Expression of β-Catenin and Cyclooxygenase 2 in Colorectal Carcinoma: An Immunohistochemical Study

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Abstract The Wnt/β-catenin pathway plays an important role in the genesis of familial adenomatous polyposis, the most common form of inherited colorectal carcinoma (CRC). Also, the inflammatory bowel diseases (IBDs) predispose to cancer development; and cyclooxygenase 2 (COX-2) seems to be pivotal in their pathogenesis. This study aimed to investigate the relationship between the expression of COX-2 protein and β-catenin in colorectal cancer. The study enrolled 45 patients, all of whom underwent surgery and immunohistochemical staining of tissue specimens for COX-2 and β-catenin was done. Correlation between the two modulators and their relationship with clinicopathological features were examined. In 34 cases (75.56%) of the tumor samples; β-catenin immunoreactivity was found in the cytoplasm and/or membrane compartment. On the other hand, COX-2 immunoreactivity was weakly and/or strongly positive in 32 cases (71.11%) and negative in 13 (28.89%). Positivity was detected in the cytoplasm and in the perinuclear area. Increased expression of β-catenin was correlated to Duke stage (P=0.009). Furthermore, nuclear β-catenin localization showed a correlation to the Duke stage (P=0.029) and insignificant correlation with distant metastases (P = 0.336). Positive COX-2 expression showed a significant relation to, liver metastases (P = 0.042), and Duke stage (P = 0.011) and insignificant correlation to lymph node invasion (P=0.25). These data indicate that cytoplasmic/membrane β-catenin over-expressions as well as positive COX-2 expressions are associated with a more aggressive behavior of the disease.

Keywords β-catenin, Cyclooxygenase 2, Immunohistochemical Staining, Histology, Pathology, Colorectal Cancer

1. Introduction

Colorectal cancer (CRC) is one of the most frequent malignancies and is the third most common cause of cancer-related death worldwide [1,2]. It might be related to a number of predisposing factors including the prior existence of benign tumours (adenomas), the prior existence of ulcerative colitis, inherited syndromes such as familial adenomatous polyposis, dietary factors such as a low fiber diet and so on [3]. CRC occurs either in a sporadic (75 - 80%) or in an inherited form (20-25%) [4]. A significant number of patients who undergo apparently curative operation unfortunately develop local recurrences or distant metastases leading to shorter survival [5]. Identification of factors that affect tumor aggressiveness and allow a more accurate prognosis is required. The molecular mechanisms of CRC initiation and/or progression have been only partially elucidated. The Wnt/β-catenin pathway plays a pivotal role in the genesis of familial adenomatous polyposis, the most common form of inherited CRC [1,6]. In the presence of adenomatous polyposis coli (APC) or β-catenin mutations, β-catenin is no longer phosphorylated and degraded in an ubiquitin-dependent fashion. β-Catenin can translocate to the nucleus where it associates with T-cell factor/lymphoid enhancing factor (TCF/LEF) transcription factors to stimulate the expression of genes involved in cell proliferation [4,5]. Loss-of-function mutations of the DNA repair genes coupled with genome instability are the leading causes of human non-polyposis colorectal cancer, the other inherited form of CRC [7]. Mutations of the Wnt/β-catenin pathway play a causative role also in 60- 80% of sporadic cases [1,6]. Growing evidence, however, supports the notion that other pathway alterations may be relevant in colon carcinogenesis [1,7]. Inflammatory bowel diseases (IBDs) predispose to cancer development [9,10]. Cyclooxygenase 2 (COX-2) seems to be pivotal in their pathogenesis, as it is involved in the biosynthesis of prostaglandins [11]. These compounds, in turn, activate pro-inflammatory genes through the nuclear factor κB (NFκB) signaling pathway [12]. Its sustained expression stimulates cell proliferation, thus linking inflammation to cancer.

2. Objectives
The aim of the present study was to clarify the correlation between β-catenin and COX-2 with CRC evolution, through investigation of their protein expression and sub-cellular localization in tissue specimens of CRCs.

