Original contribution

Loss of expression of \( \mu \)-protocadherin and protocadherin-24 in sporadic and hereditary nonpolyposis colorectal cancers☆,☆☆

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Summary Colorectal cancer (CRC) is a neoplastic disease in which normal mucosa undergoes a process of malignant transformation due to the progressive accumulation of molecular alterations affecting proto-oncogenes and oncosuppressor genes. Some of these modifications exert their carcinogenic potential by promoting a constitutive activation of the \( \beta \)-catenin signaling proliferation pathway, and when present, loss of cadherin expression also significantly contributes to the same effect. Using a combined approach of molecular and immunohistochemical analysis, we have previously demonstrated that most sporadic CRCs exhibit a down-regulated expression of a cadherin, named \( \mu \)-protocadherin, that is generally observed in association with a higher proliferation rate and a worse prognosis. The aim of this report was to perform a comparative immunohistochemical assessment of \( \mu \)-protocadherin and a similar cadherin, named protocadherin-24, in sporadic CRC and hereditary nonpolyposis colorectal cancer. The data obtained put in evidence that double-negative CRCs, lacking both the analyzed protocadherins, are more represented among sporadic tumors, whereas double-positive CRCs, maintaining their expression, exhibit an opposite trend. As expected, loss of protocadherin expression was accompanied by nuclear localization of \( \beta \)-catenin and increased positivity of the Ki-67 proliferation marker. This finding is consistent with the different clinical evolution of the 2 considered CRC sets according to which patients with hereditary nonpolyposis colorectal cancer experience a better prognosis as compared with those affected by a sporadic CRC.

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1. Introduction

Colorectal cancer (CRC) is a neoplastic condition of great medical and social interest, and despite the considerable progress achieved in the comprehension of its molecular pathogenesis, it remains the most frequent tumor in general population and the second cause of cancer-related death in Western countries [1]. The development of CRC is a multistep process in which a series of genetic and epigenetic alterations activates proto-oncogenes and inactivates oncosuppressor genes, leading to the malignant transformation of colorectal mucosa cells [2,3]. The initial, and probably most important, among such modifications determines a constitutive activation of the β-catenin signaling pathway, resulting in an increased proliferation of CRC cells. Although mutations inactivating the APC oncosuppressor gene represent the main factor responsible for β-catenin activation, a growing body of evidence suggests that loss of cadherin expression, believed to occur as a consequence of epigenetic events, significantly contributes to the same effect, playing also an important role in the acquisition of an invasive phenotype [4,5]. In this regard, the assessment of E-cadherin involvement has led to controversial results in the past, indicating that, at best, the expression of this cadherin is lost in a limited number of CRCs. On the contrary, data obtained in our laboratory using a combined approach of microarray meta-analysis and immunohistochemical evaluation allowed us to demonstrate that most CRCs undergo a downregulated expression of a distinct cadherin, namely μ-protocadherin, that was observed together with higher levels of the Ki-67 proliferation marker and a reduced probability to achieve a 5-year disease-free survival [6,7].

Functional studies, carried out to better characterize the biological role of this adhesion molecule, put in evidence that, as observed for other cadherins, the oncosuppressive property of μ-protocadherin is due to the capacity to sequester β-catenin in a submembrane location, inhibiting its nuclear translocation and the subsequent proliferation effect mediated by the transcription of its target genes [8,9]. Consistently with these findings, treatment of CRC cells with pharmacological inhibitors of the β-catenin pathway, such as mesalazine (5-ASA) or FH535, strongly upregulates μ-protocadherin expression disclosing an involvement of this cadherin in the response to drugs that are potentially endowed with an anti-CRC chemoprevention activity [10]. It is interesting to consider that a couple of reports published some years ago suggested the existence of a similar cadherin, called protocadherin-24, that shares some of the biological properties already ascribed to μ-protocadherin including its particular expression pattern (restricted to the liver, kidney, and colon) and its capacity to inhibit β-catenin [11,12]. Based on this premise, the aim of our article was to better characterize the similarity of μ-protocadherin with protocadherin-24 and to perform an immunohistochemical survey to assess their expression in 2 separate sets of sporadic CRCs and hereditary nonpolyposis colorectal cancers (HNPCCs). The results obtained confirmed the relationship between μ-protocadherin and protocadherin-24, indicating that double-negative samples (lacking the expression of both protocadherins) prevail among sporadic CRCs, whereas double-positive tumors (maintaining the expression of both protocadherins) are more represented in HNPCC. Notably, this particular expression pattern is in perfect agreement with the different clinical outcomes of the 2 considered groups of malignancies according to which HNPCC tumors experience a better prognosis as compared with sporadic CRCs.

