Tissue Microarray-determined Expression Profiles of Cyclooxygenase-2 in Colorectal Adenocarcinoma: Association with Clinicopathological Parameters

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Abstract

Accumulated evidence reveals that increased cyclooxygenase-2 (COX-2) is involved in the development of colorectal cancer. Our purpose was to quantify COX-2 expression in colorectal cancers using tissue microarray analysis and look for an association with clinicopathological stage. Immunohistochemical analysis of COX-2 was performed in tissue microarray slides containing 90 specimens including 32 well-differentiated, 35 moderately differentiated, and 23 poorly differentiated colorectal adenocarcinomas. All colorectal adenocarcinomas showed significant immunohistochemical expression of COX-2 when compared to normal colon epithelia. However, there was no significant difference in immunostaining scores between poorly, moderately, and well-differentiated tumors (195 ± 28, 214 ± 26 and 200 ± 24, respectively). The COX-2 immunostaining score correlated significantly with T stage (P < 0.05) but not with N or M stage. The positive expression rates of CK20 were 97% for well-differentiated, 94% for moderately differentiated, and 65% for poorly differentiated colorectal adenocarcinomas, suggesting that CK20 may not be an effective discriminator between poorly differentiated colorectal adenocarcinoma and metastatic adenocarcinoma.

Key Words: adenocarcinoma, colorectal, cyclooxygenase-2, cytokeratin 20

Introduction

Cyclooxygenase (COX) is the rate-limiting enzyme catalyzing the conversion of arachidonic acid to various products, including prostaglandins and other eicosanoids. COXs are classified as members of the myeloperoxidase family (5). Two isoenzymes, COX-1 and COX-2, have been identified as membrane proteins anchored to the endoplasmic reticulum in mammals (31). These proteins are tethered to the outer leaflet of the membrane bilayer where they have access to arachidonic acid, their substrate (2). COX-
COX-2 is constitutively expressed in virtually all tissues and regulates normal physiological functions (25). However, COX-2 is generally undetectable under physiological conditions but can be induced by a wide spectrum of growth factors and proinflammatory cytokines and contributes to pathological processes, including tumorigenesis (32).

Increased expression of COX-2 has been reported in many primary tumors such as colorectal (18, 22), lung (8), breast (11), esophageal (35), and pancreatic carcinomas (30). COX-2 is overexpressed in 70-90% of colorectal cancers (18, 22). The cumulative evidence suggests that increased COX-2 is involved in the development of colorectal cancer (4, 17, 29). For example, COX-2 participates in angiogenesis, converts procarcinogens to carcinogens, and inhibits apoptosis (23, 29). Recently, treatment with the selective COX-2 inhibitor, celecoxib, was shown to prevent colorectal cancer in patients with familial adenomatous polyposis (20). However, the results of studies relating COX-2 with prognosis of colorectal cancer or clinical pathological subgroups do not agree (7, 15, 24, 27, 33).

In this study, the expression of COX-2 was re-evaluated using a tissue microarray technique. All 90 colorectal adenocarcinoma specimens were placed in a single tissue microarray slide and evaluated simultaneously. The tissue microarray technique provides a much more reliable method for evaluating immunohistochemical expression because the staining of all the tumor tissues takes place under the same conditions (12). Our results demonstrated that higher expression of COX-2 is significantly associated with T-stage colorectal adenocarcinomas.

One core was taken from selected areas of each paraffin-embedded tumor tissue block and tissue microarray slides were constructed according to a previously published method (9). Each representative core in tissue microarray slide was 2 mm in diameter and the pathological diagnosis in each of these cases was reviewed by at least two experienced pathologists.

The diagnosis of primary colorectal adenocarcinoma was confirmed by the histopathological finding of a lesion transitional between neoplastic and normal colorectal epithelia and by immunoreactivity with cytokeratin 20 (CK20). A previous study demonstrated that most colorectal adenocarcinomas stain positive for CK20 (19). The positive immunostaining pattern to CK20 was demonstrated in tissue microarray slides (Table 1). All colorectal adenocarcinoma samples were classified on the basis of histological grading and TNM staging, and all tumors were staged according to the 1997 AJCC/TNM system.

**Materials and Methods**

Paraffin-embedded tissue blocks were retrieved from the Department of Pathology, Tri-Service General Hospital. Totally 90 primary colorectal adenocarcinomas were selected, including 32 well-differentiated, 35 moderately differentiated, and 23 poorly differentiated adenocarcinomas.

