hMLH1, hMSH2 and Cyclooxygenase-2 (Cox-2) in Sporadic Colorectal Polyps

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Abstract. Background: Colorectal adenomatous polyps are known as premalignant lesions. Mutations in the mismatch repair (MMR) enzymes hMLH1, hMSH2 and hMSH6 are recognized causes of hereditary non-polyposis colorectal cancer and act by inducing a mutator phenotype characterized by microsatellite instability (MSI). MSI is also detected in sporadic colorectal cancers. Cox-2 is an inducible enzyme that regulates prostaglandin synthesis and it is overexpressed at sites of inflammation, in colorectal adenomatous polyps and cancer. The aim of this study was to evaluate the immunoeexpression of hMLH1, hMSH2 and Cox-2 in polyps resected through colonoscopy, and to examine their association with clinicopathological characteristics (age, gender, location, size, histology and grade of dysplasia). Patients and Methods: One hundred and sixty-seven colonic polyps, 6 normal colonic mucosa samples, and 23 samples of colorectal adenocarcinoma were used in this study. All patients had no family history of colorectal cancer. The samples were prospectively collected and immunostained for hMLH1, hMSH2 and Cox-2 using the ABC-immunohistochemistry technique with amplification by biotinylated tyramide. The mean age was 60.2±13.8 years (range 21-90 years) and 77 (55.8%) were men. Results: Tubular adenomas were present in 81.4%, tubulous-villous in 15.9%, serrated in 1.8%, and villous in 0.9%. The majority of the adenomas were located in the rectosigmoid region (63.5%), followed by ascendent in 14.2%, cecum in 7.5%, descendent in 8.2% and transverse in 6.7%. Low-grade dysplasia was detected in 59.6% of the adenomas. Loss of hMLH1 and hMLH2 immunoeexpression was observed in 20% and 15.5% of the adenomas, respectively. Cox-2 expression was found in 9% of the adenomas, and in 40% of the adenocarcinomas. Moreover, Cox-2 immunoeexpression was associated with the multiplicity of adenomas in the same patient (p=0.001). There was no association between marker immunoeexpression and gender, age, location, size, histology or grade of dysplasia. Conclusion: Loss of hMLH1 and hMLH2 immunoeexpression in adenomas is relatively frequent in patients without colorectal cancer family history. Cox-2 is overexpressed in colorectal adenomatous polyps and adenocarcinomas, and its positivity in adenomas may indicate a higher risk for multiple lesions.

Malignant tumors that befall the colon and the rectum each year add up to about 945,000 new cases worldwide, being the fourth most common cancer worldwide and the second in developed countries. According to the National Cancer Institute (1), the estimated number of new colorectal cancer cases in Brazil in 2006 was 11,390 cases in men and 13,970 in women. These figures correspond to an estimated risk of 12 new cases per 100,000 men and 15 per 100,000 women (1).

Early detection of colorectal adenomatous polyps and localized tumors is possible and it has shown to be effective in other countries through research of occult blood in feces and endoscopic methods (1).

Several studies have concluded that there is high incidence of colorectal cancer in patients with polyps (2). About 90% of pre-invasive neoplastic lesions of colon are polyps or polyp precursors (aberrant crypt foci). There is evidence that colorectal cancer progresses from normal tissue to adenoma and carcinoma through an accumulation of genetic alterations (3-5). These genetic alterations in adenomas and carcinomas offer the opportunity to detect specific changes...
in DNA. In colorectal cancer, delineation of various stages during tumor progression offers the opportunity to intervene in the process by detecting molecular alteration.

The evolution in the knowledge of colorectal carcinogenesis is due to the studies of molecular alterations verified in polyps with atypias and in colorectal carcinomas. The identification and characterization of the genetic changes in the malignant transformation process have progressed rapidly over the last three decades. The predominant changes include deletions, rearrangements and mutations leading to either deactivation or activation of specific target genes. Two major classes of genes are involved: oncogenes and suppressor genes. Oncogenes are activated or deactivated genes whose products promote cell growth. Tumor suppressor genes normally regulate cell growth (6).