2. Materials and methods

2.1. Patients identification

A group of 70 patients with sporadic CRC was identified through the archive of Unit of Pathology of Modena. The tumor samples were taken by patients undergoing colorectal surgical resection. The work was carried out in agreement with the Declaration of Helsinki, obtaining the informed consent from patients. All histologic preparations were examined by the pathologist (L. L.) by selecting the block containing both the neoplasia and the normal mucosa to be used as an internal control. The tumor stage was determined according to the TNM classification of the American Joint Committee on Cancer [13]. Forty-one male and 29 female patients with 7 carcinomas in stage I, 26 in stage II, 24 in stage III, and 13 in stage IV were studied. The onset age ranged from 42 to 88 years, with an average age of 65 years. The neoplasia was localized to the right colon in 16 cases, in 32 to the left colon, and 22 to the rectum. Recurrences and metastasis after surgery were established by the investigation of clinical files and pathological reports of patients.

In addition, 15 cases of hereditary colorectal carcinoma (HNPCC or Lynch syndrome), previously studied and with mutations known in the genes of mismatch repair [14-16], were analyzed, with 10 male and 5 female patients, of which 3 carcinomas in stage I, 7 in stage II, and 5 in stage III. The onset ages ranged from 19 to 75 years, with an average age of 47 years. The neoplasia was localized to the right colon in 9 cases and to the left colon. The germ-line mutations were 7 in the MLH1, 6 gene in the MSH2 gene, and 2 in the MSH6 gene.

2.2. Immunohistochemical analysis

For each case, a representative paraffin-embedded block containing tumor tissue and normal mucosa was sectioned at 4 μm. Immunoperoxidase staining was run with the Benchmark XT Automatic Staining System using diaminobenzidine as chromogen (Roche, Monza, Italy) and using the View DAB Detection Kit (Roche). At the end of the reaction, slides were counterstained with hematoxylin. Mouse monoclonal antibodies against β-catenin (Roche), antib-MLH1 (Roche), and antihuman E-cadherin (clone NCH-38; Dako, Glostrup, Denmark) were used using prediluted commercially available preparations. Mouse monoclonal antihuman Ki-67 antibody (MIB-1;
Dako) was used at 1:200 dilution, and rabbit polyclonal anti-human $\mu$-protocadherin and protocadherin-24 antibodies (Sigma-Aldrich, Milan, Italy) were used at dilution 1:100. Positive controls for both protocadherins were represented by proximal tubules of the kidney and enterocytes of small and large intestines. Therefore, we used the normal intestinal mucosa adjacent to the tumor present in each analyzed case as a positive internal control, and we considered the lymphoid tissue normally present in the lamina propria as an internal negative control. The presence of immune-reactive neoplastic cells stained for $\mu$-protocadherin and protocadherin-24 was graded as follows: 0, negative (absence of membranous staining in all neoplastic cells or <10%), and positive in more than 10% of neoplastic cells. According to previous reports, expression of $\beta$-catenin and E-cadherin in normal colon epithelium was restricted to cell membrane. Altered expression of $\beta$-catenin

