

## Immunohistochemical analysis of candidate gene product expression in the duodenal epithelium of children with coeliac sprue

I Barshack, I Goldberg, Y Chowers, B Weiss, A Horowitz, J Kopolovic

### Abstract

**Background**—Coeliac sprue is a chronic disease, in which there is a characteristic mucosal lesion of the small intestine and impaired nutrient absorption, which improves upon the withdrawal of wheat gliadins and related grain proteins from the diet. Biopsy specimens demonstrate diffuse enteritis with pronounced atrophy or total loss of villi. There is also a long term risk of malignant disease.

**Aims**—To compare the immunorexpression of DCC (deleted in colon cancer), p53, E-cadherin, and  $\beta$ -catenin in the duodenal mucosa of children with coeliac disease with that seen in children with no evidence of small intestinal disease.

**Methods**—To gain more insight into the genetic and immunohistochemical alterations of the duodenal epithelium in coeliac disease, 21 endoscopic biopsies from children with coeliac disease and 10 duodenal biopsies from children without coeliac disease were immunohistochemically evaluated for p53, DCC, E-cadherin, and  $\beta$ -catenin.

**Results**—DCC expression was not reduced in patients with coeliac disease compared with those without coeliac disease. p53 positive nuclear immunostaining was seen in seven of the 21 patients with coeliac disease. Positive nuclear staining was seen mainly in the deep and the lateral aspects of the crypts. All patients in the control group were negative for p53. In nine and three of the 21 patients with coeliac disease, respectively, the immunohistochemical expression of E-cadherin and  $\beta$ -catenin was reduced. However, both E-cadherin and  $\beta$ -catenin immunostaining in the control group was not altered.

**Conclusions**—E-cadherin and  $\beta$ -catenin were reduced in the duodenal epithelium of children with coeliac disease when compared with normal mucosa. p53 was overexpressed in the duodenal mucosa of patients with coeliac disease. The reduced expression of E-cadherin and  $\beta$ -catenin and p53 overexpression may contribute to the morphological changes seen in the small intestinal mucosa in coeliac disease.

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Keywords: coeliac disease; p53; deleted in colon carcinoma gene; E-cadherin;  $\beta$ -catenin

Coeliac sprue is a chronic disease, in which there is a characteristic mucosal lesion of the small

intestine and impaired nutrient absorption, which improves on withdrawal of wheat gliadins and related grain proteins from the diet.<sup>1</sup> The mucosa of patients with coeliac sprue can be flat or normal. Biopsy specimens demonstrate diffuse enteritis with pronounced atrophy or total loss of villi. The surface epithelium shows vacuolar degeneration, loss of the microvillous brush border, and intraepithelial lymphocytes.<sup>2,3</sup> There is a long term risk of malignant disease. Most of these malignancies are intestinal lymphomas, mainly T cell lymphoma<sup>4,5</sup>; however, there is also an increased frequency of small intestinal adenocarcinoma.<sup>5</sup>

The proliferation rate is increased in the small intestinal epithelium of patients with coeliac disease,<sup>6,7</sup> although apoptosis is even higher.<sup>8</sup> In addition, lymphocytic infiltration, crypt hyperplasia, villous atrophy, and mucosal permeability are all increased, whereas mucosal integrity is reduced.<sup>1</sup> Compared with inflammatory changes, much less is known about the genetic and immunohistochemical alterations that take place in the epithelium of these patients.

p53 overexpression and mutations have been shown repeatedly in adenomas and carcinomas of the gut,<sup>9</sup> hyperplastic polyps,<sup>10</sup> and non-dysplastic gastrointestinal epithelium associated with chronic reparative processes, as is seen in ulcerative colitis.<sup>11</sup> p53 mutation is an important genetic event implicated in colon and small intestine carcinogenesis. Reduced immunohistochemical expression of the deleted in colorectal cancer (DCC) protein was noted in frank colon carcinoma,<sup>12,13</sup> whereas adenomas and normal colonic epithelium were found to express normal amounts of the DCC protein.<sup>13</sup> However, loss of heterozygosity at the DCC gene locus was recently noted in ulcerative colitis, with high grade dysplasia being associated with colorectal carcinoma.<sup>11</sup>

