Study on the Expression and Significance of TGF-β1, p-ERK1/2 and K-ras in Colorectal Cancer Using Tissue Microarray Technique

Xin HU1
Yu-ting KUANG2
Mao-min SUN3
Ying-ying WANG1
Yu-juan ZHANG1
Ling-ling GUO1
Shou-li WANG1

1 Department of Pathology, Medical School of Soochow University, Suzhou 215123, Jiangsu Province, China.
2 Department of Surgery, The First Hospital Affiliated to Soochow University, Suzhou 215123, Jiangsu Province, China.
3 Department of Anatomy, Medical School of Soochow University, Suzhou 215123, Jiangsu Province, China.

Corresponding to: Shou-li WANG
Tel: 86-512-6588 0129
Fax: +86-512-6588 0103
E-mail: wangsoly112@hotmail.com

OBJECTIVE This study aimed to explore the expression and significance of transforming growth factor β1 (TGF-β1), extracellular signal-regulated kinases 1/2 (ERK1/2), and K-ras in colorectal cancer (CRC) using tissue microarray technology.

METHODS The expressions of TGF-β1, ERK1/2, and K-ras in colon cancer cells taken from the specimens of 92 CRC patients (stage I: 16 cases, stage II: 28 cases, stage III: 24 cases, and stage IV: 24 cases) were analyzed using tissue microarray technology and immunohistochemistry, and compared with those of 20 normal colon tissue samples.

RESULTS High immunoreactive scores (IRS) of TGF-β1, p-ERK1/2, and K-ras protein in CRC were obtained, which were 66.3% (61/92), 59.8% (55/92), and 48.9% (45/92), respectively, and those in normal epithelial cells of colon were 10% (2/20), 20% (4/20), and 30% (6/20), respectively (P < 0.05). The expressions of TGF-β1 and ERK1/2 in CRC at stage I were 37.5% and 31.3%, respectively, and those in CRC at stage IV were 83.3% and 79.3%, respectively, with statistically significant differences. No significant relationship was found between K-ras expression and tumor stages (P > 0.05).

CONCLUSION High level expressions of TGF-β1 and ERK1/2 are closely related to the clinical stages of colon cancer and cross-talk may exist between the 2 signal pathways.

KEY WORDS: colorectal cancer, TGF-β1, ERK1/2, K-ras, tissue microarray technique.

Introduction

Colon cancer is one of the most common malignant tumors, and its incidence is the third to fourth highest in China and has increased yearly. In recent years, the 5-year survival rate of colon cancer has risen as the integrated treatment with surgery, radiotherapy and chemotherapy has been applied for the patients. However, the primary cause of death in the patients after the treatment is metastasis, which has been affected by multiple factors[1,2]. At present, evaluation of the prognosis for a patient with tumor is mainly based on the TNM (Tumor-Node-Metastasis) system. However, a number of patients who are considered to be at the same stage/grade have distinctly different survival time. The application of new bimolecular might potentially supplement traditional staging. Tissue microarray (TMA)
technology is employed by arranging a number of tiny tissue sections neatly on one carrier and its advantage is that small amount of samples convey a large amount of information[3,4]. A broad spectrum of research has shown that tissue microarray technology has been widely used in tumor pathology research and in biomolecular screening[4,5].

The transforming growth factor-beta1 (TGF-β1) is known to regulate a large number of biological processes, such as proliferation, differentiation and survival. In recent years, increased evidence supports the notion that signaling cross-talk between TGF-β and other pathways plays crucial roles in the pathogenesis of a variety of diseases[6]. Mitogen-activated protein kinase (MAPK), including Erk1/2, JNK1/2/3, and p38/ MAPKs, are essential for a variety of cellular events. Activated MAPKs phosphorylate a battery of nuclear transcription factors with diverse functions in regulating proliferation, survival, migration, and so on[7]. One of the best characterized trigger for this MAPK pathway is Ras activation, which propagates signals from a number of ligand- or self-activated receptor tyrosine kinases (RTKs)[8]. In addition, MAPKs can also be regulated by TGF-β stimulation[9], which was consistent with our previously published studies[10]. This study employed the method of immunohistochemistry (S-P) combined with tissue microarray technology to explore the expressions of transforming growth factor β1 (TGF-β1), extracellular signal-regulated kinases 1/2 (ERK1/2), and K-ras in 92 wax-embedded colorectal cancer (CRS) specimens, and then analyzed the correlation between these biomarkers and the clinical stages of the tumor.

