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• BRIEF REPORTS •

Inactivation of PTEN is associated with increased angiogenesis and VEGF overexpression in gastric cancer

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Abstract

AIM: To investigate the expression of PTEN/MMAC$_1$/TEP$_1$ and vascular endothelial growth factor (VEGF), their roles in biologic behavior and angiogenesis and their association in gastric cancer.

METHODS: Immunohistochemical staining was used to evaluate the expression of PTEN, VEGF and microvascular density (MVD) on paraffin-embedded sections in 70 patients with primary gastric cancer and 24 patients with chronic superficial gastritis (CSG). Expression of PTEN, VEGF and MVD were compared with clinicopathological features of gastric cancer. The relationship between expression of PTEN, VEGF and MVD as well as the relationship between PTEN and VEGF expression in caner cells were investigated.

RESULTS: PTEN expression significantly decreased (t = 3.98, P<0.01) whereas both VEGF expression and MVD significant increased (t = 4.29 and 4.41, respectively, both P<0.01) in gastric cancer group compared with CSG group. PTEN expression was significantly down-regulated (t = 1.95, P<0.05) whereas VEGF expression (t = 2.37, P<0.05) and MVD (t = 3.28, P<0.01) was significantly up-regulated in advanced gastric cancer compared with early-stage gastric cancer. PTEN expression in gastric cancer showed a negative association with lymph node metastasis (t = 3.91, P<0.01), invasion depth (t = 1.95, P<0.05) and age (t = 4.69, P <0.01). MVD in PTEN-negative gastric cancer was significantly higher than that in PTEN-positive gastric cancer (t = 3.69, P<0.01), and there was a negative correlation between PTEN expression and MVD (γ = -0.363, P<0.05). VEGF expression was positively associated with invasion depth (especially with serosa invasion, t = 4.69, P<0.01), lymph node metastasis (t = 2.31, P<0.05) and TNM stage (t = 3.04, P<0.01). MVD in VEGF-positive gastric cancer was significantly higher than that in VEGF-negative gastric cancer (t = 4.62, P<0.01), and there was a positive correlation between VEGF expression of and MVD (γ = 0.512, P<0.05). VEGF expression in PTEN-negative gastric cancer was significantly stronger than that in PTEN-positive gastric cancer (t = 2.61, P<0.05), and there was a significantly negative correlation between the expression of VEGF and PTEN (γ = -0.403, P<0.05).

CONCLUSION: Our results imply that inactivation of PTEN gene and over-expression of VEGF contribute to the neovascularization and progression of gastric cancer. PTEN-related angiogenesis might be attributed to its up-regulation of VEGF expression. PTEN and VEGF could be used as the markers reflecting the biologic behaviors of tumor and viable targets in therapeutic approaches to inhibit angiogenesis of gastric cancers.

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INTRODUCTION

Gastric cancer is one of the tumors with a relatively high incidence and mortality in the digestive system. Although many advanced measures have been taken to improve the outcome of patients suffering from gastric cancer, up to now, there has been no radical progress in reducing its incidence and mortality. This could be due to the fact that the underlying mechanism in tumorigenesis and progression of gastric cancer is still poorly understood. Many studies have demonstrated that tumor suppressor genes, such as *p*53, play an important role in oncogenesis and progression of various malignancies. Recently, many investigators have been interested in the role of PTEN/MMAC$_1$/TEP1, a novel tumor suppressor gene, located on chromosome band 10q 23.3. Accumulated evidence has suggested that inactivation of PTEN/MMAC$_1$/TEP1 gene was implicated in the carcinogenesis and progression of various tumors[1-10]. Loss of PTEN expression was dominantly attributed to the inactive alteration of PTEN gene, including mutations, deletions, loss of heterozygosity (LOH) and promoter methylation in various malignancies[11,12], meaning that the expressive intensity of PTEN could almost embody the status of *PTEN* gene.

Several studies have strongly implied that PTEN was associated with tumor-induced angiogenesis[13-16]. However, less information regarding the role of PTEN in gastric cancer, to our knowledge, was available. The present study was designed to investigate the role of PTEN in tumorigenesis and progression of gastric cancer and its association with angiogenesis and VEGF expression.

