Analysis of p53 and vascular endothelial growth factor expression in human gallbladder carcinoma for the determination of tumor vascularity

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INTRODUCTION

Angiogenesis refers to the formation of new blood vessels from a pre-existing vascular network. Adequate vascularization is critically required for the growth and metastasis of human solid tumors. Angiogenesis is influenced by multiple factors such as growth factors, extracellular matrix proteins, and cell adhesion molecules, as well as by the imbalance between angiogenesis-stimulating factors and inhibiting factors. One of the most important angiogenesis-stimulating factors is VEGF, a diffusible endothelial cell-specific mitogen that induces endothelial-cell proliferation and increases vascular permeability. There is evidence that it may promote a degradative environment that facilitates migration of endothelial cells by the conduction of plasminogen activators and collagenase. VEGF plays a major role in regulating angiogenesis. Most tumors produce high levels of VEGF. Neutralization of VEGF leads to a marked inhibition of angiogenesis and tumor growth. However, there is little information about the genetic changes associated with angiogenesis. So far, most studies have stressed the role of oncogene activation and tumor suppressor gene (TSG) inactivation in promoting aberrant tumor-cell proliferation. Although it remains unclear whether any of these genetic alterations can trigger the disruption of control of angiogenesis, some in vitro studies have demonstrated the important role played by the p53 tumor suppressor gene in controlling tumor angiogenesis. In the present study, we have examined p53 and VEGF expressions as well as microvessel count (MVC) in human gallbladder carcinoma tissues to investigate the involvement of the p53 gene in regulation of tumor angiogenesis and its clinical significance.

BASIC RESEARCH

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Abstract

AIM: To examine the expression of p53 and vascular endothelial growth factor (VEGF) as well as microvessel count (MVC) and to investigate the role of VEGF as an angiogenic marker and the possible role of p53 in the regulation of angiogenesis in human gallbladder carcinoma.

METHODS: Surgically resected specimens of 49 gallbladder carcinomas were studied by immunohistochemical staining for p53 protein, VEGF, and factor VIII-related antigen. VEGF expression and mutant p53 expression were then correlated with Nevin stage, differentiation grade, MVC, and lymph node metastasis.

RESULTS: Positive p53 protein and VEGF expressions were found in 61.2% and 63.3% of tumors, respectively. p53 and VEGF staining status was identical in 55.1% of tumors. The Nevin staging of p53- or VEGF-positive tumors was significantly later than that of negative tumors. The MVC in p53- or VEGF-positive tumors was significantly higher than that in negative tumors, and MVC in both p53- and VEGF-negative tumors was significantly lower than that in the other subgroups.

CONCLUSION: Our findings suggest that p53-VEGF pathway can regulate tumor angiogenesis in human gallbladder carcinoma. Combined analysis of p53 and VEGF expression might be useful for predicting the tumor vascularity of gallbladder cancer.
MATERIALS AND METHODS

Clinical materials
Forty-nine histologically proven gallbladder carcinomas were selected. All patients were surgically treated at the Department of General Surgery of the First and Second Hospitals affiliated to China Medical University, Shenyang, China, but did not receive chemotherapy or anti-angiogenesis therapy before surgery. The cases included 24 males and 25 females. The average age of the males and females was 62 years and 55 years, respectively.

Six cases had papillary adenocarcinoma (12.2%), 43 cases had tubular adenocarcinoma (87.8%), 22 cases had well-differentiated tumor (44.9%), 17 cases had moderately differentiated tumor (34.7%), 10 cases had poorly differentiated tumor (20.4%). Nevin stage (Table 1) was determined based on clinical materials: 19 cases of S1, S2, and S3, and 30 cases of S4 and S5. Twenty-seven cases (55.1%) had lymph node metastasis (+), 22 cases (44.9%) had no lymph node metastasis (-). In each case, all available sections stained with hematoxylin and eosin were reviewed.

Immunohistochemical study of p53 and VEGF
Four micrometer thick sections from the formalin-fixed and paraffin-embedded tissues were placed on the poly-L-lysine-coated slides for immunohistochemistry.