3. Material and Methods

3.1. Patient Histories and Tissue Samples

Forty five systematic random samples from patients who had undergone surgical resection (starting from 2004 to 2009) at the Department of Surgery, Zagazig University hospitals, were investigated in this study. Informed consent was obtained from patients for their tissues to be used in research. The specimens were obtained immediately after surgical resection, fixed in 10% neutral buffered formalin and then embedded in paraffin blocks. Ten samples were obtained from the proximal colon; and thirty-five from the distal colon. Each sample was matched with the distant non-neoplastic mucosa removed during the same surgery, usually 15 to 20 cm away from the border of the main tumor lesion. Only in 11 cases was the so-called transitional mucosa, that is the mucosa closer (less than 3 cm) to the tumor mass, available for analysis. All patients were selected at their first diagnosis; and none had received chemotherapy or radiation therapy before resection, or referred associated with IBD. CRCs were classified according to International Union Against Cancer criteria and were recorded as adenocarcinoma with a variable degree of differentiation. Staging at the time of diagnosis was based on the tumor-nodes-metastasis system. A median follow-up for 10 months (2-24 months) was applied to all 45 patients who received conventional postoperative treatment.

3.2. Immunohistochemistry

Serial 4-µm sections were mounted on poly-L-lysine-coated slides, de-paraffinized, rehydrated, and microwaved for 15 minutes at high power in 10 mmol/L citrate buffer (pH 6.0) to unmask the epitopes. Endogenous peroxidase was quenched using 3% H2O2 for 20 minutes. Slides were then washed in phosphate-buffered saline (pH 7.5) and incubated with the corresponding antibodies containing 5% normal bovine blocking serum in phosphate-buffered saline. The primary antibodies used were anti–COX-2 antibody (Dako Cytomation, Glostrup, Denmark) for 2 hours at room temperature 1:200 and anti–β-catenin antibody (Dako Cytomation, Glostrup, Denmark) for 2 hours at 37°C 1:200. A standard labeled streptavidin-biotin-peroxidase complex (LSAB+ System-HRP kit; Dako Cytomation, Glostrup, Denmark) was used to amplify the immunoreaction. Tissues were stained for 5 minutes with DAB (3,3’-diaminobenzidine) chromogen and counterstained with Meyer's hematoxylin, dehydrated, and cover-slipped. Each experiment was performed in duplicate. Primary antibodies were omitted in negative controls. Sections from breast carcinoma immunostained with β-catenin; and sections from colon cancer with strong immunostaining for Cox-2 were used as positive controls.

3.3. Evaluation of Immunohistochemistry

Immunostaining for each marker was graded by a semi-quantitative method based on a scale that takes into account the intensity and distribution of the staining. The intensity was scored as follows: 1 (weak), 2 (moderate), or 3 (strong). The percentage of positive cells was scored as follows: 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), or 4 (76-100%). The 2 scores were combined to obtain the final one: negative (0-2), weakly positive (3-5), or strongly positive (6-7). For statistical analysis, positive cases include both weakly and strongly positive. Membrane, cytoplasm, and/or nuclear signal localization was independently evaluated using light microscopes (Olympus, CX31 binuclear) and classified for each marker.

3.4. Statistical Analysis

Data were analyzed by using SPSS software. Association between the expression levels of the proteins investigated and the clinicopathological parameters was assessed using the χ2 test and Fisher exact test. The P-value < 0.05 was considered to be significant.

4. Results

| Table 1. Clinico-pathological data |
|-----------------------------------|
| Item                             | No. | %    |
| Age                              |     | Mean: 57.5 |
| Age                               |     | SD: 10.2  |
| Sex:                             |     |       |
| Male                             | 34  | 75.55%|
| Female                           | 11  | 24.44%|
| Total                            | 45  | 100%  |
| Histopathological diagnosis:     |     |       |
| Well-differentiated               | 13  | 28.89%|
| Moderately-differentiated         | 30  | 66.66%|
| Poorly- differentiated            | 2   | 4.44% |
| Total                            | 45  | 100%  |
| Depth of invasion:               |     |       |
| Submucosa                        | 6   | 13.33%|
| Muscularis propria               | 37  | 82.22%|
| Serosa                           | 2   | 4.44% |
| Total                            | 45  | 100%  |
| Lymph node invasion              | 11  | 24.44%|
| Distant metastasis               | 10  | 22.22%|
| Dukes stages:                    |     |       |
| A                                | 4   | 8.88% |
| B                                | 20  | 44.44%|
| C                                | 11  | 24.44%|
| D                                | 10  | 22.22%|
| Total                            | 45  | 100%  |
4.1. Clinicopathological Results