Recently, genes not related to oncogenes or suppressor genes have been found to be implicated in carcinogenesis. These genes compose a family of genes commonly known as mismatch repair genes (MMR) that predispose individuals to colorectal cancer. These alterations are responsible for hereditary non-polyposis colorectal cancer (HNPCC), popularly known as Lynch syndrome (7, 8). A number of genes are involved in MMR such as: hMSH2, hMLH1, hPMS2, hMSH3 and hMSH6 (2). Mutations of these genes produce microsatellite instability (MSI) sequences. The loss of MMRs leads to a greatly elevated frequency of point mutations (mutator phenotype) and MSI. In most of the colorectal cancers in patients with HNPCC, MSI occurrence has been used as biomarkers for the detection of Lynch syndrome.

Cyclooxygenase-2 (Cox-2) is an inducible enzyme that regulates prostaglandin synthesis and is overexpressed at sites of inflammation, in colorectal adenomatous polyps and cancer, and may be an early event in colorectal carcinogenesis. Cox-2 is a major molecular target for cancer chemoprevention utilizing COX inhibitors that may reduce the incidence of colonic adenomas (9).

Thus, the aim of this research was to investigate hMLH1, hMSH2 and Cox-2 immunoeexpression in sporadic colorectal polyps and adenocarcinoma.

**Patients and Methods**

One hundred and thirty-eight patients who had had colorectal polyps resected by colonoscopies from 2002 to 2004 were enrolled in this investigation. Seventy-seven patients were men (55.8%) and the mean age was 60.2±13.8 years (range 21-90 years). Control samples comprised 6 cases of normal colonic biopsy, 20 hyperplastic polyps and 23 colorectal adenocarcinomas. Clinicopathological parameters including age, gender, location, size, number, histology and grade of dysplasia of the adenomas were recorded.

**Histopathological and immunohistochemical evaluation.**

Histological slides (H&E) were reviewed to confirm the histopathological diagnosis of colorectal lesions and corresponding formalin-fixed paraffin-embedded tissue blocks were sectioned for immunohisto-chemical analysis. Five to six unstained 4 µm blank histological sections were cut from each designated block. Two blanks were used for hMLH1 and hMSH2 (hMLH1 – clone G168-728, Pharmigen, San Diego, CA, USA; hMSH2 – clone G219-1129, Pharmigen, San Diego, CA, USA) and one blank for Cox-2 (clone CX-294, Dako, Carpinteria, CA, USA), using the ABC-immunohistochemistry technique with amplification by biotinylated tyramide (Dako Cytomation CSA II, Carpinteria, CA, USA). Two blanks were used for hMLH1 and hMSH2 (hMLH1 – clone G168-728, Pharmigen, San Diego, CA, USA; hMSH2 – clone G219-1129, Pharmigen, San Diego, CA, USA) and one blank for Cox-2 (clone CX-294, Dako, Carpinteria, CA, USA), using the ABC-immunohistochemistry technique with amplification by biotinylated tyramide (Dako Cytomation CSA II, Carpinteria, CA, USA). The immunohistochemical technique was performed as previously described (10-16). Briefly, immunodetection involved the use of 4 µm-thick formalin-fixed paraffin-embedded tissues, treated with 4% hydrogen peroxide (H₂O₂) in methanol for 35 minutes to eliminate endogenous peroxidase activity. The sections were placed in a microwave oven for 10 minutes for antigen retrieval, rinsed in phosphate-buffered saline (PBS) and incubated with 10% normal horse serum to block non-specific binding. Upon removal of the serum, the primary monoclonal antibody was applied at room temperature. Following further washing with PBS, sections were incubated with biotinylated antimouse immunoglobulin for 30 minutes. After washing twice with PBS, the sections were treated with Vectastain Elite horseradish peroxidase complex (Vector Laboratory, Burlingame, CA, USA) for 30 minutes. Following another rinse with PBS, the sections were incubated with diaminobenzidine 0.05% and 0.04% H₂O₂ for 20 minutes. After a final wash with distilled water, the sections were counterstained with Harris Alum Hematoxylin (Eng Scientific Inc. Clifton, NJ, USA), dehydrated through graded alcohol and xylene, and mounted with Permount (Fisher Scientific, Fair Lawn, NJ, USA).