Catenins are cytoplasmic proteins associated with E-cadherin, a major mediator of cell–cell adhesion. Downregulation of  $\beta$ -catenin expression is associated with malignant transformation.<sup>14</sup> In previous studies, reduced immunostaining for E-cadherin was seen in colonic adenomas and carcinomas.<sup>15</sup> Moreover, downregulation of E-cadherin was seen in areas of ulceration and reparative epithelium of the gastrointestinal tract.<sup>16</sup> Loss of normal membranous E-cadherin as visualised by immunohistochemical stains was also seen in inflammatory bowel disease, although no changes were found in  $\beta$ -catenin expression in the same series,<sup>17</sup> suggesting that proliferative and reparative processes may result in E-cadherin

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reduced immunostaining. In a recent study, reduced cadherin–catenin complex expression was also seen in adult patients with coeliac disease.<sup>18</sup>

Our study aimed to evaluate the role of p53, DCC, E-cadherin, and  $\beta$ -catenin in the genesis of mucosal alterations in coeliac disease.

### Materials and methods

Duodenal endoscopic biopsies from a total of 21 children with coeliac disease were selected from the repository of the department of pathology, Sheba Medical Center. In all cases, circulating anti gliadin or antiendomesial antibodies (or both) were detected and all biopsies were the first diagnostic biopsy of untreated children. All biopsies showed the histopathological criteria for coeliac disease, namely: the presence of villous atrophy, increased intraepithelial lymphocytes, and abnormal enterocytes.<sup>19</sup> Ten normal control biopsies were obtained from patients who had no evidence of small intestinal disease. The average age of the 12 female and nine male patients with coeliac disease was 7.8 years (range, 14 months to 16 years) and the average age of the six boys and four girls in the control group was 10.1 years (range, 19 months to 15 years).

### ANTIBODIES

Primary antibodies used in immunohistochemical staining were: monoclonal anti-p53, clone 1801 (Zymed, San Francisco, California, USA); monoclonal anti-DCC, clone G97–449 (PharMingen, San Diego, California, USA); monoclonal anti-E-cadherin, clone 4A2C7 (Zymed); monoclonal anti- $\beta$ -catenin, clone CAT-5H10 (Zymed).

### IMMUNOHISTOCHEMICAL ANALYSIS

Paraffin wax embedded sections (4  $\mu$ m thick) of all tissues were thaw mounted on to Fisherbrand Super Frost Plus slides. After air drying at 37°C for 16 hours and incubation for 30 minutes at 60°C, dewaxing, and rehydration, slides were separated into two groups for antigen exposure. The slides to be stained for E-cadherin were placed in 10mM citrate buffer (pH 6.0) and heated twice for five minutes in a 750 W microwave oven. After each five minute interval the buffer was replaced and the oven was reactivated for five minutes. When the two cycles were completed, the slides were allowed to cool down to room temperature (RT) for 10 minutes. The slides were rinsed for five minutes in Tris buffered saline (TBS; 0.05M Tris/HCl, 0.1M NaCl, pH 7.6) containing 0.1% bovine serum albumin (BSA) and 0.05% Tween 20 (BSA-TBS-Tween). Slides for p53, DCC, and  $\beta$ -catenin staining were placed in jars containing 10mM citrate buffer (pH 6.0).

The jars were placed into a plastic pressure cooker filled with 1 litre of water. The pressure cooker was placed in the microwave oven. The oven was set at 750 W for 20 minutes to achieve boiling and for another 10 minutes to maintain boiling. The remainder of the procedure was the same as for E-cadherin. To reduce background signals, all slides were incubated at RT with 10% non-immune goat serum for 15 minutes, followed by CAS block (Zymed) for 30 minutes. For E-cadherin,  $\beta$ -catenin, and DCC staining, the antibody was applied to the slides and incubated at RT for 60 minutes, and p53 staining was performed at 4°C for 16 hours. Staining was performed with labelled avidin–biotin.<sup>20 21</sup>

Control sections comprised serial sections from the same blocks that were similarly processed except that no primary antibodies were added. In addition, five colorectal cases known to show reduced DCC expression were used as controls for DCC staining.