Patients' age ranged from 35 to 80 years, with a mean of 57.5 years. Sex composition was 34 men and 11 women. Thirteen cases were well-differentiated; 30 cases were moderately differentiated; and only 2 cases were poorly differentiated adenocarcinoma. Six tumors invaded the submucosa; 37 invaded the muscularis propria; and 2 invaded the serosa and the adjacent organs. Eleven patients had regional lymph node invasion, whereas 34 were negative. Distant metastases were found in 10 patients. According to Duke’s classification [13], stages A, B, C, and D were detected in 4, 20, 11, and 10 patients, respectively (Table 1).

4.2. Immunohistochemical Results

In the adjacent non-neoplastic colonic mucosa, β-catenin was distributed between the cell membrane and the cytoplasm. In 34 cases (75.56%) of the tumor samples; similar or increased protein level were predominantly found. In 27 cases of them (79.4%), there were increased protein levels in the cytoplasm and/or membrane compartment (C/M) (Figs 1, 2). In 7 cases (15.56%), the protein was essentially localized in the nucleus (N) (Fig 3). In 11 cases (24.44%), the protein was not detected (Fig 4). In the non-neoplastic mucosa adjacent to the tumor mass, negative immunostaining reaction for COX-2 was observed. In tumor specimens, COX-2 immunoreactivity was weakly and/or strongly positive in 32 cases (71.11%) (Fig 5), and negative in 13 cases (28.89%). Positivity was detected in the cytoplasm and in the perinuclear area. Using the F test, (2-tailed); increased expression of β-catenin was correlated to Duke staging ($P = 0.009$) (Table 2). Furthermore, nuclear β-catenin localization showed an association to the Duke stage ($P = 0.002$) and insignificant association with distant metastases ($P = 0.33$) compared with the group with cytoplasmic/membrane localization (Table 2). Positive COX-2 expression showed a significant relation to, liver metastases ($P = 0.04$), and Duke staging ($P = 0.011$) and insignificant association to lymph node invasion ($P = 0.25$) (Table 3). These data indicate that cytoplasm/membrane β-catenin over-expression and positive COX-2 expression are related to a more aggressive behavior of the disease; most patients passed the period of 10-months survival except 5 patients; 2 of whom with stage C, and 3 with cancer stage D who died at the end of the 10th month.

| Parameters                | β-catenin score | Localization | P-value | C/M | N | Total | P-value |
|---------------------------|-----------------|---------------|---------|-----|---|-------|---------|
| Depth of invasion         |                 |               |         |     |   |       |         |
| No. | %   | No. | %  | No. | %  |
| pT1 | 2   | 33.3 | 4  | 66.7 | 6  |
| pT2 | 1   | 25  | 3  | 75  | 4  |
| pT3 | 29  | 87.9 | 4  | 12.1 | 33 |
| pT4 | 2   | 100 | 0  | 0   | 2  |
| Total | 34  | 11  | 45 |     |     |
| Lymph node metastasis     |                 |               |         |     |   |       |         |
| No. | %   | No. | %  | No. | %  |
| Absent | 28  | 82.4 | 6  | 17.6 | 34 |
| Present | 6   | 54.5 | 5  | 45.5 | 11 |
| Total | 34  | 11  | 45 |     |     |
| Distant metastases        |                 |               |         |     |   |       |         |
| No. | %   | No. | %  | No. | %  |
| Absent | 30  | 78.9 | 8  | 21.1 | 38 |
| Present | 4   | 57.1 | 3  | 42.9 | 7  |
| Total | 34  | 11  | 45 |     |     |
| Duke stage                |                 |               |         |     |   |       |         |
| No. | %   | No. | %  | No. | %  |
| A | 1   | 25  | 3  | 75  | 4  |
| B | 13  | 65  | 7  | 35  | 20 |
| C | 10  | 90.9 | 1  | 9.1  | 11 |
| D | 10  | 100 | 0  | 0.00 | 10 |
| Total | 34  | 11  | 45 |     |     |