Materials and Methods

Materials

Ninety-two CRS and 20 normal colon tissue samples were obtained from the Affiliated Hospital of Suzhou University. The clinical data such as sex, mean age, tumor location, and grading are shown in Table 1. All specimens were embedded in paraffin conventionally, cut into 4-mm-thick sections and then stained with hematoxylin-eosin. One mm in diameter of tissue was used after being collected by needle (Beecher Instruments).

Conduction of tissue microarray

After conducting anti-off-microarray process by polylysine, the H&E-stained sections were observed under the microscope to confirm the location of the tumor tissue and then marked the areas of the tumor location in the tissue wax block corresponding to the location observed in the H&E sections. Each recipient wax block was designed with the dimensions of 8 × 7 to form an array organized into 56 points, and there were a total of 2 recipient wax blocks. Tissue columns with 1 mm in diameter and 4 mm in height taken from the marked positions of the donor wax block, were placed into the corresponding holes of acceptor block. After that, the tissue columns were inoculated in an incubator at 60°C for about 1 h, and then cooled at room temperature so as to make sections of wax blocks. Next, the sections were mounted on 10% polylysine-treated glass slides, dried at room temperature, and fully baked in dry oven at 65°C for 30 min. Finally, they were incubated at 60°C overnight.

Immunohistochemistry staining

Sections were deparaffinized, rehydrated, and incubated with 0.3% hydrogen peroxide (H₂O₂) in methanol for 30 min to block the endogenous peroxidase activity. The slides were then exposed to rabbit anti-TGF-β1, ERK1/2 (Santa Cruz Biotechnologies, USA) and K-ras (Abcam, UK) diluted at 1:50 in a buffer containing 10% fetal bovine serum. After incubation with the primary antibody for 30 min, the slides were incubated with biotinylated anti-immunoglobulins for 20 min and then incubated with biotinylated peroxidase-streptavidin complex (ABC-kit, DAKO Cytomation, Glostrup, Denmark) for another 20 min. The sections were then stained for 5 min with 3, 3’-diaminobenzidine (DAB) freshly prepared in 0.05 M Tris-HCl buffer at pH7.6. After counterstaining with haematoxylin, the sections were dehydrated and mounted. The expression levels of these biological markers were classified according to the following grading system: a positive rate was categorized as 0 (no positive cells), 1 (positive cells ≤ 25%), 2 (25% < positive cells ≤ 50%), and 3 (positive cells > 50%). The stain intensity was categorized as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Each category was multiplied to get a total immunoreactive score (IRS). IRS 0-3 reflected low expression of the biomarkers, whereas IRS 7-9 meant high expression.

Statistical analysis

The data were presented with mean and standard deviation. Analyses were carried out using SPSS 10.0 (SPSS Inc, Chicago, L, USA). The differences between the groups were compared using the paired Student’s t test. A P value less than 0.05 was considered significant.

Results

Clinicopathological features

The clinical characteristics of the 92 patients are shown in Table 1. The patients included 28 females and 64 males, with the mean age of 65.25 years (ranging from 49 to 81) when receiving initial colorectal surgery. The degrees of tumor differentiation of the patients were defined based on the American Joint Committee on Cancer[11], which were demonstrated as follows: high grade in 11, middle grade in 68, low grade in 9, and unknown grade in 4.
The quality of tissue microarray
After staining the samples with conventional H&E, the normal colon tissue and the tissue microarray of colon cancer were observed to have representative histological structures under a microscope (Fig.1). The immunohistochemical stain of the most microarray samples was representative (Figs.2-4), with the exception of a few parts of tissue microarray that dropped during restoring antigen in immunohistochemical staining.

The expressions of TGF-β1, p-ERK1/2 and K-ras in CRC
Fig.2-4 illustrates representative examples of TMA immunostaining for TGF-β1, p-ERK1/2, and K-ras in CRC. The normal colonic epitheliums showed weak stain of TGF-β1 and p-ERK1/2 and a negative to weak stain of K-ras in their membrane and cytoplasm. There was strong cytoplasmatic expression of TGF-β1 in cells of CRC (Fig.2). Apart from staining in tumor cells, a slight positivity was further found in inflammatory cells. We compared p-ERK1/2 and K-ras expression profiles in CRC and normal epithelium tissue. p-ERK1/2, and K-ras in cancer cells were stained more intensely than those were observed in normal epithelial cells ($P < 0.05$). Immunohistochemical scores and the results of the statistical analysis are presented in Table 2.