MATERIALS AND METHODS

Subjects

Surgical specimens of 70 patients with histologically confirmed primary gastric adenocarcinoma and 24 patients with chronic superficial gastritis and duodenal ulcer were obtained from Department of General Surgery, Affiliated Hospital, Luzhou Medical College and Department of General Surgery, West China Hospital, Sichuan University in 1996-2000. There were 56 male and 14 female patients in gastric cancer group with age range from 31 to 66 (mean 45) years while there were 21 male and three female patients in chronic superficial gastritis group with age

range from 28 to 43 (mean 35) years. All patients with gastric cancer had received radical resection or palliative surgical treatments, but no one had ever accepted chemotherapy and radiotherapy as well as biotherapy before operation.

Evaluation of clinicopathological features

In gastric cancer group, 18 tumors were categorized histologically as well-differentiation and 52 poor-differentiations. Eight tumors were categorized as early-stage gastric cancer and 62 advanced gastric cancer. Thrifty two patients had serosa invasion, and 46 had lymph node metastasis. According to the criteria set out by the Union of International Caner Commission (new TNM stage, UICC for gastric cancer, 1985), 20 tumors were categorized as stage I, 16 stage II , 30 stage III and four stage IV.

Antibodies and reagents

Rabbit anti-PTEN polyclonal antibody, rabbit ant-VEGF polyclonal antibody, mouse anti-CD34 monoclonal antibody and streptavidin-biotin-peroxidase (KIT-9710, UltraSensitive S-P for mouse or rabbit) as well as DAB reagents were all purchased from Maixin Corporation (Fuzhou, Fujian province, China).

Immunohistochemical staining

Five-micron paraffin-embedded sections were dewaxed in xylene, dehydrated in ethanol. Endogenous peroxidase activity was blocked by incubation of samples in a 3% solution of hydrogen peroxide in methanol and heated in pressure-cooker for 50 s to retrieve antigens. After washing with PBS, the samples were incubated for 60 min with primary antibody against CD34, PTEN or VEGF at 37 ℃, for 60 min with the biotin-conjugated second antibody and for 10 min with the third antibody streptavidin-peroxidase at room temperature, and then the immunoreactive products were stained with DAB and counter-stained with methyl green subsequently. PBS was used instead of the primary antibodies for negative controls.

Evaluation of PTEN and VEGF immunostaining

PTEN and VEGF protein expression in benign and malignant gastric epithelium cells was assessed according to the score graded as the percentage of positive immunoreactive cells and the score graded as positive immunoreactive intensity, and the sum of both was used to reflect the level of PTEN and VEGF protein expression. The score graded as percentage of positive immunoreactive cells was defined as follows: <10% as 0, 10-25% as 1, 25-50% as 2, and ⩾50% as 3. The score graded as positive immunoreactive intensity was defined as follows: negative stain (equal to background) as 0, weak positive stain (weak yellow) as 1, positive stain (yellow) as 2, and strong positive stain (brown) as 3. The sum of scores less than or equal to 2 (⩽2) was defined as negative PTEN ($PTEN^-$) or VEGF ($VEGF^-$) protein expression, and more than or equal to 3 (⩾3) as positive PTEN ($PTEN^+$) or VEGF ($VEGF^+$) expression.

Microvascular density counting

Microvascular density (MVD) was determined according to the criterion introduced by Weidner[17]: any separated single vascular endothelium cell or cluster of endothelium cells and microvascular tube with diameter less than 8 erythrocytes were counted. Briefly, the stained sections were screened at ×100 magnifications under a light microscope (Olympus) to identify four regions with the highest number of microvessels, which were then counted at ×200 magnifications, and the average was used to reflect MVD.

Statistical analysis

All results were expressed as mean±SD. Statistic software SPSS 11.5 for Windows was used to analyze the results using Student *t* test and Pearson correlation analysis. The accepted level of significance was $P<0.05$ (Two-tailed).

RESULTS

Characteristics and comparison of PTEN and VEGF expression and MVD in gastric cancer with those in chronic superficial gastritis

PTEN and VEGF expression were demonstrated to localize in cytoplasm of gastric glandular epithelium and tumor cells (Figures 1, 2). In gastric cancer, the positive immunoreactive signal in invasive front region was weaker for PTEN, but stronger for VEGF and microvessels (Figure 3) than that in the fundic region of tumor. There was a significant down-regulation of PTEN expression and a significant up-regulation of both VEGF and MVD in gastric cancer in comparison with those in chronic superficial gastritis (Table 1).