<table>
<thead>
<tr>
<th>Patients' Distribution</th>
<th>Normal (%)</th>
<th>Altered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age</td>
<td>60.2±13.8</td>
<td>^</td>
</tr>
<tr>
<td>Gender</td>
<td>74.4</td>
<td>25.6</td>
</tr>
<tr>
<td>Men</td>
<td>74.6</td>
<td>25.4</td>
</tr>
<tr>
<td>Women</td>
<td>36</td>
<td>24</td>
</tr>
<tr>
<td>Number of polyps</td>
<td>Single</td>
<td>36</td>
</tr>
<tr>
<td>Multiple</td>
<td>69.5</td>
<td>31</td>
</tr>
<tr>
<td>Size (mm)</td>
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<tr>
<td>&gt;10 to 20 mm</td>
<td>59.6</td>
<td>34.8</td>
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<tr>
<td>&gt;20 mm</td>
<td>40.4</td>
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<td>Histology</td>
<td>Low</td>
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</tr>
<tr>
<td>High</td>
<td>1.8</td>
<td>0</td>
</tr>
<tr>
<td>Site</td>
<td>Right colon</td>
<td>21.7</td>
</tr>
<tr>
<td>Left colon</td>
<td>14.9</td>
<td>27.4</td>
</tr>
<tr>
<td>Rectosigmoid</td>
<td>63.5</td>
<td>18.4</td>
</tr>
</tbody>
</table>

P-value: ^Student’s t-test; *Pearson Chi-square test; #Fisher’s exact test.
alcohols to xylene, and coverslipped. Sections of colorectal adenocarcinoma were used as positive controls and primary antibody was replaced by PBS for negative controls. The stainings were scored semiquantitatively on a graded scale of 0 through 4 for both intensity and distribution by two investigators in a blinded analysis. (13-16). In normal tissues, hMLH1 and hMSH2 immunoexpression showed a strong nuclear staining. On the other hand, Cox-2 positivity was revealed by cytoplasmatic staining. hMLH1 and hMSH2 were considered altered when there was a reduced immunoexpression or complete absence of the staining. Lymphocytes and normal adjacent epithelium exhibited strong nuclear staining for hMSH2 and hMLH1, and served as positive internal controls for staining of these proteins. Cox-2 positivity was observed in tissues as brown cytoplasmatic staining.

**Statistical analysis.** Clinicopathological characteristics of the lesions and immunohistochemical alterations were compared using Fisher’s exact probability test and Pearson’s Chi-square test for qualitative data, and Student’s t-test for quantitative data, with a two-tailed p-value at the 5% level considered significant.

**Results**

The histology showed 81.4% tubular adenomas, 15.9% tubular-villous, 1.8% serrated type and 0.9% villous adenomas. The adenomas were located in the rectosigmoid area in 63.5%, in the transverse in 14.2%, in the descendent in 8.2%, in the cecum in 7.5%, and in the ascendant in 6.7% of the cases. High-grade dysplasia occurred in 40.4% of the cases.

There was no loss of hMLH1 and hMSH2 immunoexpression in the normal mucosa samples, or in the hyperplastic polyps. Cox-2 immunoexpression was negative in the normal mucosa and hyperplastic polyps.

There was loss of expression of hMLH1 and hMSH2 in 20% and 15.5% of the adenomas, respectively. Cox-2 was positively expressed in 9% of the adenomas. There was loss of expression of the hMLH1, hMLH2 and Cox-2 in adenocarcinomas in 20%, 10%, and 40% of cases, respectively. hMLH1, hMSH2 and Cox-2 immunoexpressions are shown in Figures 1 to 3. The distribution of the clinicopathological characteristics of 138 patients with colorectal adenomas and their association with the immunohistochemical markers are shown in Tables I to III.

There was no association between the immunomarkers and age, gender, size of the adenoma, location, histology, a grade of dysplasia of the adenomas. There was a greater number of multiple polyps in patients with adenomas than in patients.
with hyperplastic polyps. Positive Cox-2 immunoexpression was shown in almost 90% of multiple polyps of the same patient. Thus, there was an association between the Cox-2 positivity and the presence of multiple polyps.

Discussion

Adenomatous polyps in colorectal carcinogenesis have a significant contribution as early tumoral preinvasive lesions. Some studies have observed greater risks of colon cancer development among adenoma carriers, while other studies have demonstrated risk reduction when carrying out polypectomie (17, 18). Histopathological studies have observed adenocarcinoma foci in adenomatous polyps as well as adenoma foci in specimens of adenocarcinomas (19). So, the study of adenomatous polyps may generate information regarding colorectal carcinogenesis development and progression.