Immunohistochemical staining was performed by the streptoavidin-biotin method. In brief, sections were deparaffinized and incubated with 3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. The sections were treated twice with microwave at 500 W for 5 min each time in 10 mmol/L sodium citrate (pH 6.0). After washing with PBS, the sections were incubated in 10% normal rabbit or goat serum for 20 min to reduce non-specific antibody binding. The antibodies used were mouse monoclonal antibody (MAb) against human p53 protein (Maxin-Bio Co., Fuzhou, China) in 1:100 dilution at 4 °C overnight, and a rabbit polyclonal antibody against human VEGF (A-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 1:50 dilution at 4 °C overnight. After washing thrice with PBS, the sections were incubated with biotinylated rabbit anti-mouse or goat anti-rabbit immunoglobulin G (Maxin-Bio Co., Fuzhou, China) for 30 min, washed thrice again with PBS, treated with streptavidin-peroxidase reagent for 30 min and then washed thrice with PBS again. Finally, the specimens were incubated in PBS containing diaminobenzidine and 1% hydrogen peroxide for 5 min and counterstained with hematoxylin. PBS was substituted for each primary antibody as negative control.Slides were examined by two investigators without the knowledge of the corresponding clinicopathologic data. p53 immunoreactivity was assessed as being positive only when tumors exhibited intense nuclear staining, and reactivity was categorized into negative expression (less than 10% positive tumor cells) and positive expression (at least 10% positive tumor cells). Immunostaining for VEGF was considered positive when unequivocal staining of cell membrane or cytoplasm was observed in more than 10% of tumor cells.

Microvessel staining and counting
Microvessel staining and counting were performed as described previously. Briefly, intratumoral microvessels were highlighted by immunostaining with a mouse MAb against factor VIII-related antigen (F-VIII RAg) (Maxin-Bio Co., Fuzhou, China) in 1:100 dilution and incubated at 4 °C overnight, after pre-digestion with 0.1% (v/v) trypsin at 37 °C for 20 min. Any single brown-stained cell or cluster of endothelial cells that was clearly separated from adjacent vessels, tumor cells and other connective tissues was considered as a microvessel. The stained sections were screened at ×40 fields to identify the regions of the high-stage gallbladder cancer.

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Table 1 Nevin staging system for gallbladder cancer

| Stage | Definitions |
|-------|-------------|
| 1     | Tumor invades mucosa only |
| 2     | Tumor invades muscularis and mucosa |
| 3     | Tumor invades subserosa, muscularis, and mucosa |
| 4     | Tumor invades all layers of gallbladder wall plus cystic lymph node |
| 5     | Tumor invades into liver bed or distant spread |

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Figure 1 Microvessel distribution (A) and expression of VEGF (B) and p53 (C) in gallbladder carcinoma tissue.
The average MVC in 49 cases of gallbladder carcinoma was \((35 \pm 12)/\text{HP}\). MVC was markedly higher in cases of Nevin stage S4-S5 than in those of S1-S3 \((P<0.01)\). MVC in moderate-poor differentiation group was higher than that in good differentiation group \((P<0.05)\). MVC in patients with lymph node metastasis was significantly higher than that in those without lymph node metastasis \((P<0.01, \text{Table 2})\).

The positive rate of VEGF expression was 63.3% in these 49 cases and was higher in cases of Nevin stage S4-S5 (76.7%) than in those of S1-S2 (42.1%) \((P<0.05)\). The positive rate of VEGF expression was not correlated with tumor differentiation \((P>0.05)\). With regard to the association with lymph node metastases, the positive rate of VEGF expression was significantly higher in patients with metastasis than in those without metastasis \((P<0.05, \text{Table 3})\).

The positive rate of mutant p53 expression was 61.2% in these 49 cases and was lower in cases of Nevin stage S1-S3 (47.4%) than in those of S4-S5 (70.0%), the difference was statistically significant \((P<0.05)\). The positive rate of mutant p53 expression was not correlated with tumor differentiation \((P>0.05)\). With regard to the association with lymph node metastases, the positive rate of p53 expression was significantly higher in patients with metastasis than in those without metastasis \((P<0.05, \text{Table 4})\).