Table 1 Comparison in PTEN and VEGF expression and MVD between chronic superficial gastritis (CSG) group and gastric cancer (GC) group (mean±SD)

Groups	*n*	PTEN	VEGF	MVD
CSG	24	3.13±2.3	1.25±1.16	29.6±9.9
GC	70	1.34±1.75[b]	2.94±1.80[b]	48.3±19.9[b]

[b]$P<0.01$ *vs* CSG group.

Association of PTEN and VEGF expression and MVD with clinicopathological profiles in gastric cancer

As showed in Table 2, The down-regulation of PTEN expression was closely associated with the older patients (>35 years), invasion depth and lymphatic metastasis of tumor, and a downtrend of PTEN expression was observed with the increase of invasion depth of tumor, especially in advanced stage of gastric cancer (⩾T_2) (Table 2). The up-regulation of both VEGF expression and MVD were significantly associated with the invasion depth, lymphatic metastasis and TNM stage, but none of them was associated with the histological differentiation of gastric cancer (Table 2).

Table 2 Association of PTEN and VEGF expression and MVD with clinicopathologic profiles in gastric cancer (mean±SD)

Variables	*n*	PTEN	VEGF	MVD
Age (yr)				
⩽35	22	2.64±1.81	2.77±1.96	46.23±17.61
>35	48	0.83±1.33[b]	3.02±1.76	49.20±20.05
Gender				
Male	56	1.32±1.69	3.00±1.91	48.45±20.39
Female	14	1.71±1.82	2.71±1.73	47.54±12.72
Differentiation				
Well	18	1.56±1.82	3.33±1.88	45.94±17.08
Poor	52	1.35±1.69	2.81±1.86	49.07±20.72
Invasion depth				
T_1	8	2.50±1.73	1.5±1.91	27.94±12.1
⩾T_2	62	1.26±1.69[a]	3.13±1.82[a]	50.9±19.31[b]
<T_3	38	1.47±1.81	2.08±1.20	39.6±16.6
⩾T_3	32	1.31±1.66	3.97±1.15[a]	58.6±18.9[b]
LN metastasis				
Positive	46	0.87±1.36	3.57±1.77	53.9±18.2
Negative	24	2.42±1.93[b]	1.74±2.07[a]	37.5±19.3[b]
TNM Stage				
I+II	36	1.83±1.81	2.51±1.29	39.6±18.3
III+IV	34	0.97±1.55	3.41±1.18[b]	57.5±17.7[b]

[a]$P<0.05$, [b]$P<0.01$ *vs* different variables.

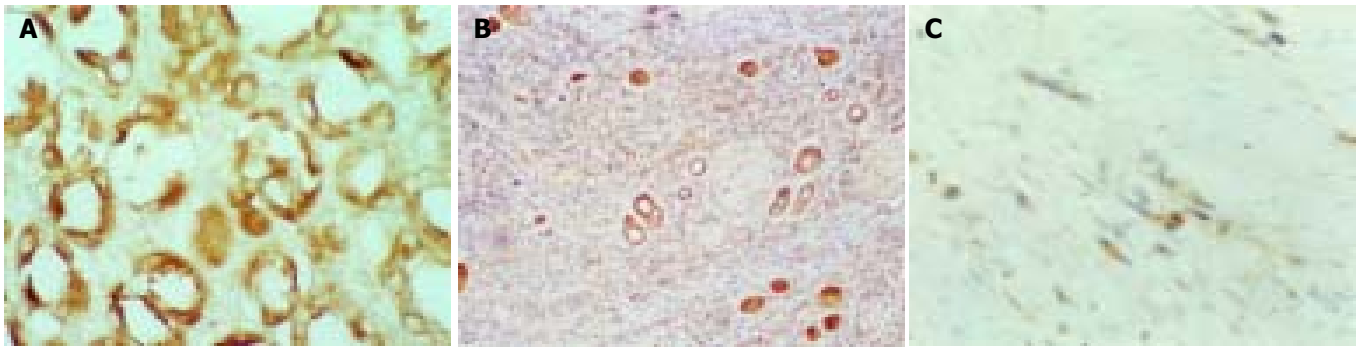

Figure 1 Expression of PTEN. A: Strongly positive expression in bening gastric glandular epithelium cells (Immunohistochemical staining, S-P×400). B: Positive expression in moderately differentiated cancer cells (Immunohistochemical staining, S-P×100). C: Weakly positive expression in poorly differentiated cancer cells. (Immunohistochemical staining, S-P×400).

