AN IMMUNOHISTOLOGIC STUDY ON KI67, P53, P21 AND CEA EXPRESSION IN COLORECTAL CARCINOCENESIS

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Key Words: CEA, colorectal carcinogenesis, immunohistochemistry, Ki67, p21, p53, topographic expression.

Abstract

The immunohistological expression for Ki67, p53, p21 and CEA was studied in 10 normal colonic mucosa, 18 adenomas, 13 ca in adenomas, 12 sm cancers and 39 advanced cancers, respectively. In normal mucosa, Ki67 staining cells were topographically expressed in the crypts. Both p21 and CEA were inversely expressed in the upper crypts. On the other hand, p53 could not be detected. This topographic expression was abrogated in all examined tumors. The immunoreactivity was positively correlated with the progression of the tumor during normal → adenoma carcinoma sequence. The immunoreactivity for p21 expression tended to decrease in relation to the progress of malignancy. Cellular CEA expression showed apical cell membrane staining in the normal mucosa, but abnormal cellular CEA staining patterns such as depolarized cytoplasmic staining and extra stromal staining tended to increase during the progression of malignant transformation. These results indicate that loss of normal topographic expression and alteration of immunoreactivity may play an important role in colorectal oncogenesis.

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Introduction

A variety of cell proliferation kinetic studies on the gastrointestinal mucosa have shown that the normal colonic mucosa is structurally composed of the cell proliferating zone in the lower crypt and non-proliferating functional zone in the upper crypt (Lipkin 1965, Kanemitsu, Koike et al 1985). This normal condition may be a precisely ordered genetic event, but still remains to be resolved. Colorectal carcinogenesis is known to result from genetic alterations that disorder normal mechanisms controlling cell proliferation and cell death. One biological feature of a tumor is characterized by the cell proliferating activity. Ki 67 immunoexpression has been extensively investigated in tumor growth during the progress of adenoma carcinoma sequence (Kanavaros, Stefanaki et al 1999). Most previous studies have revealed that Ki 67 LI positively correlates with the progress of the neoplasm. A few investigators focused on the topologic expression of Ki 67 immunostaining cells, emphasizing that Ki 67 immunostaining topologically expressed in the normal mucosa, but this normal pattern was abrogated in adenoma and cancer (EL-Deiry, Tokino et al 1995). p53 gene is known to play an important role in the multistep process of colorectal carcinogenesis. The action of wild type p53 is considered to have two pathways, one is G1 cell cycle arrest in response to DNA damage, and the other is the programmed cell death due to irreparably damaged DNA (Lowe, Schmitt et al 1993). Mutation of p53 contributes to loss of this normal function, resulting in a selective growth advantage for neoplastic cells. Wild type p53 transcriptionally induces p21 cip/waf1, p21 inhibits the cell cycle progress in the G1-S transition by blocking cyclin and cyclin dependent kinase and inhibits PCNA dependent DNA repair as p53 dependent pathway (Walczak, Kordek et al 2000). p21 expression develops during cell differentiation in a p53 independent pathway (Girland, Slomp et al 1999). p21 expression is topographically compartmentalized in the upper crypts of normal mucosa, but this normal pattern is abrogated in the neoplasia (Cho, Roe et al 2000). Alteration of p21 expression plays an important role in promoting tumorigenesis (Holland, Elder et al 2001). CEA has been widely used as a valuable tumor marker in the diagnosis and treatment of colorectal cancer. Recently, CEA has been demonstrated to be a member of the immunoglobulin gene superfamily and to function as intercellular adhesion molecule. Over expression of CEA develops in tumorigenesis, contributing to the disruption of normal tissue architecture and inhibiting differentiation of tumor cells.
This study was undertaken to investigate the immunohistological expression of Ki 67, p53, p21 and CEA in the normal colon, adenoma, carcinoma in adenoma (ca in adenoma), early cancer invading sm layer (sm ca) and advanced colorectal cancer, respectively, and to define if any significant topological expression and or the rate of positive expression developed during the progress of adenoma carcinoma sequence.

**Materials and Methods**

All the materials were obtained from surgically resected specimens with definite pathological diagnosis, including 10 normal colonic mucosa, 18 adenomas, 13 ca in adenomas, 12 sm carcinomas and 39 advanced cancers, respectively. Each formalin fixed and paraffin embedded tissue was dewaxed in xylene and rehydrated in graded alcohol. Slides were treated with 1% hydrogen peroxide in methanol for 15 min to block endogeneous peroxidase. After washing in phosphate buffered saline (PBS, pH 7.4), slides were boiled in 10 mM citrate buffered saline (pH 6.0) for 15 min to retrieve antigens. All primary and secondary antibodies and streptavidin biotin peroxidase complex were obtained from DAKO Japan, Kyoto. Each primary antibody was Ki 67 antigen (MIB-1, 1:50), p53 (DO-7, 1:50), p21 (1:25) and CEA, 11-7C (1:25), respectively.