<table>
<thead>
<tr>
<th>Differentiation status</th>
<th>Tumor</th>
<th>Intensity</th>
<th>% Staining</th>
<th>Total score</th>
<th>Positive rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well differentiated (n = 33)</td>
<td>3.0 ± 0.2*</td>
<td>83 ± 3*</td>
<td>259 ± 19*</td>
<td>96.9%</td>
<td></td>
</tr>
<tr>
<td>Moderately differentiated (n = 31)</td>
<td>2.9 ± 0.2*</td>
<td>81 ± 5*</td>
<td>246 ± 20*</td>
<td>93.5%</td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated (n = 21)</td>
<td>1.9 ± 0.4*</td>
<td>47 ± 9*</td>
<td>161 ± 35*</td>
<td>65.2%</td>
<td></td>
</tr>
<tr>
<td>Normal colon epithelia (n = 10)</td>
<td>1.0 ± 0.4</td>
<td>16 ± 6</td>
<td>33 ± 26</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Immunostaining patterns for cytokeratin 20 in colorectal adenocarcinoma. * Indicates significant difference in cytokeratin 20 expression between the tumor and normal colorectal epithelia (P < 0.05).

Tissue microarray slides were dewaxed in xylene, rehydrated in alcohol, antigen retrieved in 0.01 M sodium citrate buffer (pH 6.0) at 100°C for 30 min, and then immersed in 3% hydrogen peroxide for 5 min to suppress endogenous peroxidase activity. After 3 rinses (each for 5 min) in phosphate buffered saline (PBS), sections were incubated for 1 h at room temperature with a rabbit anti-human COX-2 antibody (1: 100, Neomarker, Freemont, CA, USA) diluted in PBS. After 3 washes (each for 5 min) in PBS, the sections were incubated with the biotinylated secondary antibody (Dako, Glostrup, Denmark) for 40 min. After 3 rinses in PBS, horseradish-peroxidase conjugated streptavidin was added for 20 min at room temperature. The peroxidase activity was developed with diaminobenzidine at room temperature.

The immunoreactivity and histological appearance of all tissue microarray slides was examined in triplicate and scored by two authors concurrently. Sections...
with less than 50% of the original tissue remaining on the slides after processing were excluded and not scored. For those cores that remained intact after staining, the intensity of membranous and cytoplasmic COX-2 immunostaining was scored on a scale of 0 (no staining) to 4 (strongest intensity), and the percentage of cells staining at each intensity was estimated from 0 to 100. The percentage of cells at each intensity level was multiplied by the corresponding intensity value, and these products were added to obtain an immunostaining score ranging from 0 to 400. A positive immunostaining reaction for CK20 was defined by at least 10% tumor staining.

**Statistical Analysis**

All results are expressed as mean ± standard error of the mean (SEM). The COX-2 immunostaining score of each colorectal adenocarcinoma was compared with the score of normal colon epithelia. Statistical analysis was performed using the Student’s t-test between groups. A P value less than 0.05 was considered to be statistically significant. One-way analysis of variance (ANOVA) was used to determine statistical significance of differences among histopathological differentiations of colon cancer. SigmaState software (Jandel Scientific, San Rafael, CA, USA) was used to perform linear regression testing to analyze the relationship between COX-2 immunostaining score and clinicopathological parameters. In addition, survival times were calculated from the date of surgery to the date of death. Of all the included colorectal adenocarcinoma patients, 90 cases were followed up for at least two years. These cases were divided into 2 groups to compare the survival time with the COX-2 immunostaining scores. Statistical analysis of survival time was done using the Kaplan-Meier survival test.

**Results**

CK20 expression in Colorectal Cancer Totally, 32 cases of well-differentiated, 35 cases of moderately differentiated, and 23 cases of poorly differentiated colorectal adenocarcinoma were selected and constructed into tissue microarray for study (Figure 1). Previous studies have demonstrated that most colorectal adenocarcinomas stain positively for CK20 (28). However, 97% of well-differentiated, 94% of moderately differentiated, and 65% of poorly differentiated colorectal adenocarcinomas were CK20-positive in tissue microarray sections (Table 1 and Figure 2, panel C, F, and I). These results suggest that CK20 alone may not be an effective marker for diagnosing metastatic lesions of poorly differentiated colorectal adenocarcinoma.
COX-2 Expression in Colorectal Cancer

The staining intensity and percentage of cells immunostained for COX-2 in colon adenocarcinoma are listed in Table 2 and Figure 2 (panels B, E, and H). The COX-2 was located in the cytoplasm and on the nuclear membrane. The COX-2 immunostaining scores were all significantly higher in well-, moderately, and poorly differentiated tumors compared to normal colon epithelia.

Table 2. COX-2 immunostaining scores in colorectal adenocarcinoma.