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**Table 1. Summary of clinical characteristics of the 92 patients undergoing surgery for colorectal cancer.**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Stage I ($n = 16$)</th>
<th>Stage II ($n = 28$)</th>
<th>Stage III ($n = 24$)</th>
<th>Stage IV ($n = 24$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>12:4</td>
<td>18:10</td>
<td>17:7</td>
<td>17:7</td>
</tr>
<tr>
<td>Mean age, years (range)</td>
<td>65.4 (56-75)</td>
<td>67.4 (49-81)</td>
<td>65.0 (59-71)</td>
<td>63.2 (53-74)</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>3</td>
<td>4</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Cecum</td>
<td>11</td>
<td>16</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sigmoid</td>
<td>2</td>
<td>8</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Grading</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G2</td>
<td>7</td>
<td>24</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>G3</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Unknown</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
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</table>


**Table 2. Frequency of different levels of biological marker expression**

<table>
<thead>
<tr>
<th>Items</th>
<th>$n$</th>
<th>IRS of TGF-β1 cases (%)</th>
<th>IRS of p-ERK1/2 cases (%)</th>
<th>IRS of K-ras cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>CRC</td>
<td>92</td>
<td>16 (17.4)</td>
<td>61 (66.3)*</td>
<td>18 (19.6)</td>
</tr>
<tr>
<td>Normal colonic epithelium</td>
<td>20</td>
<td>14 (70.0)</td>
<td>2 (10.0)</td>
<td>12 (60.0)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>64</td>
<td>12 (18.8)</td>
<td>42 (65.6)</td>
<td>11 (17.2)</td>
</tr>
<tr>
<td>Female</td>
<td>28</td>
<td>4 (14.3)</td>
<td>19 (67.9)</td>
<td>7 (25.0)</td>
</tr>
<tr>
<td>Degree of differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>9</td>
<td>2 (22.2)</td>
<td>4 (44.4)</td>
<td>3 (33.3)</td>
</tr>
<tr>
<td>G2</td>
<td>68</td>
<td>12 (17.6)</td>
<td>32 (47.1)</td>
<td>13 (19.1)</td>
</tr>
<tr>
<td>G3</td>
<td>11</td>
<td>2 (18.2)</td>
<td>5 (45.5)</td>
<td>2 (18.2)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>16</td>
<td>5 (31.3)</td>
<td>6 (37.5)**</td>
<td>6 (37.5)</td>
</tr>
<tr>
<td>II</td>
<td>28</td>
<td>6 (21.4)</td>
<td>16 (57.1)</td>
<td>8 (28.6)</td>
</tr>
<tr>
<td>III</td>
<td>24</td>
<td>3 (12.5)</td>
<td>19 (79.2)</td>
<td>2 (8.3)</td>
</tr>
<tr>
<td>IV</td>
<td>24</td>
<td>2 (8.3)</td>
<td>20 (83.3)</td>
<td>2 (8.3)</td>
</tr>
</tbody>
</table>

$*P < 0.05$, vs. normal colonic epithelium; $**P < 0.05$, vs. stage IV.
Fig. 1. H&E staining results of typical micro-lattice point made by microarrays (H&E stain, × 40).

Fig. 2. Expression of TGF-β1 in CRC tissue. A, IHC stain, × 40; B, IHC stain, × 100; C, IHC stain, × 200.

Fig. 3. Expression of p-ERK1/2 in CRC tissue. A, IHC stain, × 40; B, IHC stain, × 100; C, IHC stain, × 200.

Fig. 4. Expression of K-ras in CRC tissue. A, IHC stain, × 40; B, IHC stain, × 100; C, IHC stain, × 200.
The relationship between clinical parameters and each of TGF-β1, p-ERK1/2, K-ras protein expression

A high IRS of TGF-β1, p-ERK1/2, and K-ras protein expression was observed in 61 (66.3%), 55 (59.8%) and 45 (48.9%) of the CRC samples, respectively. There was a statistically significant difference in TGF-β1 and ERK1/2 expression in CRC at stage I 37.5% and 31.3% versus CRC at stage IV 83.3% and 79.3%. K-ras in cancer cells was stained more intensely than that in normal epithelial cells (P < 0.05), however, no significant differences were found between K-ras expression and the level of tumor differentiation and tumor stages (P > 0.05).