After washing in TBS, slides were incubated with biotinylated rabbit anti-mouse IgG at room temperature for 30 min, then with streptavidin biotin peroxidase complex for 30 min at room temperature. The slides were washed in TBS and peroxidase activity was developed with 3-3' diamono-benzidine tetrahydrochloride, and then counter-stained with hematoxylin. For negative control, normal rabbit IgG was used instead of the primary antibody.

The percentage and the distribution of each positively staining cell were examined at 200 or 400 magnification power. Each labeling index was calculated by the percentage of the positive staining cells in 10 crypts of each normal mucosa and in 10 high power fields of each tumor, respectively. The assessment for topographic immunoeexpression was made as follows: positive expression compartment was assessed in the normal mucosa divided into 5 parts along the crypt and in the tumor divided into 3, respectively. Immunostaining reactivity for p53 was graded as follow, - negative, 0<+≤5%, 5<+≤20%, 20<++≤50%, 50<+++≤100%, respectively; p21 was graded as follows:
- negative, 0<±≤5%, 5<+≤10%, 10<++≤20%, 20<+++,
respectively. More than 5% immunostaining was defined as positive expression for p21, comparing with normal mucosa.
Statistical analysis was performed by the chi-square test or by Fisher’s exact test.

Results

**Ki 67 immunohistological expression**

**Ki 67** immunohistological expression was positive in all the specimens examined. In the normal mucosa, the positive immunostaining cells were topologically restricted to the lower crypts (Figure 1). The distribution pattern of positively expressing compartment for Ki 67 is shown in Figure 2. Each positive compartment in the lower 1/5, 2/5, 3/5 and 4/5 of the crypts was 5%, 33%, 57% and 5% respectively. On the other hand, positive staining cells were diffusely distributed not only in the adenomas, but also in any other tumors. Normal topographical expressing pattern for Ki 67 was abrogated in all examined tumors. As shown in Table I, the mean LI of normal mucosa was 19.5±3.5% which was significantly lower than adenomas with a mean average of 44.2±9.2%. The LI of Ki 67 expression significantly increased in relation to the progress of the tumorigenesis.

**P53 immunohistological expression.**

No p53 staining cells were observed in the normal mucosa. The positive p53 staining cells were scattered in 3 of 18 adenomas and strong p53 staining cells were diffusely distributed in advanced cancers as shown in Figure 3. Three of 18 adenomas showed weak p53 expression graded as ± in 2 and + in one adenoma, respectively. p53 immunoreactivity tended to increase in relation with the progress of malignant transformation. The p53 expression was defined as positive when p53 expressed more than 5% of cells, the rate of positive expression was 5.6% in adenomas, 61.5% in ca in adenomas, 41.7% in sm cancers, 71.8% in advanced cancers, respectively. These findings strongly suggest that p53 expression might play an important role in the conversion of adenoma into cancer.
Figure 1: (LEFT) Ki67 immunostaining cells were topographically compartmentalized in the lower crypts of the normal mucosa (×50). From Shigetoshi Kato et al. 18- 8-10-18-03-337. Annals of Cancer Research and Therapy, Vol. 11(2003). © 2003 by PJD Publications Ltd.

Figure 3: (RIGHT) Positive p53 staining cells were diffusely distributed in advanced cancer (×200). From Shigetoshi Kato et al. 18- 8-10-18-03-337. Annals of Cancer Research and Therapy, Vol. 11(2003). © 2003 by PJD Publications Ltd.
Figure 4: (LEFT) Positive p21 staining cells were topologically compartmentalized in the upper crypts (×100). From Shigetoshi Kato et al. 18- 8-10-18-03-337. Annals of Cancer Research and Therapy, Vol. 11(2003). © 2003 by PJD Publications Ltd.

Figure 6: (RIGHT) CEA staining cells in the normal mucosa were topographically expressed in the upper crypts (×50). From Shigetoshi Kato et al. 18- 8-10-18-03-337. Annals of Cancer Research and Therapy, Vol. 11(2003). © 2003 by PJD Publications Ltd.
Fig. 2 The distribution pattern of Ki67 expressing compartment in the normal crypts

- Number of crypts (n=100)
- Labeling index 19.5±3.5(%)

Fig. 5 The distribution pattern of p21 expressing compartment in the normal crypts

- Number of crypt (n=100)
- Labeling index 27.7±3.4(%)
Table I  Labeling index of Ki67 Immunoexpression in the normal mucosas and colorectal tumors