Table 2 Clinicopathologic characteristics of MVC in gallbladder carcinoma (mean ± SD)

| Characteristics         | n  | MVC       |
|-------------------------|----|-----------|
| Tumor differentiation   |    |           |
| Good                    | 22 | 30 ± 11   |
| Moderate-poor           | 27 | 38 ± 11*  |
| Nevin staging           |    |           |
| S1, S2, S3              | 19 | 26 ± 11   |
| S4, S5                  | 30 | 40 ± 8    |
| Lymph node metastasis   |    |           |
| Yes                     | 27 | 40 ± 10   |
| No                      | 22 | 28 ± 11   |

\(^{a}P<0.05\) vs good group; \(^{b}P<0.01\) vs “S1, S2, S3” group; \(^{c}P<0.01\) vs yes group.

Table 3 Clinicopathologic characteristics of VEGF expression in gallbladder carcinoma

| Characteristics         | n  | +     |
|-------------------------|----|-------|
| Tumor differentiation   |    |       |
| Good                    | 22 | 15    |
| Moderate-poor           | 27 | 16    |
| Nevin staging           |    |       |
| S1, S2, S3              | 19 | 8     |
| S4, S5                  | 30 | 23    |
| Lymph node metastasis   |    |       |
| Yes                     | 27 | 21    |
| No                      | 22 | 12    |

\(^{a}P<0.05\) vs “S1, S2, S3” group; \(^{b}P<0.05\) vs yes group.

Table 4 Clinicopathologic characteristics of mutant p53 expression in gallbladder carcinoma

| Characteristics         | n  | +     |
|-------------------------|----|-------|
| Tumor differentiation   |    |       |
| Good                    | 22 | 13    |
| Moderate-poor           | 27 | 17    |
| Nevin staging           |    |       |
| S1, S2, S3              | 19 | 9     |
| S4, S5                  | 30 | 21    |
| Lymph node metastasis   |    |       |
| Yes                     | 27 | 20    |
| No                      | 22 | 10    |

\(^{a}P<0.05\) vs “S1, S2, S3” group; \(^{b}P<0.05\) vs yes group.

Table 5 Relationship between MVC and presence of p53 and VEGF (mean ± SD, \(n\)%)

| Patients, VEGF(+) | VEGF(-) | MVC       |
|-------------------|---------|-----------|
| p53(+)            | 27 (55.1%) | 3 (6.1%) | 30 (61.2%) |
| p53(-)            | 4 (8.2%) | 15 (30.6%) | 19 (38.8%) |
| Total             | 31 (63.3%) | 18 (36.7%) | 49 (100%) |

\(^{a}P<0.05\) vs others; \(^{b}P<0.05\) vs “VEGF(+)” group; \(^{c}P<0.05\) vs “p53(+)” group.

Statistical analysis

The relationship between p53 or VEGF expression and MVC was evaluated by \(\chi^{2}\)-test, and the relationship between p53 and VEGF expression and various clinicopathologic factors was examined by the \(\chi^{2}\) test. \(P<0.05\) was considered statistically significant.

RESULTS

Expression of VEGF, p53, and MVC

The microvessels in malignant tissues were heterogeneously distributed. These neovascular areas occurred anywhere within the tumor but most frequently at the tumor margins (Figure 1A). The immunoreactive regions of VEGF were located in cytoplasms or membranes of the gallbladder carcinoma cells (Figure 1B). The immunoreactive regions of mutant p53 were located in the nuclei of the gallbladder carcinoma cells (Figure 1C).

Clinicopathologic characteristics of MVC, VEGF, and p53 expressions

The average MVC in 49 cases of gallbladder carcinoma was \((35 \pm 12)/\text{HP}\). MVC was markedly higher in cases of Nevin stage S4-S5 than in those of S1-S3 \((P<0.01)\). MVC in moderate-poor differentiation group was higher than that in good differentiation group \((P<0.05)\). MVC in patients with lymph node metastasis was significantly higher than that in those without lymph node metastasis \((P<0.01, \text{Table 2})\).