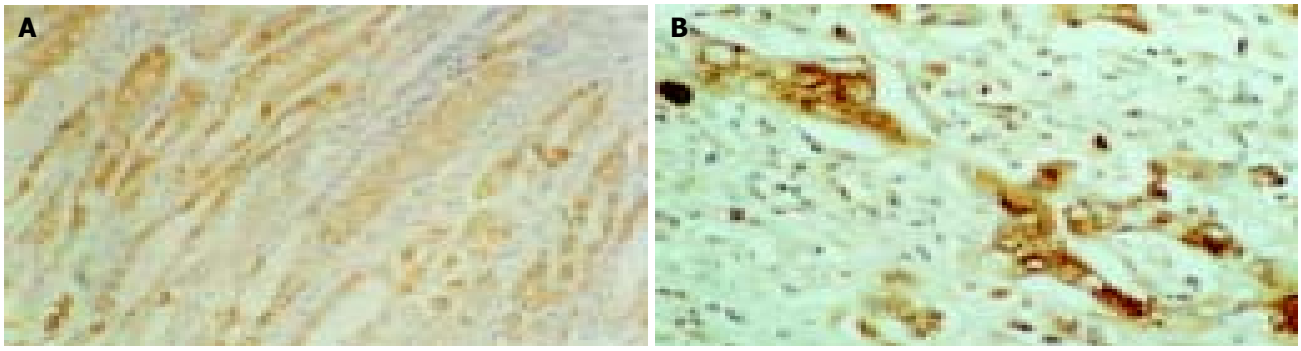

Figure 2 Expression of VEGF. A: Weakly positive expression in benign gastric glandular epithelium cells. B: Strongly positive expression in poorly differentiated cancer cells. (Immunohistochemical staining, S-P×400).

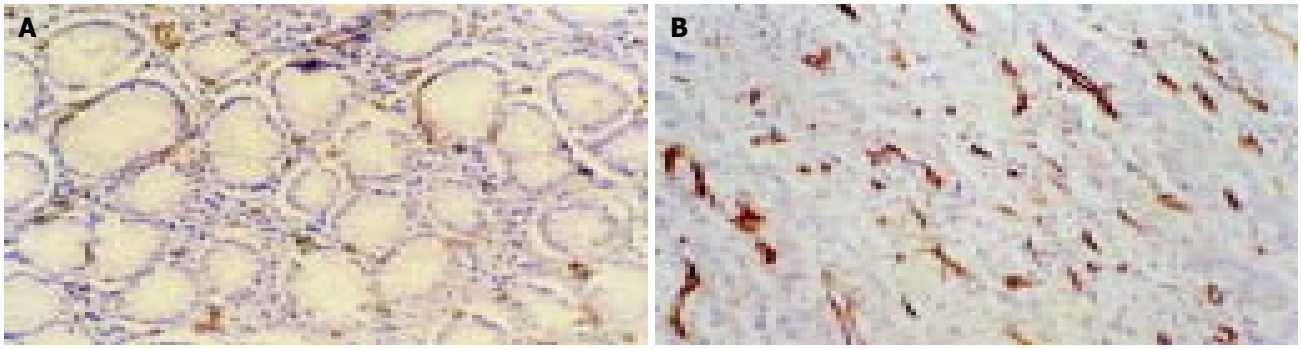

Figure 3 MVD in benign / malignant gastric tissues. A: Lessand weakly stained microvessel labeled by CD34 was regularly distributed among the benign gastric epithelium gland (Immunohistochemical staining, S-P×400). B: Increased and strongly stained microvessel labeled by CD34 was irregularly infiltrated in poorly differentiated adenocarcinoma (Immunohistochemical staining, S-P×200).