Fig. 1  A, Microarray analysis of protocadherin-24 mRNA expression performed in 2 publicly available CRC data sets (right and left). Results are presented as bar histogram indicating the analyzed sample groups on x-axis and the signals detected on y-axis. B, QRT-PCR analysis of protocadherin-24 mRNA expression in CaCo2 cells treated with FH535 and 5-ASA. Results are presented as bar histogram indicating the compound concentrations on x-axis and the expression variations (relative quantity) on y-axis. Asterisks indicate statistical significance. NCM, normal colorectal mucosa; CRA, colorectal adenoma; CRC, colorectal carcinoma; IBD, inflammatory bowel diseases.
was contemplated when 10% of tumor cells or greater showed nuclear or cytoplasmic immunoreactivity [17]. Loss of membranous expression of E-cadherin was considered in cases exhibiting either no immunoreactivity or 10% of tumor cells or less with positive membranous staining. Detection of proliferative activity was carried out using an anti–Ki-67 antibody, and basal cells of the normal colonic crypts were used as an internal positive control. Ki-67 labeling index was determined by counting the number of positive nuclei for 1000 tumor cells in 10 consecutive fields chosen randomly in nonnecrotic areas of the tumor. Statistical analysis of immunohistochemical data was performed using the χ² test and the Student t test.

2.3. DNA microarray analysis

Microarray analysis of protocadherin-24 messenger RNA (mRNA) expression was carried out, as already described for μ-protocadherin [6], using 2 distinct publicly available data sets downloaded from Gene Expression Omnibus: the first, provided by Galamb et al [18], contained 8 normal colorectal mucosa (NCM) samples, 15 colorectal adenomas (CRAs), 15 CRCs, and 15 cases of inflammatory bowel diseases, and the second, provided by Sabates-Bellver et al [19], contained 32 NCM samples and 32 CRCs. mRNA expression was assessed as signal and indicated as the mean ± SEM values.

2.4. Cell cultures and treatment

The CaCo2 CRC cell line was obtained from ATCC (Rockville, MD) and cultured as already described [9]. FH535 (Sigma-Aldrich) and 5-ASA (SofarFarm, Milan, Italy) were respectively dissolved in dimethyl sulfoxide and complete medium, added to cell cultures at the final concentrations, and were incubated for 96 hours, as indicated in Results and in the figures. Control cells were exposed to an equivalent amount of vehicle. All the experiments were repeated at least

![Image: Expression of μ-protocadherin (A, original magnification ×100) and protocadherin-24 (B, ×100) in normal colorectal mucosa. For both protocadherins, the intensity of staining increases from the bottom to the top of the crypts and from the lateral to the apical borders of enterocytes. C and D, The same staining performed on transversally sectioned crypts (both ×400); μ-protocadherin and protocadherin-24 are again, respectively, presented on the left and on the right.](image-url)
2.5. RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted with the Qiagen total RNA purification kit (Qiagen, Valencia, CA) and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). cDNA synthesis was performed using 100 ng of RNA for each sample with the High Capacity cDNA Retrotranscription Kit (Invitrogen, Milan, Italy). Quantitative real-time polymerase chain reaction (QRT-PCR) was then conducted using an ABI PRISM 7900 detection system (Applied Biosystems, Milan, Italy). All primers and probes used for mRNA amplification were designed by Applied Biosystems. Each cDNA sample was run in triplicate using the Taqman Universal PCR Master Mix (Invitrogen), and GAPDH was used as an endogenous control. Quantification of RT-PCR signals was performed using the Ct relative quantification method as described [21]. Statistical analysis was accomplished with the Student t test.

3. Results

3.1. DNA microarray analysis of protocadherin-24 mRNA expression in NCM and colorectal tumors

To provide a preliminary assessment of protocadherin-24 mRNA expression in colorectal tumors, we analyzed the microarray profiles of a number of NCM samples and CRA, CRC, and inflammatory bowel disease cases contained in 2 distinct publicly available data sets downloaded from Gene Expression Omnibus (see Materials and methods for more details). The results of this analysis showed an about 2-fold reduction of signal detected in CRA (P < .001) and CRC (P < .05) as compared with NCM (Fig. 1A), disclosing an expression pattern closely reminding that obtained for μ-protocadherin under the same experimental conditions [6].