<table>
<thead>
<tr>
<th>Lesion</th>
<th>L.I of Ki67</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal mucosa</td>
<td>19.5±3.5% (n=10)</td>
</tr>
<tr>
<td>adenoma</td>
<td>44.2±9.2% (n=18)</td>
</tr>
<tr>
<td>ca in adenoma</td>
<td>55.0±6.8% (n=18)</td>
</tr>
<tr>
<td>sm cancer</td>
<td>56.6±9.1% (n=12)</td>
</tr>
<tr>
<td>advanced cancer</td>
<td>66.2±8.6% (n=39)</td>
</tr>
</tbody>
</table>

Table II  p21 expression pattern in the colorectal tumors

<table>
<thead>
<tr>
<th>p21 expressing pattern</th>
<th>superficial type (%)</th>
<th>deeper type (%)</th>
<th>diffuse type (%)</th>
<th>positive rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>adenoma (n=18)</td>
<td>13 (72.2)</td>
<td>1 (5.6)</td>
<td>14 (77.8)</td>
<td></td>
</tr>
<tr>
<td>ca in adenoma (n=13)</td>
<td>6 (46.2)</td>
<td>2 (15.4)</td>
<td>8 (61.5)</td>
<td></td>
</tr>
<tr>
<td>sm cancer (n=12)</td>
<td>3 (25)</td>
<td>1 (8.3)</td>
<td>2 (16.7)</td>
<td>6 (50)</td>
</tr>
<tr>
<td>advanced cancer (n=39)</td>
<td>12 (30.8)</td>
<td>5 (12.8)</td>
<td>8 (20.5)</td>
<td>25 (64.1)</td>
</tr>
</tbody>
</table>
p21 immunohistological expression

p21 staining cells were topographically limited to the lower crypts in the normal mucosa (Figure 4). As shown in Figure 5, p21 expressing compartment locating at the upper 1/5, 2/5, 3/5 and 4/5 of the normal crypts was 5%, 38%, 51% and 6%, respectively. This normal topographic pattern was abrogated at the tumor-epithelial glandular level in the tumors. The p21 expression pattern in each of the colorectal tumors is shown in Table II. More than 5% of p21 positive cells in the upper 2/3, lower 2/3 and all parts of the tumors were defined as superficial, deep and diffuse type respectively.

As shown in Table II, the superficial expression type was developed in 13 (72.2%) of 18 adenomas and 46.2% of 13 ca in adenomas respectively. But this expression type markedly decreased to 30.81% in the advanced cancers, additionally advanced cancers developed abnormal expressing pattern including 5 deep expression type and 8 diffuse expression type, respectively.

The rate of positive p21 expression was 100% in the normal mucosa, 72.5% in adenoma, 61.5% in ca in adenomas, 50% in sm cancers and 64.1% in advanced cancers, respectively. These findings may suggest that loss of normal topographical pattern for p21 is an early event during the progress of tumorigenesis and promotes the progression of tumor transformation.

CEA immunohistological expression

CEA expression was positively observed in all examined normal mucosa and any tumors. CEA staining cells were topologically distributed at the upper crypts in the normal mucosa as like p21 expressing pattern (Figure 6). CEA expressing compartment in the upper 2/5, 3/5 and 4/5 of the normal crypts was 23%, 51% and 26%, respectively. This topographical expression was abrogated in all examined tumors. In regarding to cellular staining pattern for CEA expression, CEA expressing cells in the normal mucosa were mainly stained at their apical membrane. The cellular staining pattern in 18 adenomas showed apical type in 10 and cytoplasmic type in 8, respectively. Apical type was defined as cellular staining limited to apical side of cytoplasm and apical cytoplasmic type defined as whole cytoplasm was stained with more strong staining of apical side. Cytoplasmic type and or cytoplasmic stromal type developed during the
progress of malignancy. Cytoplasmic type was diffusely stained cytoplasm with loss of polar CEA expression and cytoplasmic and stromal type was cytoplasmic staining with additional stromal staining. In 39 advanced cancers, apical type was found only in one and the remaining 38 cellular staining type were apical cytoplasmic type in 19, cytoplasmic type in 15, and cytoplasmic stromal type in 4, respectively. These findings suggest that loss of the normal topological expression for CEA may be an early event during colorectal neoplasia and functions in the progress of tumor growth in association with abnormal intracellular CEA expression.

Discussion

It is well known that the gastrointestinal proliferating epithelial cells are compartmentalized in the lower crypts and move toward the upper differentiating crypts and desquarate from their tops. This constitutional mechanism may be regulated by a series of genetic controls, but this is not still fully understood. Recently, colorectal neoplasms are believed to result from a series of genetic alterations that disrupt normal mechanisms controlling cell growth (Vogelstein, Fearon et al 1988).