Correlation of PTEN and VEGF expression with MVD in gastric cancer

MVD was significantly higher in PTEN negative (n = 54) than in PTEN positive gastric cancer (n = 16) (52.7±19.6 *vs* 33.4±13.2, t = 3.69, P<0.01), and significantly higher in VEGF positive (n = 52) than in VEGF negative gastric cancer (n = 18) (52.71±19.59 *vs* 29.78±12.9, t = 4.62, P<0.001). There was a significantly negative correlation between MVD and the intensity of PTEN expression (γ = -0.363, P<0.05) and a positive correlation between MVD and the intensity of VEGF expression (γ = 0.512, P<0.01).

Correlation between VEGF and PTEN expression

The intensity of VEGF expression was significantly higher in PTEN negative than in PTEN positive gastric cancer (3.18±1.53 *vs* 2.15±2.19, t = 2.61, P<0.05). Furthermore, There was a significantly negative correlation between VEGF and PTEN expression (γ = -0.403, P<0.05).

DISCUSSION

PTEN/MMAC$_1$/TEP$_1$-encoding product, a dual-specificity protein-phospholipid phosphatase, which was involved in regulation of a variety of signal transduction pathways through dephosphorylation, including down-regulation of the activity of the focal adhesion kinase (FAK) to inhibit cell adhesion, invasion and metastasis[18-20], disabling of phosphatidylinositol 3-kinase (PI3'k)/Akt signal pathway to accelerate cell apoptosis and inhibit cell proliferation and inhibition of MAPK signal pathway to restrain cell differentiation[21-23]. Some studies[18-20,24-27] have demonstrated that anti-sense or deletion of PTEN gene significantly up-regulates the ability of cell proliferation, adhesion and migration, accompanied by increased activity of FAK and PI3'k/Akt, whereas over-expression of PTEN protein in wild-type PTEN transfected cells is detected, with a resultant cell cycle arrest, increased cell apoptosis, decreased potential in cell mitosis, proliferation, adhesion and migration, and the

concomitant decrease of FAK and PI3'k/Akt activities, suggesting that PTEN regulates negatively the growth, invasion and metastasis of tumors, and then the inactivation of PTEN greatly contributes to the tumorigenesis and progression of tumors.

So far, little information on the expression and role of *PTEN* gene in gastric cancer is available. Recently, Byun *et al.*[28] showed an abnormally low expression with only in 36% (20/55) gastric cancer tissues and 33% (5/15) cell lines while none of 71 cases with non-cancer tissues showed a decreased expression. The LOH of *PTEN* gene reached at 47% (14/30) and was closely linked to low expression of *PTEN m*RNA in gastric cancer. Furthermore, the rate of LOH was significantly higher in advanced gastric cancers (63%) than that in early-stage tumors (18%), and in poorly differentiated tumors (69%) than in well- or moderately differentiated tumors (29%). Fei *et al.*[29] compared PTEN expression in 26 gastric cancer, 21 first-degree relatives of gastric cancer patients and 12 healthy individuals by RT-PCR and immunohistochemistry, and found that PTEN expression was significantly decreased in gastric cancer group and the first-degree relatives group compared with those in matched non-malignant gastric tissues and healthy control group. Kang *et al.*[30] screened 310 cases with gastric carcinoma, and found that 62 cases lost PTEN expression, and the loss of PTEN expression was linked to the promoter methylation of *PTEN* gene, which was significantly associated with tumor depth and size, lymphatic invasion, advanced stage, pTNM stage, and patients' survival, implying that loss of PTEN expression is involved in the pathogenesis of gastric cancer. Nevertheless, there are some discrepant and conflict findings. One study from Japan did not find any mutation and promoter methylation of *PTEN* gene as well as the alteration of *m*RNA in gastric cancer cell lines and primary tumor tissues[31]. Another study showed that the mutation rate of *PTEN* gene was not significantly increased in human advanced gastric cancer[32], suggesting that PTEN does not participate in gastric carcinogenesis and progression as a tumor suppressor gene. In current study, PTEN expression was only slightly decreased in early gastric cancer cells but significantly decreased in advanced gastric cancer cells, compared with that in benign gastric epithelial cells. PTEN expression decreased as the invasion density increased, and lower PTEN expression was observed in gastric cancer with lymph node invasion and in TNM III/IV stage. Our results were very similar to those reported by Kang *et al.*[30], suggesting that loss of PTEN expression is a relatively later molecule event in the pathogenesis of gastric cancer, and thus plays more important role in the progression than in oncogenesis of gastric cancer. The malignant gastric epithelial cells with loss of PTEN expression may hold the characteristics with a high aggressive and metastatic potential, and thus PTEN can be considered as an objective and reliable marker reflecting the pathobiological behaviors of gastric cancer. Moreover, Lee *et al.*[33] showed that loss of PTEN expression was significantly associated with poor gastric carcinoma prognosis. In addition, we also observed a significantly decreased expression of PTEN protein in the older patients (>35 years) with gastric cancer compared with the younger patients, implying that PTEN may be more implicated in the gastric carcinogenesis in the elder. In other words, there might hold dissimilar tumorigenetic mechanisms between the older and younger patients with gastric cancer.