The etiology of colorectal cancer is heterogeneous, with environmental influences and genetic involvement. In approximately 80% of colorectal cancer cases it seems that the disease is sporadic without any evidence of hereditary commitment. The genetic contribution to colorectal cancer includes an increase of risk in individuals with a familial history and families with autosomic dominant genetic alterations (20). The identification of pre-malignant lesions is an indispensable requirement for the screening and prevention of colorectal cancer. It has been known for many years that many colorectal cancers arise from pre-existing adenomas, usually as a result of gene mutation in the APC gene. In the analysis of different genetic alterations in the adenoma-carcinoma progression, Fearon and Volgestein considered the genetic model of colorectal carcinogenesis to be a multiple step process (21).

In this investigation, regarding all kinds of adenomas, the loss of hMLH1 and hMSH2 immunoexpression was 20% and 15.5%, respectively. Cox-2 was expressed positively in 9% of the adenomas in general. In the adenocarcinomas, there was a loss of hMLH1 and hMSH2 immunoexpression in 20% and 10%, respectively. Cox-2 was positive in 40% of the colorectal adenocarcinomas. These findings of hMLH1 immunoexpressions in colonic adenomatous polyps and adenocarcinomas, and Cox-2 positivity in patients without colorectal cancer family history, indicates the relatively frequent role of MSI and Cox-2 alterations in sporadic colorectal cancer.

There were no immunoexpression alterations of the studied markers in the hyperplastic polyps, suggesting the benign behavior of these lesions.

Figure 1. hMLH1 positive nuclear immunoexpression in an adematous polyp (x400).
Figure 2. hMSH2 immunoexpression in an adenomatous polyp (x400).

Figure 3. Cox-2 cytoplasmic immunoexpression in a tumor (x400).
Evidence to support a role for the mismatch repair genes human mutL homolog 1 (hMLH1) and human mutS homolog 2 (hMSH2) in the etiology of colorectal cancer has come from linkage analysis, segregation studies and molecular biologic analysis (4, 22, 23). More recently, carriers of potentially pathogenic mutations in the hMLH1/hMSH2 genes have consistently been shown to be at a greatly increased risk of developing colorectal cancer compared with the general population (6). Immunohistochemistry can be a method used in adenomatous polyps and colon hyperplasia considering the prevention and treatment of pathologies associated with them.

The loss of hMLH1 immunoeexpression was observed in cases of MSI, but not in cancers with microsatellite stable (MSS) (22). The inactivation of the genes involved in the DNA of MMR is associated with MSI in colorectal cancer. Herman and co-authors demonstrated that the hypermethylation of CpG islands of hMLH1 is found in most sporadic colorectal cancers with MSI and is, almost always, associated with the loss of the expression of the hMLH1 gene (23). The result of the study suggests that MSI in sporadic colorectal cancer frequently results from the epigenic inactivation of hMLH1 in association with the methylation of DNA (23).

We found a greater number of multiple polyps in patients with adenoma (44 out of 113), than in patients with hyperplastic polyps (p=0.012). In 88.8% of the cases, Cox-2 immunoeexpression was positive in the multiple polyps (p=0.001). Thus, Cox-2 immunoeexpression may be useful to indicate the presence of multiple polyps in the colon.

The increased expression of Cox-2 has been found in gastrointestinal adenomatous polyps of animal models of adenomatous polyposis (24-27), in patients of familial adenomatous polyposis (28-30) and in sporadic adenomas in human patients (31-35). These findings suggest that Cox-2 plays a significant role in the promotion and development of colorectal cancer and has stimulated the development of Cox-2 usage as a preventive agent for colorectal cancer (32). In invasive carcinomas the expression of Cox-2 was increased in the adenoma portion of the tumor and was detected in 62% of the cases. Expression of Cox-2 in malignant epithelial cells was detected in only 23% of invasive carcinoma cases (22). It has been concluded that an increased expression of Cox-2 can be an early event in carcinogenesis of colorectal cancer (36).

Therefore, in conclusion, the results of this study showed that loss of hMLH1 and hMLH2 immunoeexpression in adenomatous polyps was relatively frequent in patients without colorectal cancer family history. Moreover, Cox-2 was overexpressed in sporadic colorectal adenomatous polyps and adenocarcinomas, and its positivity in adenomas may indicate a higher risk for multiple lesions.

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References


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