<table>
<thead>
<tr>
<th>Differentiation status</th>
<th>Intensity</th>
<th>% Staining</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well differentiated (n = 32)</td>
<td>3.1 ± 0.2*</td>
<td>57 ± 5*</td>
<td>200 ± 24*</td>
</tr>
<tr>
<td>Moderately differentiated (n = 35)</td>
<td>2.9 ± 0.2*</td>
<td>72 ± 6*</td>
<td>214 ± 26*</td>
</tr>
<tr>
<td>Poorly differentiated (n = 23)</td>
<td>2.7 ± 0.2*</td>
<td>71 ± 7*</td>
<td>195 ± 28*</td>
</tr>
<tr>
<td>Normal colon epithelia (10)</td>
<td>2.2 ± 0.3</td>
<td>28 ± 12</td>
<td>46 ± 14</td>
</tr>
</tbody>
</table>

Data are means ± standard error of the mean (SEM) of immunostaining score for cyclooxygenase-2 (COX-2) in colorectal adenocarcinoma. * Indicates significant difference in COX-2 expression between tumor and normal colorectal epithelia (P < 0.05).

Fig. 2. Hematoxylin and eosin staining of poorly differentiated (A), moderately differentiated (D), and well-differentiated (G) colorectal adenocarcinoma, and normal colon epithelia (J); immunostaining for cyclooxygenase-2 in poorly differentiated (B), moderately differentiated (E), and well-differentiated (H) colorectal adenocarcinoma, and normal colon epithelia (K); and immunostaining for cytokeratin 20 in poorly differentiated (C), moderately differentiated (F), and well-differentiated (I) colorectal adenocarcinoma, and normal colon epithelia (L). Original magnification X 400.
poorly differentiated adenocarcinomas than in normal colon epithelia. However, there was no significant difference in immunostaining score between well, moderately, and poorly differentiated adenocarcinomas (195 ± 28, 214 ± 26 and 200 ± 24, respectively).

**Expressions of COX-2 with Clinicopathological Parameters**

In Figure 3, linear regression testing was performed to analyze the relationship between COX-2 immunostaining score and clinical TNM stage. The COX-2 immunostaining score was significantly correlated with the T stage ($P < 0.05$) but not with N or M stage.

Among the 90 colorectal adenocarcinoma cases with 2-year follow up, one-half of cases had a higher level of COX-2 expression (immunostaining score ≥ 160) and the other half cases had lower score (score < 160). Using COX-2 scores as variable parameters, higher scores were associated, but not significant with higher mortality (Figure 4, $P = 0.3$).

**Discussion**

We have demonstrated that COX-2 is over-expressed in colorectal adenocarcinomas and that CK20 may not be effective as an immunomarker for lesions of metastatic poorly differentiated colorectal adenocarcinoma. In addition, increased COX-2

![Fig. 3. Clinicopathological correlation with cyclooxygenase-2 immunostaining scores. * Indicates statistical significance of the linear regression testing ($P < 0.05$).](image)

![Fig. 4. Overall survival of 90 patients with colorectal adenocarcinoma. Higher cyclooxygenase-2 immunostaining scores were associated with worse survival. Survival rates were analyzed using the Kaplan-Meier survival test ($P = 0.3$).](image)
expression in colorectal adenocarcinomas is associated with advanced T stage.

Cyclooxygenase, previously named “prostaglandin G/H synthase”, has attracted increasing attention in recent cancer studies because of the observation that colorectal cancer incidence is reduced in patients taking non-steroidal anti-inflammatory drugs (13). Studies also showed that COX-2 mRNA expression is upregulated (6) and that increased COX-2 induces angiogenesis and suppresses apoptosis in colorectal tumors (23, 29). These findings identify COX-2 as a new target of cancer treatment.

Several clinical trials of COX-2 inhibitors have been carried out in patients with colorectal cancers (1). Recent clinical results suggest that COX-2 inhibitors in combination with conventional chemotherapy agents prolong survival of patients with metastatic colorectal cancer (10). However, whether COX-2 expression is an independent prognostic factor in colorectal cancer remains controversial. Several studies indicate a possible prognostic role for COX-2 expression in colorectal cancer patients (15, 26, 31), whereas others show no relationship between COX-2 expression and clinicopathological parameters in individual cases (14, 32, 33).

Previous immunostaining intensity measurements were unreliable because of study-to-study variation in environmental and/or amplification conditions. The tissue microarray technique overcomes this problem by simultaneously measuring staining intensity of all the tissues on the same slide (12) and has been used to quantitate staining in a large number of samples (16). Thus, our immunostaining procedure has produced more reliable results because it was carried out under the same conditions and on a single tissue microarray slide.

Though CK20 has been considered a useful tool in detection of micrometastasis in lymph node or peripheral blood of colorectal adenocarcinomas (21, 19, 34), only 65% of poorly differentiated colorectal adenocarcinomas stained positive for CK20, suggesting that less differentiated cancer cells may lose their ability to express CK20. Therefore, the use of CK20 to detect micrometastases of colorectal cancer might result in false negative results.

In conclusion, COX-2 is overexpressed in colorectal adenocarcinomas. Increased COX-2 expression correlates with advanced T stage. Therefore, pharmacological agents targeted to COX-2 may be beneficial for the treatment of colorectal cancer.

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References


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