Angiogenesis is prerequisite for progressing tumor growth, invasion and metastasis. MVD in tumor tissue is unanimously considered as a better parameter to reflect the level of the neovascularization of tumor. VEGF, one of the most powerful pro-angiogenic factors, is dominantly involved in all the process including vascular endothelial cell mitosis, proliferation, adhesion and migration. VEGF expression is induced in a hypoxia-inducible factor alpha (HIF-1alpha)-dependent way through activation of the PI3 kinase signaling pathway[34]. VEGF and MVD are all involved in prognosis in various carcinomas[35-38]. Our results revealed a significantly increase in MVD and VEGF expression in gastric cancer cells, and both the VEGF expression and MVD were significantly associated with the invasion depth, lymphatic metastasis and pTNM stage. Furthermore, MVD was significantly correlated with the intensity of VEGF expression. These results suggest VEGF is dominantly involved in the neovascularization of gastric cancer, and thus facilitates the tumor's growth, invasion and metastasis.

Several studies[13-16] have suggested that PTEN is implicated in the regulation of tumor's angiogenesis, and loss of PTEN expression is closely associated with the increased neovascularization in various malignancies *in vitro* and *in vivo*. However, information on the relationship between PTEN protein expression and neovascularization in gastric cancer is scarce. Zheng *et al.* recently showed MVD was negatively related to PTEN expression in gastric cancer. In the present study, we also observed that MVD in PTEN negative gastric cancer was markedly higher than that in PTEN positive gastric cancer, and there was a significantly negative correlation between MVD and PTEN expression in gastric cancer tissue, implying that loss of PTEN expression is highly implicated in the neovascularization of tumor in gastric cancer.

The mechanism of PTEN-related angiogenesis is not well known. Recent *in vitro* studies[39-41] have suggested that loss of PTEN expression significantly up-regulates VEGF expression *via* modulation of HIF-1alpha expression and VEGF-mediated pro-angiogenic signaling through PI3'K/Akt-dependent signaling transduction pathway to enhance the anti-apoptotic, proliferative, and chemotactic activity of endothelial cells and the ability of tube formation. Jiang *et al.* screened the expression of *PTEN* and *HIF-1alpha* mRNA and VEGF protein in human colorectal tumor tissues, and observed a negative correlation of *PTEN* expression with *HIF-1alpha* (γ = -0.36, P< 0.05) and VEGF (γ = -0.48, P<0.05) and a positive correlation between VEGF and *HIF-1alpha* (γ = 0.71, P<0.01). In this study, we also found a significantly increase in VEGF expression in PTEN negative gastric cancer compared with PTEN positive gastric cancer, and that the intensity of VEGF expression was negatively associated with PTEN expression. Taking all these findings together, we postulate that VEGF is a key downstream molecule for PTEN function in carcinogenesis and progression in a wide range of human carcinomas including gastric cancer. Therefore, inactivation of *PTEN* gene could contribute to gastric tumor progression by directly functioning on tumor cells to enhance the ability of growth, anti-apoptotic, invasion and metastasis through up-regulation of PI3'K/Akt signaling transduction pathway, by up-regulating VEGF expression in tumor cells, which enhances the activity of tumor-derived angiogenesis and functions on vascular endothelial cells to increase angiogenesis in tumor tissues. Based on these data, it is concluded that PTEN and VEGF are reliable targets in the therapeutic approach for the inhibition of angiogenesis in gastric cancer.

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