Ki 67 immunostaining is considered to be a reliable method to accurately assay the growth fraction of the tumor because Ki 67 nuclear antigen has been expressed in the proliferating cells during the cell cycle of G1, S, G2 and M phase but not in the quiescent cells of G0 (Gerdes, Lemke et al 1984). Previous studies have shown that Ki 67 expressing proliferating cells are restricted to one third and or a half of the lower crypts. However, only a few authors emphasized that this topographic Ki 67 expression was a precisely ordered genetic event in the normal mucosa (El-Deiry, Tokino et al 1995). This normal topographical pattern was abrogated not only in adenomas but also in colorectal cancers. In this study, the normal mucosa was divided into 5 compartments in order to more precisely analyze the topographic Ki 67 expression. 95% of the Ki 67 expressing compartment was observed in the lower 3/5 of the normal crypts. On the other hand, this normal topographical pattern was abrogated even in adenomas but also in other examined tumors as reported by other investigators (Kanavaros, Stefanaki et al 1999). Previous studies revealed that Ki 67 LI was significantly higher in adenoma than
in normal mucosa and tended to increase in relation with progressing tumor during the colorectal carcinogenesis (Saleh, Jackson et al 2000). Our study revealed that each Ki 67 LI in normal mucosa, adenomas, ca in adenomas, sm cancers and advanced cancers was 19.5%, 44.2%, 55.0%, 56.6% and 66.2%, respectively. These findings strongly suggest that loss of normal topographic expression of Ki 67 may be an early event and plays an important role for colorectal carcinogenesis. p53 is known to be the most common genetic alteration in human cancers and especially develops in over 50% of colorectal cancers. Alteration of p53 has an important role in the induction of colorectal cancers as described in introduction.

In this study, no positively staining cell for p53 was observed in the normal mucosa. This negative p53 immunohistological expression coincided with previous studies because wild type p53 was too short a half life to immunohistologically detect (Walczak and Kordek 2000). Our result revealed that each p53 expression in adenoma, ca in adenoma, sm cancer and advanced cancers was 5.6%, 61.5%, 41.7% and 71.8%, respectively. This finding agrees with the previous reports that p53 plays an important role in the conversion of adenoma to malignant transformation and that p53 overexpression correlated with the progressive growth of the tumor during adenoma carcinoma sequence (Ohue, Tomita et al 1994). Regarding the topographic p53 expression, no conclusion was obtained because no positive staining cell was observed in the normal mucosa. Previous study has shown that p21 expression was topographically compartmentalized in the upper normal crypts and this normal topographic expression was abrogated in the colorectal tumors (El-Deiry, Tokino et al 1995). A few authors emphasized topographically inverse relationship between Ki 67 and p21 expressing compartment in the normal mucosa. They supposed that p21 played a critical role in negative control of the proliferating compartment in the normal mucosa and this topographical p21 expression was probably independent of p53 pathway. The above described p21 expression develops during cellular differentiation. In this study, 94% of the p21 expressing compartments were observed in upper 3/5 of normal crypts as reported by previous studies. This topographical p21 expression pattern was abrogated in the colorectal tumors which were observed at the level of the epithelial gland in our
study. However, when the tumors were divided into three compartments and p21 expressing compartments were analyzed, superficial p21 expressing type was seen in 12 (66.7%) of 18 adenomas and in 6 (46.2%) of 13 ca in adenomas. This superficial expression type similar to normal mucosa markedly decreased in accordance with the progressing malignancy. In the advanced cancer, abnormal p21 expressing type such as deep expression pattern and diffuse expression pattern were observed. Our results suggest that loss of normal topographical pattern of p21 expression occurred early in the neoplastic process.

CEA is considered to be expressed in a various tissues and to function as intercellular molecule for formation of tissues (Ilantzis, Jothy et al 1997). Deregulation of CEA expression might contribute to development of malignant transformation. In the normal colonic mucosa, CEA expressing cells were weakly stained at luminal surface of cell membrane. Alteration of CEA expression tends to increase in relation to the progress of the malignancies. Several experimental and clinical studies described that CEA expression function to inhibit differentiation of some kinds of cells and or to disturb tissue architecture leading to favorable malignant growth. In this study, CEA expression in the normal mucosa was topographically observed in the upper crypts as reported by many other investigators. This topographical normal expression pattern was totally abrogated in all examined tumors. Although all normal mucosal cells showed polar CEA expression, approximately over half of adenoma still developed polarity of CEA expression similar to that of normal mucosa. However, sm cancers and advanced cancers tended to increase depolarized CEA expression such as cytoplasmic staining pattern and/or stromal staining pattern. Our results suggest that loss of normal topographic CEA expression may be the early event during the carcinogenesis and depolarized CEA expression may endow favorable malignant growth against transformed cells.

References