Immunostaining was scored as positive for p53 when nuclear staining was seen in more than 5% of epithelial cells. DCC, E-cadherin, and  $\beta$ -catenin expression was thought to be altered when cytoplasmic and/or membranous staining was reduced or absent. Immunoeexpression was assessed by comparison with an internal control section of normal small intestine. Any immunohistochemical stain that showed reduced expression was repeated to limit false negative results.

### Results

#### DCC

In normal small intestine tissue, immunoreactivity was mainly cytoplasmic and uniform within the epithelium. DCC expression was not greatly reduced in the patients with coeliac disease (table 1).

#### p53

p53 was not expressed in normal duodenal specimens. p53 positive nuclear immunostaining was seen in seven of the 21 coeliac biopsies (table 1). Positive nuclear staining was found mainly in the deep and lateral aspects of crypts, and less frequently in scattered cells (fig 1).

#### E-CADHERIN

In normal duodenal biopsies, immunoeexpression was membranous, on the lateral cell wall, and with minimal cytoplasmic expression. We noted a pronounced reduction of immunostaining in nine of the 21 coeliac biopsies (table 1). The loss of immunostaining was more pronounced at the surface than in the crypt bases (fig 2).

#### $\beta$ -CATENIN

In normal duodenal biopsies, immunostaining was predominantly uniformly membranous and weakly cytoplasmic; staining was greatly reduced in three of the 21 coeliac biopsies (table 1). No overlap was found between cases showing loss of E-cadherin and  $\beta$ -catenin expression.

Table 1 Immunohistochemical expression of p53, E-cadherin,  $\beta$ -catenin, and DCC in patients with and without coeliac disease

	p53 expression	Reduced E-cadherin expression	Reduced $\beta$ -catenin expression	Reduced DCC expression
Coeliac patients	7/21	9/21	3/21	0/21
Non-coeliac cases	0/10	0/10	0/10	0/10

DCC, deleted in colorectal carcinoma.

### Discussion

We found that p53 was overexpressed in seven of 21 patients with coeliac disease. The positive p53 stained cells were located in the deep and lateral aspects of elongated regenerative crypts. Similarly, p53 overexpression was seen in non-dysplastic colonic epithelium associated with chronic reparative processes, as is seen in inflammatory bowel disease.<sup>11</sup> Overexpression of p53 was also reported in Crohn's associated carcinomas, adenomas, and dysplastic mucosa.<sup>22</sup> In addition, it has recently been shown

that p53 is overexpressed in adenomas and adenocarcinomas of the small intestine.<sup>23</sup> However, coeliac related regenerative changes are usually not associated with dysplasia.<sup>24</sup> To date, no data have been published concerning coeliac related adenocarcinomas of the small intestine and p53 expression, although it may be involved in the rare events that lead to neoplastic changes.

Expression of the DCC protein in colorectal tumours is related to tumour progression and metastasis, but is not regularly found in