3.2. QRT-PCR analysis of protocadherin-24 mRNA expression in FH535– and 5-ASA–treated CaCo2 cells

To evaluate the capacity of β-catenin inhibitors to modulate protocadherin-24 mRNA expression, we performed a QRT-PCR analysis in CaCo2 cells upon a 96-hour incubation with FH535 or 5-ASA. As shown in Fig. 1B, both treatments determined a strong increase of the analyzed parameter, appearing more evident at the highest concentrations of the 2 compounds and revealing an approximately 13-fold and 15-fold up-regulation, respectively, with FH535 and 5-ASA (P < .05). Again, protocadherin-24 mRNA exhibited a pattern of expression very similar to that of μ-protocadherin, suggesting the existence of a biological correlation between the 2 considered protocadherins that we planned to better characterize in the subsequent investigation.

3.3. Immunohistochemical evaluation of μ-protocadherin and protocadherin-24 expression in sporadic CRC and HNPCC

The purpose of our study was to perform a comparative assessment of μ-protocadherin and protocadherin-24 expression that was conducted by immunohistochemical analysis on 70 sporadic CRCs and 15 HNPCCs with known germ-line mutations affecting the MMR genes [14-16]. To gain additional information, the same evaluation was also extended to other antigens such as β-catenin to determine its subcellular localization, Ki-67 to estimate the proliferation levels of tumor samples, and E-cadherin as a control.

The analysis of NCM put in evidence that the physiological pattern of protocadherin-24 expression was virtually identical to that of μ-protocadherin (Fig. 2). In fact, for both these 2 proteins, the staining appeared to be localized at the lateral borders

| Table Results of immunohistochemical analysis performed in sporadic CRC and HNPCC cases |
|---------------------------------|---------------------------------|----------------|----------------|----------------|
| CRC cases                       | Protocadherin expression        | n (%)          | β-c expression (n) | Ki-67 positivity (%) |
|                                 |                                 |                | Mb | Nc | Mb | Nc | β-c | Global |
| Sporadic (70)                  | μ-protocadherin                 | +             | 26 (37) | 19 | 7 | 46 | 68 | 52 |
|                                |                                 | −             | 44 (63) | 1  | 43 | 60 | 68 | 68 |
| Protocadherin-24               | +                                | 23 (33) | 16 | 7 | 50 | 61 | 53 |
|                                | −                                | 47 (67) | 4  | 43 | 46 | 69 | 67 |
| HNPCC (15)                     | μ-protocadherin                 | +             | 11 (71) | 9  | 2 | 39 | 65 | 44 |
|                                | −                                | 4 (29)  | 2  | 2 | 55 | 70 | 62 |
| Protocadherin-24               | +                                | 10 (67) | 8  | 2 | 41 | 65 | 45 |
|                                | −                                | 5 (33)  | 3  | 2 | 47 | 70 | 56 |

NOTE. Expression of Ki-67 is reported as percentage of positive cells calculated in CRC cases exhibiting Mb β-c, Nc β-c, or both (global).
Abbreviations: +, positive immunohistochemical staining; −, negative immunohistochemical staining; n, absolute number of cases; β-c, β-catenin; Mb, membranous staining of β-catenin; Nc, nuclear staining of β-catenin.
and the apical extrusion zone of enterocytes. In addition, its intensity remarkably increased from the former to the latter site of the cells and from the bottom to the top of the crypts. In this regard, it is worth to underline that all the analyzed samples of NCM included in our study exhibited this expression pattern both for μ-protocadherin and for protocadherin-24.

Interestingly, 63% of sporadic CRCs and only 29% of HNPCCs exhibited a loss of μ-protocadherin expression, and similarly, protocadherin-24 was not expressed in 67% of sporadic CRCs and in 33% of HNPCCs (Table). Representative cases of positive and negative staining for μ-protocadherin and protocadherin-24 are shown in Fig. 3A–D.