Discussion

The tissue microarrays technique is a valuable tool for high-throughput analysis, which allows various clinical tissue specimens of the individual are arranged on one slide, and with the technique, it is possible for scientists to conduct research. This technique deeply influences primary research, clinical research, and applied research in science and technology through its advantages, such as high-throughput, time saving, low-error rates, and wide-ranging uses[4,5].

There are 2 preparation methods of tissue microarray: one is manual operation and the other is semi-automatic operation, which uses tissue microarray instrument. For the semi-automatic operation, the cost is relatively higher than the manual one as the samples are required to be fully prepared for the tissue microarray instrument. The principal methods to prepare the samples for making manual tissue microarray are the wax method and the frozen section method. The frozen section method is seldom used as the quality of microarray made in this way is not very good. Therefore, the wax section method has been commonly used, currently. The Department of Pathology in West China University of Medical Sciences once developed a TMA construction technique without prefabricating recipient blocks, but used double-sided adhesive tape attached to x-ray film as an adhesive platform on which the tissue cores were placed securely[12]. But in the course of making TMA by hand, the operation on some key procedures, such as selecting the target region of the tissue, preparing the wax carrier, post-processing tissue microarray wax blocks, and mounting the microarray all directly affect the quality of tissue microarray.

In this study, tissue microarray was prepared with paraffin tissue samples taken from colorectal surgery for the 92 CRC cases, punctured the appropriate sites chosen to make TMA, and immunohistochemically stained it by S-P method. As shown in Fig.1, the normal colon tissue and the tissue microarray of colon cancer exhibited clear histological structures, and the tissue microarray also had a clear staining background. The positive positioning was simple for half-quantitative analysis. The principal improvements of technology for this project have been made as follows: (1) Renew all of the relevant reagents for fixing tissue samples. (2) Observe carefully the morphological structure of each sample after staining, and select representative (according to the grading standards of pathological morphology) sectors for group selection. (3) At room temperature above 35°C to make the wax block tough enough for paracentesis. (4) Tissues occupy many locations in the microarray so that the site of the array is easily identified and that prevent dropping pieces in bioremediation process of antigen, which makes the label unclear. (5) Hyperthermia boil and the microwave are employed for antigen bioremediation. The immunohistochemical technique S-P combined with tissue microarray technique was employed in this study to investigate the expressions of TGF-β1, p-ERK1/2, and K-ras protein in colorectal cancer, and the correlation between the expressions and the clinical tumor stages was studied in order to find out the evidence for the screening and to predict the prognosis of CRC patients through the related biological molecules.

TGF-β1 is a group of growth factors closely related to carcinogenesis[15]. In the early stage, the TGF receptors (TβR I, TβR II) and Smad proteins change their activities in signaling pathway, in turn causing a loss of the restraining effects on cell growth, therefore, the cells grow without control and as a result, tumor develops[14]. In the late stage, TGF-β1 can promote tumor cell invasion and metastasis by means of the non-Smads signaling pathway[15]. Recent studies suggest that extracellular signal-regulated kinase (ERK1/2) plays a central role in tumor invasion[16,17]. As shown in the results, the positive stain of TGF-β1 was primarily located in the cytoplasm and membrane of the tumor cells and inflammatory cells. Semi-quantitative analysis showed that the expression of TGF-β1 in CRC was higher than that in normal colon tissue (P < 0.05). In addition, a significant relationship was found between TGF-β1 expression and the CRC stages (P < 0.05). The high level expression of TGF-β1 is closely related with the prognosis of the CRC patients, and TGF-β1 may potentially affect the development of colorectal cancer.

The results of IHC from this research showed that the stain of p-ERK1/2 and K-ras in CRC was stronger than those in normal epithelial cells of colon. There was a statistically significant difference in TGF-β1 expression between CRC at stage I and CRC at stage IV. However, there was no statistically significant difference in K-ras expression between CRC at stage I and CRC at stage IV. We hypothesized that TGF-β1 may promote tumor invasion via activation of the ERK1/2 pathway. According to a study reported in the literature[18], TGF-β1 can activate ERK signaling pathway through direct phosphorylation of ShcA, suggesting that some cross-talk may exist between the TGF-β1 signaling pathway and the ERK signaling pathway, and this probably produces mutually antagonistic effects.
Conflict of interest statement

No potential conflicts of interest were disclosed

References