Table 2. Association between β-catenin expression, and some clinicopathological parameters
Table 3. Association between COX-2 expression, and some clinico-pathologic parameters

| Parameters                  | COX-2 |
|-----------------------------|-------|
|                             | Positive | Negative | Total |
| **Depth of invasion**       |         |          |       |
| No. | % | No. | % |
| pT1 2 | 33.3 | 4 | 66.7 | 6 |
| pT2 1 | 25 | 3 | 75 | 4 |
| pT3 27 | 81.8 | 6 | 18.2 | 33 |
| pT4 2 | 100 | 0 | 0.0 | 2 |
| Total 32 | 71.1 | 13 | 28.9 | 45 |
| **Lymph node metastasis**   |         |          |       |
| Absent 26 | 76.5 | 8 | 23.5 | 34 |
| Present 6 | 54.5 | 5 | 45.5 | 11 |
| Total 32 | 71.1 | 13 | 28.9 | 45 |
| **Distant metastases**      |         |          |       |
| Absent 22 | 57.9 | 13 | 42.1 | 38 |
| Present 10 | 100 | 0 | 0.0 | 10 |
| Total 32 | 71.1 | 13 | 28.9 | 45 |
| **Duke stage**              |         |          |       |
| A 0 | 0.00 | 4 | 100 | 4 |
| B 15 | 75 | 5 | 25 | 20 |
| C 9 | 81.8 | 2 | 18.2 | 11 |
| D 8 | 80 | 2 | 20.0 | 10 |
| Total 32 | 71.1 | 13 | 28.9 | 45 |

Figure 1. Photomicrograph of a case of moderately differentiated adenocarcinoma of the colon, stained with anti β-catenin immunohistochemical staining (strong cytoplasmic and membranous reaction) (Meyer’s hematoxylin counter stain, 400 X)

Figure 2. Photomicrograph of a case of moderately differentiated adenocarcinoma of the colon, stained with anti β-catenin immunohistochemical staining (strong membranous reaction) (Meyer’s hematoxylin counter stain, 400 X)

Figure 3. Photomicrograph of a case of moderately differentiated adenocarcinoma of the colon (with mucinous changes), stained with anti β-catenin immunohistochemical staining (strong nuclear reaction) (Meyer’s hematoxylin counter stain, 400 X)

Figure 4. Photomicrograph of a case of moderately differentiated adenocarcinoma of the colon, stained with anti β-catenin immunohistochemical staining (weak cytoplasmic and membranous reaction) (Meyer’s hematoxylin counter stain, 400 X)
factor (TCF/LEF) family to form a bipartite complex [16,17].

transcription factors of the T-cell factor/lymphoid enhancing-
catenin interacts with
the cytoplasm. Stabilized β
specification of different tissues [15].  The ce

kinase-Wnt signaling promotes inactivation of glycogen synthase
limited and undergoes rapid degradation. Concomitantly,
established epithelium during development and adult stages of life [18].

Recent studies in rodents illuminate the role of canonical
Wnt signaling in proliferation of normal intestinal
epithelium during development and adult stages of life [18].

β-catenin expression was elevated in about 79.4% of the
cases; only in about 20% of which was the protein
predominantly located in the nucleus. This result mostly
agreed with the studies reporting that mutations of the
Wnt/β-catenin pathway and consequent accumulation of
β-catenin in the nucleus were found in 60% - 80% of
sporadic CRCs [1].

COX-2 expression was also monitored in the current study
because it is a key enzyme in the production of
prostaglandins, hence activating the inflammatory response. In
IBDs, in addition, over-expression of the inducible
isoform COX-2 has been shown to occur at multiple stages
of colon carcinogenesis allowing for elevated prostaglandin
synthesis to occur in the tumor microenvironment [19].