3.4. Immunohistochemical evaluation of β-catenin, Ki-67, and other antigens in sporadic CRC and HNPCC

Assessment of β-catenin was especially focused on the correlation between its subcellular localization and the expression pattern of the 2 investigated protocadherins. In this regard, among sporadic CRCs, virtually all cases lacking μ-protocadherin expression (43/44) and most cases lacking protocadherin-24 expression (43/47) exhibited a nuclear staining of β-catenin (Table). Consistently, in the same set of patients, a membranous positivity of β-catenin was observed in 19 of 26 cases maintaining μ-protocadherin expression and 16 of 23 expressing protocadherin-24. Evaluation of HNPCCs disclosed a similar trend in cases expressing the 2 protocadherins, whereas cellular β-catenin seemed equally distributed between the membrane and the nucleus in cases lacking their expression (Table). Fig. 3 also shows examples of CRC with membranous or nuclear localization of β-catenin (Fig. 3E and F).
Fig. 5  Bar histogram representing the percentages of CRC cases characterized by the loss of $\mu$-protocadherin and/or protocadherin-24 expression in sporadic (light gray) and HNPCC (dark gray) tumors. Reported values were calculated on the total number of CRCs, respectively, belonging to the 2 considered sample sets (sporadic and HNPCC). Asterisks indicate statistically significant differences between sporadic CRC and HNPCC.
Fig. 6  Bar histogram representing the extent of Ki-67 expression (A, cell percentage) and β-catenin nuclear localization (B, case percentage) in CRC cases belonging to the various phenotype categories already indicated in Fig. 5. Asterisks indicate statistically significant differences between sporadic CRC and HNPCC.
Evaluation of Ki-67 positivity was measured as mean percentage and related to the expression pattern of the 2 protocadherins and to the subcellular distribution of β-catenin. No substantial differences emerged in this analysis by the comparison between sporadic CRC and HNPCC, and considering the 2 sets of patients all together, Ki-67 positivity averaged 67% in μ-protocadherin–negative tumors and 51% in those positive for its expression. Superimposable results were obtained for protocadherin-24 where the same value was 66% in negative cases and 51% in positive cases.

Loss of E-cadherin expression was detected in only 4 of the 70 analyzed sporadic CRC cases, all characterized by a normal expression of μ-protocadherin and protocadherin-24, combined with a location of β-catenin on the plasmatic membrane. Conversely, none of the analyzed HNPCC cases exhibited an absence of E-cadherin signal (not shown).

Based on the strict association, observed in sporadic CRC, between the loss of MLH1 expression and the onset of neoplasia in the proximal colon, all cases of our study exhibiting that location were also subjected to an immunohistochemical assessment of MLH1 protein. The data obtained by this subset of patients demonstrated that only 1 among a global number of 16 analyzed samples showed a complete loss of MLH1 expression.

### 3.5. Numerical/statistical elaboration and interpretation of data derived by immunohistochemical analysis

A relevant finding arose by the evaluation of how the 2 protocadherins were distributed and reciprocally combined in the various examined CRC cases. This approach, in fact, put in evidence that double-negative cases (lacking expression of both protocadherins) were 53% in sporadic CRC and only 13% in HNPCC (Fig. 4). Consistently, double-positive cases (expressing both protocadherins) resulted in 14% and 54%, respectively (Fig. 4). Both of these comparisons were statistically significant with P values of less than .05 (Fig. 5). No substantial differences were instead noted for single μ-protocadherin–negative cases (10% in sporadic CRC versus 13% of HNPCC) and single protocadherin-24–negative cases (23% in sporadic CRC versus 20% of HNPCC).

Interestingly, within the subset of sporadic CRC patients characterized by a location of the neoplasia in the proximal colon (globally 16), 5 cases maintained the expression of both protocadherins, and among them, 1 was MLH1 negative, indicating that probably loss of MLH1 protein was not the only factor promoting this phenotype.

Mean values of the Ki-67 labeling index seemed similar in all the mentioned CRC case comparisons (Fig. 6A). Despite this, double-negative CRCs exhibited higher percentages of Ki-67 expression as compared with double-positive CRCs (69% versus 48%, \(P < .05\)), with single negative/positive cases placed in an intermediate situation.

Distribution of nuclear β-catenin positivity in the same CRC categories showed, in general, a good correlation with the expression pattern of Ki-67 (Fig. 6B). Not surprisingly, in HNPCC, β-catenin localized less frequently in the nuclear compartment of cancer cells, in agreement with the more limited involvement of its pathway that has been demonstrated in such tumors.