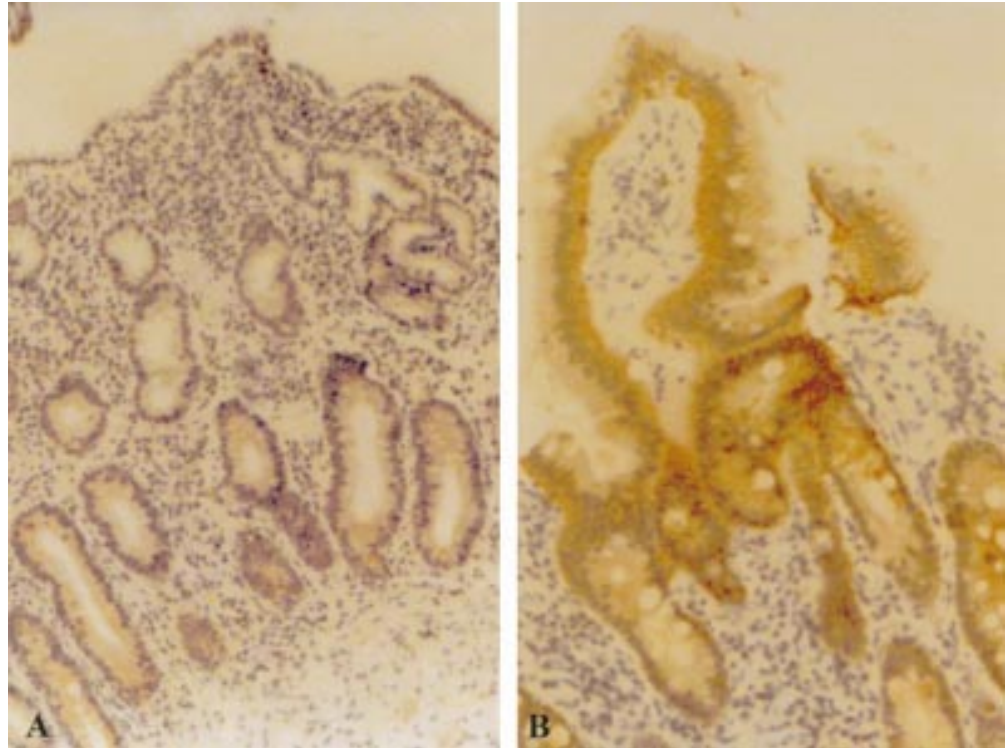


Figure 1 p53 overexpression in the mucosa of a child with coeliac disease at (A) low and (B) high magnification compared with (C) negative p53 staining in normal mucosa.

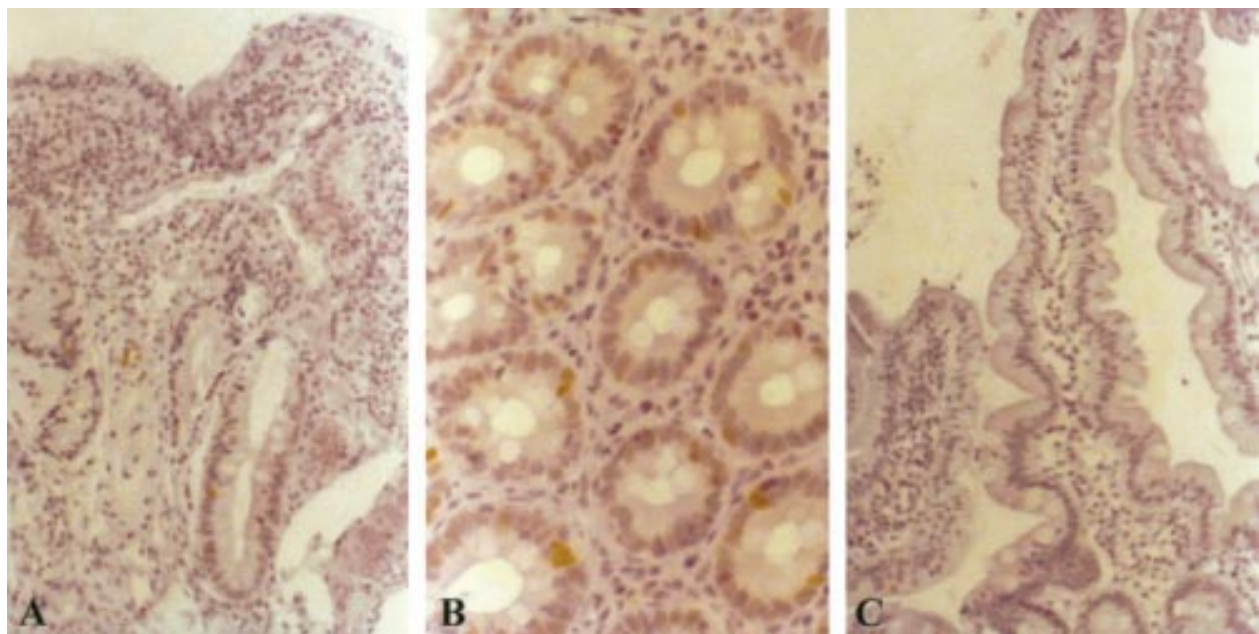


Figure 2 (A) Reduced E-cadherin immunopexpression in the mucosa of coeliac disease (mainly in the surface epithelium) compared with (B) E-cadherin staining in normal mucosa.

non-dysplastic regenerative changes.<sup>11–13</sup> In our study, we found no appreciable differences in the expression of DCC between the coeliac group and the controls.

