to be significant higher in Dukes stage A or B than that of Dukes stage C or D. For HIF-1$\alpha$ expression, there was a significant difference between Dukes stage A and B, and the level of HIF-1$\alpha$ expression was found to be significantly higher in Dukes stage C+D than that of Dukes stage A or B. The VEGF expression had similar results as HIF-1$\alpha$ expression. In colorectal adenocarcinoma, decreased levels of PTEN were significantly associated with increased expression of HIF-1$\alpha$ mRNA ($r=-0.36$, $P<0.05$) and VEGF protein ($r=-0.48$, $P<0.05$) respectively. The levels of HIF-1 were positively correlated with VEGF expression ($r=0.71$, $P<0.01$).

**CONCLUSION:** Loss of PTEN expression and increased levels of HIF-1$\alpha$ and VEGF may play an important role in carcinogenesis and progression of colorectal adenocarcinoma.

## INTRODUCTION

So far, the mechanism of colorectal oncogenesis is not fully understood. Recent studies[1-6] have reported on the association of a tumor suppressor gene PTEN with the oncogeneses of several type cancer and cancer cell lines and on PTEN playing an important role in the tumor progression and metastases. Moreover, hypoxia-inducible Factor-1 alpha (HIF-1$\alpha$) is a transcription factor identified as being activated by hypoxia, and plays a central role in tumor angiogenesis[7-19]. Therefore, this study was undertaken to investigate the expression and relationship between PTEN and HIF-1$\alpha$ and VEGF in colorectal carcinogenesis.

## MATERIALS AND METHODS

### Materials
The specimens of colorectal cancer were surgically obtained from 71 patients at the Hospital of Wuhan University between 1996 and 1997, and 71 patients were catalogued by histological subtypes as follows: 62 cases of colorectal adenocarcinoma (17 cases in Dukes stage A, 18 stage B, 20 stage C and 7 stage D) and 9 patients of colorectal adenoma (5 cases of tulubo-villous adenoma, 2 tubular adenoma and 2 villous adenoma).

### Antibodies and reagents
A rabbit VEGF polyclonal antibody was purchased from Neomark International Corporation (Taipei, Taiwan), PTEN mRNA, HIF-1$\alpha$ mRNA *in situ* hybridization and immunohistochemical repairase were purchased from Boster Corporation ( Wuhan ,China), and S-P reagent was purchased from Maixin Corporation (Fuzhou, China).

### Immunohistochemical technique
Sections were dewaxed in xylene, taken thorough ethanol, and then incubated with 3 % hydrogen peroxide to block endogenous peroxidase activity. Sections then were repaired by repairase for 10 minutes, and the procedure of immunohistochemical determination was performed according to the manufacturer's instruction. A rabbit polyclonal antibody was a dilution of 1:50.

### In situ hybridization
Frozen sections were cut onto slides, briefly air-dried, and stored at -80 ℃. Prior to use they were fixed in 4 % paraformaldehyde in PBS for 20 min, washed in PBS, and treated with proteinase K (0.0005 %) in 0.1 M Tris/0.05 M EDTA, pH 8.0 for 5 min at 37 ℃. Slides were rinsed in 0.2 % glycine in water, postfixed in 4 % paraformaldehyde/0.1 M NaOH/0.1 M NaAc for 5 min, rinsed in 0.1 M triethanolamine

(TEA), pH 8.0 for 3 min, acetylated for 10 min in 0.25 % acetic anhydride/0.1 M TEA, pH 8.8, washed in 2×SSC, and dehydrated. RNA probe was then hybridized to the sections at 60 ℃ for 16 hrs in 50 % formamide/10 % dextran sulfate/0.15 M NaCl/1×Denhardt' s solution/0.01 M Tris· Cl, pH 8.0/0.01 M DTT with 0.5 mg/ml tRNA. Sections from each tumor were always hybridized to sense probes as a control for specificity. The slides were next rinsed in 4×SSC and incubated at 37 ℃ for 30 min with 0.1 mg/ml RNaseA in 0.5 M NaCl/0.01 M Tris· Cl, pH 8.0/1 mM EDTA. They were then desalted, dehydrated through graded ethanols, and coated with emulsion. Following exposure at 4 ℃ for 5 days, emulsion was developed and fixed, and sections were stained with hematoxylin and eosin. To analyse image scanning, we obtained value of absorbance.

### Statistical analysis

All results were expressed as the means ±SD. Statistical analyses, including the Chi-square test and correlated Spearman test, were carried out with the software package SPSS10.0. A $P$ value of <0.05 was considered statistically significant.

## RESULTS

### Characteristics of PTEN and HIF-1a expression in colorectal adenoma and adenocarcinoma

PTEN mRNA and HIF-1α mRNA expression was brown granular, and localized in cytoplasm of tumor cells (Figures 1, 2). HIF-1α mRNA expression was mainly localized in cytoplasm of tumor cells, frequently observed in margin of tumor necrotic zones. VEGF expression was mainly localized in cytoplasm of tumor cells, also observed in endothelial cell of blood vessel (Figure 3). Expression of PTEN mRNA, HIF-1α mRNA and VEGF protein was detected in 7, 4 and 3 cases of 9 colorectal adenomas respectively. PTEN mRNA expression was significantly higher ($P$<0.05) in adenomas than that in adenocarcinoma, but HIF-1α mRNA expression was significantly lower ($P$<0.05) in adenomas than that in adenocarcinoma. There was a significant difference ($P$<0.05) in VEGF expression between colorectal adenomas and adenocarcinoma.