Negative COX-2 was noticed to be significantly associated
with very low, if any, distant metastases. Those with high
β-catenin expression and elevated COX-2 showed more
frequent distant metastases and Duke C and D. A similar
relationship was also observed in the cases with nuclear
β-catenin accumulation. An increase of COX-2 in the distant
non neoplastic mucosa, the transitional mucosa and primary
tumor was found in patients with liver metastases. A key role
in this process is thought to be played by Wnt-5a, a member
of the Wnt family of secreted growth factors, which regulate
tumor cell migration and macrophage proteolytic activity
[20]. The role of Wnt-5a and other members of the
non-canonical Wnt pathway in cancer progression is,
however, still debated [21]. In summary, the data presented
here indicate that both COX-2 and β-catenin play a pivotal
role in the genesis of colorectal cancers. Nevertheless, a
direct link between these two key pathways has remained
elusive. Previous reports showed that one of the bioactive
products of COX-2, prostaglandin E2, activates components
of the canonical Wnt signaling system [22]. On the other
hand, there is an observation that accumulation of β-catenin
induces COX-2 expression in articular chondrocytes [23].
Kazem et al. suggested the use of both β
-catenin and COX-2 as a marker of tumor progression and poor prognosis [24].

Some epidemiologic studies suggested that
anti-inflammatory drugs (NSAIDs) have chemopreventive
effects and reduce the incidence of mortality from GIT
cancers [17].

One potential limitation of the study is the fact that we
were not able to look for so large number of tissue samples,
as the enrolled 45 patients were the available cases in our
institute along the period of the study. This might reflect a
relatively low incidence of CRCs in our region, that
represent a leading cause of death in the Western world [25].
There are many predisposing factors for such cases [3].
Dietary habits among populations, such as high levels of
saturated fats have been implicated in occurrence of CRCs
[26]. Recently, it has been suggested that there is a potential
value of phytic acid extracted from rice bran in reducing
colonic cancer risk in rats [27]. Observations of the
expression patterns of the oncogenic genes may only
represent a starting point for a thorough investigation of the
functions of these genes in pathogenesis of CRCs.

5. Discussion
This study investigated the expression profile of β-catenin
and COX-2 in colonic cancer and tumor-genesis in a series of
sporadic CRCs. We reported here a significant correlation
between β-catenin and increased COX-2 levels, and tumor
metastatic progression. β-catenin is a component of the Wnt
signaling pathway which controls the specification,
maintenance and activation of intestinal stem/progenitor
cells. Deregulation of this pathway due to either genetic or
epigeneic defects can potentially result in the development
of familial and/or sporadic epithelial cancers [14]. The
functional versatility of Wnt/β-catenin signaling are seen
through its action in cancer stem cells. These cells require
β-catenin in mediating the response to Wnt signaling for
specification of different tissues [15]. The cellular β-catenin
resides mainly at sites of intercellular (adherens)
junctions where it interacts with E-cadherin and α- catenin. In the
absence of Wnt signal, the cytoplasmic pool of β- catenin is
limited and undergoes rapid degradation. Concomitantly,
Wnt signaling promotes inactivation of glycogen synthase
kinase-3 beta (GSK3β) causing β- catenin to accumulate in
the cytoplasm. Stabilized β-catenin interacts with
transcription factors of the T-cell factor/lymphoid enhancing
factor (TCF/LEF) family to form a bipartite complex [16,17].
TCF/LEF provide the DNA-binding specificity, while
β-catenin provides trans-activation domains. This bipartite
complex then leads to the expression of Wnt target genes,
such as c-myc and cyclin D1, well known for their role in cell
proliferation and oncogenesis [12]. β-catenin participates in
trans-activation by recruiting two other transcriptional
co-factors, CBP/p300 acetyltransferase and the
chromatin-remodeling protein Brg-1, to TCF target gene
promoters [11,12]. Nuclear accumulation of β-catenin is
considered a hallmark of activated canonical Wnt signaling.
Recent studies in rodents illuminate the role of canonical
Wnt signaling in proliferation of normal intestinal
epithelium during development and adult stages of life [18].

Figure 5. Photomicrograph of a case of poorly-differentiated
adenocarcinoma of the colon; stained with anti-COX-2
immunohistochemical staining (weak cytoplasmic and membranous
reaction) (Meyer’s hematoxylin counter stain, 400 X)
6. Conclusion

The study indicated that cytoplasmic/membrane β-catenin over-expressions as well as positive COX-2 expressions are associated with a more aggressive behavior of the disease. However, it is still controversial, regarding whether Wnt/β-catenin is the target for expression of COX-2 or the reverse. More future studies including larger numbers of patients and in correlation with therapy are recommended.

Conflict of Interest

The authors declare that no conflicts of interest exist.

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