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The positive rate of mutant p53 expression was 61.2% in these 49 cases and was lower in cases of Nevin stage S1-S3 (47.4%) than in those of S4-S5 (70.0%), the difference was statistically significant \((P<0.05)\). The positive rate of mutant p53 expression was not correlated with tumor differentiation \((P>0.05)\). With regard to the association with lymph node metastases, the positive rate of p53 expression was significantly higher in patients with metastasis than in those without metastasis \((P<0.05, \text{Table 4})\).

Correlation between p53, VEGF, and MVC

Table 5 shows the relationships between MVC and the presence of p53 and VEGF. Staining status was identical in 27 of 49 tumors (55.1%), and a significant \((P<0.05)\) association between p53 and VEGF expression was demonstrated. The MVC in tumors that were p53 or VEGF positive was significantly \((P<0.05)\) higher than that in p53- or VEGF-negative tumors. Moreover, the...
mean MVC in tumors of all the subgroups that were both p53 and VEGF positive was the highest, while the mean MVC was significantly lower in patients with tumors that were both p53 and VEGF negative than in all the other subgroups.

**DISCUSSION**

In 1971, Folkman proposed that tumor growth depends on angiogenesis. Now there is considerable indirect and direct evidence that tumor growth is dependent on angiogenesis. Numerous studies showed that neovascularization is closely associated with growth, invasion, metastasis, staging and prognosis of tumors\(^1,2,8\). Our study also indicated that angiogenesis was correlated with the Nevin staging and tumor differentiation. The higher the staging is, the poorer the differentiation and the higher the level of MVC are in gallbladder carcinoma. Since MVC is of prognostic value, we investigated whether this holds true for VEGF expression as well. Our findings demonstrate that both VEGF expression and MVC are significantly elevated in patients with disease progression and depend on each other. Tumors with established poor outcome (staging, MVC) are likely to produce higher levels of VEGF, suggesting that VEGF significantly contributes to the poor outcome in these patients.

The most common genetic alteration in various cancers is loss of the p53 tumor suppressor gene function. P53 protein has various important functions in cellular integration, including cell growth control, response to DNA damage, checkpoint mechanisms during the cell cycle, regulation of transcription and control of genomic stability. It is suggested that p53 protein may play a role in suppressing angiogenesis\(^2,4,19\). In addition, it was reported that a pathway from p53 regulating VEGF induces angiogenesis in cellular transfection models\(^16\). In the present study, we explored the possibility of a relationship between aberrant p53 and VEGF expression and tumor angiogenesis in gallbladder carcinoma. We observed that VEGF-positive tumors had significantly more microvessels than VEGF-negative tumors. Our findings agree with other studies\(^12,13,26\), suggesting that VEGF-induced tumor angiogenesis plays an important role not only in tumor progression but also in metastasis. Although little is known concerning the VEGF regulatory pathways, Kieser et al.\(^14\) reported that mutant-type p53 might stimulate VEGF expression. In the present study, p53 and VEGF expression status coincided in 27 of 49 (55.1\%) tumors, and a significant association was found between the two factors. Moreover, MVC was the highest in p53 and VEGF positive tumors and the lowest in p53 and VEGF negative tumors. These results suggest not only the existence of a p53-VEGF regulatory pathway in gallbladder carcinomas, but also a possible role of such a pathway in regulating tumor angiogenesis in this type of cancer. These further suggest that tumors that have lost p53 suppressor function not only permit uncontrolled cell growth, but also possibly produce a suitable environment for hematogenous metastasis by stimulating VEGF-induced angiogenesis in the primary site of carcinoma. In contrast, tumors with preserved p53 function and angiogenic inhibitory pathway are less possible to progress, and the p53 gene may therefore contribute to better prognosis.

In conclusion, our results demonstrate that abnormal p53 accumulation is closely associated with VEGF expression and tumor vascularity in human gallbladder carcinoma. P53 plays a critical role in suppressing tumor growth by regulating tumor angiogenesis. Clinical application of combined analysis of p53 and VEGF expression may be useful for predicting the clinical staging, occurrence of metastasis in patients with this disease. More prospective studies should be made to prove our findings.

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