### Correlation between PENT, HIF-1a, VEGF expressions and dukes stages of colorectal adenocarcinoma

Table 1 shows the correlation between PENT, HIF-1α, VEGF expression and Dukes stages of colorectal adenocarcinoma. For PTEN expression, there was a significant difference among Dukes stage A, B, C and D, and the level of PTEN expression was found to be significant higher in Dukes stage A or B than that in Dukes stage C+D. For HIF-1α expression, there was a significant difference between Dukes stage A and Dukes stage B, and the level of HIF-1α expression was found to be significantly higher in Dukes stage C+D than that in Dukes stage A or B. VEGF expression had same results as HIF-1α expression.

**Table 1** Correlation between expressions of PENT, HIF-1α, VEGF and Dukes stages of colorectal adenocarcinoma

| Dukes stage | $n$ | PTEN($\bar{x}\pm s$) | HIF-1 ($\bar{x}\pm s$) | VEGF($\bar{x}\pm s$) |
|---|---|---|---|---|
| A | 17 | 0.1782±0.0271[ab] | 0.1029±0.0457[ac] | 0.1207±0.0436[ac] |
| B | 18 | 0.1583±0.0397[b] | 0.1656±0.0329[c] | 0.1772±0.0514[c] |
| C+D | 27 | 0.1470±0.0524 | 0.2335±0.0748 | 0.2219±0.0803 |

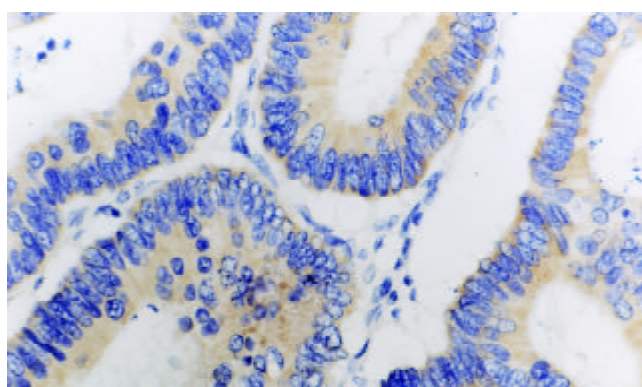[a]$P$<0.05, vs Dukes stage B; [b]$P$<0.05, vs Dukes stage C+D; [c]$P$<0.01, vs Dukes stage C+D.



**Figure 1** PTEN expression was observed in the cytoplasms of rectal tubular adenoma. Immunostaining, S-P method. ×400.
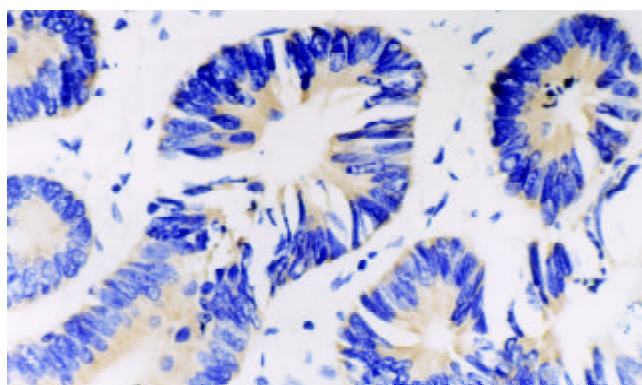


**Figure 2** HIF-1α expression was observed in rectal tubular adenoma. Immunostaining, S-P method. ×400.
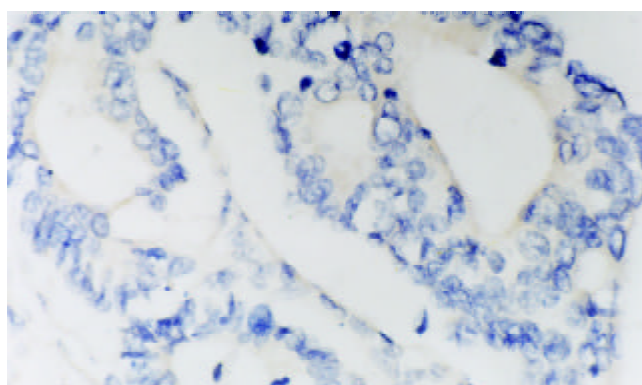


**Figure 3** Sigmoid carcinoma shows infiltration into the deep muscular layer, intermediated differentiation, in which VEGF expression was observed. Immunostaining, S-P method. ×400.

### Correlation between colorectal adenoma and adenocarcinoma in PTEN, HIF-1a and VEGF expression

The Spearman analysis showed that the level of PTEN was significantly associated with HIF-1α expression ($r$=-0.36, $P$<0.05) and with VEGF protein expression ($r$=-0.68, $P$<0.05) respectively. The level of HIF-1α was correlated with VEGF expression ($r$=0.72, $P$<0.01).