In the control cases, we found that E-cadherin was expressed uniformly along the crypt–villous axis, in a similar pattern to that described previously,<sup>18 25 26</sup> and this expression was reduced in patients with coeliac disease. The alteration was more prominent at the surface of the mucosa.

E-cadherin, a transmembrane protein of the adherens junction, has a major role in the maintenance of tissue architecture<sup>27</sup> and morphology.<sup>28</sup> It is linked to the actin cytoskeleton by several cytoplasmic proteins, one of which is  $\beta$ -catenin, a molecule that is required for the establishment of functional cadherin mediated adhesion.<sup>21</sup> It was recently shown that E-cadherin cooperates with integrins and insulin-like growth factor I to induce the migration of epithelial colonic cells.<sup>29</sup> The importance of the E-cadherin complex in the maintenance of intestinal epithelial architecture has been noted in tumours and in non-neoplastic samples, such as in the regenerative epithelium of peptic and Crohn's ulceration,<sup>30</sup> and has recently been studied in inflamed and ulcerated areas of the intestinal mucosa. In active inflammation, the expression of the E-cadherin complex was upregulated. In particular, epithelium adjacent to ulcers showed increased expression of protein and mRNA, but in ulcer associated cell lineages staining was weak or negative. Reparative epithelium growing over denuded areas also showed weaker expression.<sup>31</sup> It is unclear why coeliac biopsies, which show mainly inflamed and non-ulcerated mucosa, have reduced E-cadherin immunorexpression in the epithelium.

Cadherin–catenin complexes are involved in the establishment of cell polarity,<sup>32</sup> rearrangement of the cytoskeleton,<sup>34</sup> the formation of intercellular junctions,<sup>34</sup> and embryonic development.<sup>35</sup> A dominant negative, N-cadherin mutant mouse model showed reduced intercellular adhesion and increased cell migration, the loss of cell differentiation and polarisation, and early apoptosis.<sup>29</sup>

In addition to reduced  $\beta$ -catenin membranous expression, previous studies have found an increase in cytoplasmic and nuclear staining, thought to be related to reduced binding to E-cadherin and, therefore, storage within the cytoplasm.<sup>18</sup> We noted a reduction in  $\beta$ -catenin expression in only four of 25 cases, and this was not associated with cytoplasmic or nuclear staining.

Malignancy is much more frequent than expected in patients with coeliac disease.<sup>36–38</sup> Primary non-Hodgkin's lymphoma of the small intestine is the most common tumour, accounting for about 50% of the malignant conditions found in patients with coeliac disease.<sup>36 37</sup> Adenocarcinoma of the small intestine, carcinoma of the oesophagus, and carcinoma of the mouth and pharynx are the main other malignancies that arise with greater than expected frequency in coeliac disease.<sup>36–38</sup> Swinson *et al* found 19 cases of small intestinal

adenocarcinoma, compared with 0.23 expected cases, among 119 non-lymphomatous malignancies. Those tumours occurred predominantly in the jejunum and duodenum.<sup>37</sup> One patient with coeliac disease had villous adenoma, a possible precursor of carcinoma,<sup>39</sup> and others have been seen with multiple adenocarcinomas.<sup>40 41</sup> The neoplastic changes are probably preceded by genomic alterations in the mucosa. p53, E-cadherin, and  $\beta$ -catenin alterations may have a role later in the phenotypic changes of the neoplastic processes. Moreover, in our study, abnormal p53, E-cadherin, and  $\beta$ -catenin expression was seen in young children. Other proto-oncogenes and tumour suppressor genes such as DCC may show altered immunostaining later in the sequence of carcinogenesis.

In summary, we found altered expression of p53, E-cadherin, and  $\beta$ -catenin in a subset of children with active coeliac disease. The immunohistochemical expression of E-cadherin and  $\beta$ -catenin was reduced. These alterations probably have a role in the histomorphological characteristics of coeliac disease. Moreover, mucosal p53 overexpression and altered E-cadherin and  $\beta$ -catenin expression may play a role in the rare events that lead to neoplastic changes, mainly adenocarcinoma of the small intestine.

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