### 3.6. Correlation among immunohistochemical data and clinical features of CRC patients

Evaluation of the histopathologic parameters revealed a statistically significant relationship between immunohistochemical data and histologic grade. In fact, high-grade tumors (poor differentiated carcinomas) showed a more pronounced loss of protocadherin expression as compared with low-grade tumors (well and moderately differentiated; \(P < .05\)). A correlation was also found between the loss of protocadherin expression and the clinical stage because an increasing number of such cases were observed in the transition from stages I to IV, although this observation was not statistically significant. Similarly, we detected a correlation between the presence of metastasis after surgery and the absence of protocadherin expression, but without statistical significance (not shown).

### 4. Discussion

The purpose of this study was to assess whether expression of μ-protocadherin and protocadherin-24 is down-regulated in CRCs, focusing especially our attention on the distinction between sporadic CRC and HNPCC. The results obtained disclosed a significant discrepancy between the 2 sets of patients, characterized by a more pronounced loss of protocadherin expression in the former than in the latter. In particular, the most relevant finding of our analysis was represented by the observation that the percentage of double-negative cases lacking expression of both protocadherins seemed to be 4 times higher in sporadic CRC than in HNPCC, whereas double-positive cases expressing both protocadherins exhibited an opposite trend. In both situations, CRC cases exhibiting a consensual expression of the 2 protocadherins (double negative and double positive) largely prevailed on those characterized by expression of a single protocadherin (single negative or single positive). These data suggest the existence of a coordinated regulation in the molecular control of μ-protocadherin and protocadherin-24, which was also supported by our preliminary data of molecular analysis presented in the initial part of the Results section. Not surprisingly, in both patient groups, loss of protocadherin expression was accompanied by a more frequent localization of β-catenin in the nuclear compartment and an increased positivity of the Ki-67 proliferation marker. This observation could be conceivably ascribed to the ability of protocadherins to sequester β-catenin on plasmatic membrane, hampering its nuclear translocation, as previously suggested by Parenti et al [8] and Ose et al [12]. The lower frequency of protocadherin down-regulation observed in HNPCC is in perfect agreement with their best clinical outcome and with the different carcinogenic mechanisms operating in these tumors, related to the
impairment of MMR genes rather than the constitutive activation of the β-catenin pathway, more typical of sporadic CRCs. A recent report developed using a high-throughput approach aimed to realize a systematic view of CRC somatic mutations allowed for the identification of 2 subclasses of CRCs that were respectively defined by authors as hyper and nonhypermutated [22,23]. The hypermutated tumors (16% of all CRCs) presented a high microsatellite instability, generally due to promoter hypermethylation of the MLH1 gene and only in a 25% of cases to its somatic mutations. Both hypermutated and nonhypermutated groups exhibited a deregulation in the Wnt signaling pathway in apparent contrast with the data presented here. This divergence could, in our opinion, depend on the fact that the mentioned study did not consider hereditary CRC such as HNPCC. In addition, those that have been analyzed in our report harbor germ-line mutations of MMR genes that affected not only the MLH1 but also the MSH2 and MSH6 genes. The different genetic background of studied tumors could therefore provide an explanation for the discrepancy under discussion.

To conclude, our data demonstrate for the first time that expression of protocadherin-24 is down-regulated in CRC, mimicking the expression pattern already observed for μ-protocadherin, with which it seems to be functionally related. As a logical consequence, CRCs losing the expression of both protocadherins undergo higher proliferation levels as compared with those losing a single protocadherin or maintaining their expression. This indicates that the evaluation of panels of cadherins/protocadherins rather than single cadherins or protocadherins could help to better define the biological and clinical features of CRCs in the future. These data could contribute to obtain a detailed molecular classification of CRC favoring a more accurate diagnosis and helping to develop therapeutic strategies based on patient stratification for precision medicine.

Although of minor relevance, our study also allowed us to characterize the physiological expression pattern of protocadherin-24 in colorectal mucosa that once again paralleled that of μ-protocadherin, suggesting a role as differentiation marker for both of these adhesion molecules.

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