## DISCUSSION

PTEN (phosphatase and Tensin homologue deleted on chromosome 10) or MMAC1 (mutated in multiple advanced cancers 1) was recently reported to undergo frequent genetic alterations, including mutations and deletions in multiple advanced cancers[20-22]. PTEN located at chromosome 10q23.3

encodes a dual-specificity phosphatase that negatively regulates the phosphoinositol- 3-kinase(PI3K)/Akt (protein kinase B) pathway and mediates cell cycle arrest and apotosis[23]. PTEN protein contains sequence motifs with significant homology to the catalytic domain of protein phosphatases and to the cytoskeletal protein, tensin, and auxilin[24]. PTEN mutations and deletions were observed in a number of glioma, prostate, kidney and breast carcinoma cell lines or tumor specimens[25-27]. Rencently, Shin *et al*[28] screened the PTEN gene in 32 colorectal cancers (8 cell lines and 24 tissues), displaying microsatellite instability (MSI) and six frameshift mutations. We observed that PTEN mRNA decreased as the pathological stage increased, and was significantly associated with VEGF protein expression ($r$=-0.68, $P$<0.05) in colorectal adenoma and adenocarcinoma. These findings suggested that PTEN alteration was possibly involved in the tumor progression and formation of metastasis, and the roles of PTEN in tumor progression and metastasis may be correlated with VEGF expression. Hwang *et al*[29] observed that PTEN inhibited the tumorigenicity of B16F10 melanoma cells, and their results suggested that PTEN inhibited tumorigenicity and metastasis through regulating VEGF expression. Jiang *et al*[30] found that the overexpression of PTEN inhibited angiogenesis in chicken embryos, and that the PTEN overexpression inhibited the VEGF expression through the PI 3-kinase or Akt dependent pathway. Our results also indicated that PTEN played an important role by inhibiting VEGF expression in colorectal oncogenesis.

HIF-1 is a BHLH-PAS transcription factor that plays an essential role in $O_2$ homeostasis[31-34]. HIF-1 is a heterodimer composed of HIF-1$\alpha$ and HIF-1$\beta$ subunits[31]. HIF-1$\beta$ (also known as the aryl hydrocarbon receptor nuclear translocator) is a common subunit of multiple BHLH-PAS proteins, whereas HIF-1$\alpha$ is the unique, $O_2$-regulated subunit that determines HIF-1 activity[35,36]. HIF-1$\alpha$ activates the transcription of genes encoding transferrin, VEGF, endothelin-1 and inducible nitric oxide synthase, which are implicated in vasodilation, neovascularization, and tumor metastasis[36,37]. Many studies had identified that HIF-1$\alpha$ protein was overexpressed in multiple types of human cancer including lung, prostate, breast, and colon carcinomas, even in preneoplastic and premalignant lesions[38-42]. More importantly, Birner *et al*[43] found that the overexpression of HIF-1$\alpha$ is an important marker in precancerous lesion such as early-stage cervical cancer, cervical intra-epithelial neoplasia III, early stage lymph node-negative breast cancer. We also found that the levels of HIF-1$\alpha$ mRNA increased gradually as the pathologic stage increased, and were statistically significantly associated with VEGF expression. The same alterations were observed in other tumor tissues. Our findings further identified that HIF-1$\alpha$ and VEGF played an important role in the tumor angiogeneses and formations of metastases. In this study, we found that HIF-1$\alpha$ mRNA and VEGF were overexpressed in 4 and 3 cases of colorectal adenomas respectively, and suggested that cell hypoxia occurred prior to carciogenesis, and persisted to subsequent progression. Generally, these data suggested that HIF-1$\alpha$ overexpression may be an early stage of carciogenesis and it occurred prior to angiogenesis or invasion which morphologically confirmed. In this study, the expression of HIF-1$\alpha$ mRNA and VEGF was significantly higher in the tissues of Dukes stage C/D of colorectal adenocarcinoma than those in Dukes stage A/B, indicating that HIF was involved in the tumor invasion and formation of metastasis. Thus, we believe that the HIF-1$\alpha$ mRNA and VEGF overexpression is a strong independent prognostic marker in colorectal tumor.

That HIF-1 expression was activated and regulated by EGFR, HER2, and IGFR through PI3K/Akt/ FRAP (FKBP-rapamycin-associated protein) pathway had been identified, and a tumor suppressor gene PTEN regulated the HIF-1$\alpha$ expression and transcription activation by inhibiting PI3K/Akt/ FRAP pathway[44,45]. The loss of wild-type PTEN resulted in HIF-1$\alpha$ overexpression, and contributed to the formations of tumor angiogeneses in human prostate cancer and glioma. Our findings that the levels of HIF-1$\alpha$ were negatively correlated with VEGF expression and the level of PTEN were positively correlated with VEGF expression had further identified that loss of PTEN expression and increased levels of HIF-1$\alpha$ and VEGF may play an important role in carcinogenesis and progression of colorectal carcinoma.

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