Colorectal carcinogenesis involves a multistep progression of genetic mutations. Based on the adenoma–adenocarcinoma sequence, much research has focused on mutation detection; sequential genetic alterations have been illustrated as a linear process (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996). Although this model represents a well-known paradigm for the sequential development of cancer driven by the accumulation of genetic defects, more and more cases of carcinogenesis have been reported in contrast to the linear and clonal development of cancer (Sedivy et al., 2000). Recently, a non-linear, chaodynamic model of carcinogenesis has been suggested by others. In this model, genetic instability among cells produces a tremendous and chaotic diversity that may lead to cancer (Coffey, 1998). Furthermore, from a holistic point of view, not only genetic events may drive the onset of cancer. Instability among cells produces a tremendous and chaotic diversity that may lead to cancer (Coffey, 1998). Furthermore, from a holistic point of view, not only genetic events may drive the onset of cancer. Among these events, it seems that the accumulation of genetic alterations is more important than their order (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996), some researchers are still interested in establishing an ideal linear process. Nonetheless, there are reports indicating that the significantly low frequency of K-ras mutation is associated with superficial or non-polyloid type colorectal adenoma or carcinoma (Yamagata et al., 1994, 1995). Others and we have further demonstrated significant correlation between polyloid growth of CRC and K-ras codon 12 mutation (Chiang et al., 1998). However, with regard to the ulcerative type of color-
analysed in relation to
expression, one of the downstream targets of
investigated. Materialising matrix metalloproteinase-7 (MMP-7)
tumour suppressor gene or the
sion including E-cadherin expression, mutations of the APC
colorectal carcinomas. Factors related to altered
nin was related to different morphological growth patterns in
genes (Korinek et al
colorectal carcinogenesis because it binds the products of the
APC tumour suppressor gene. When APC is mutated, which occurs
in up to 80% of colorectal cancer, β-catenin accumulates and
translocates to the nucleus, where it binds the transcription factors
of the TCF/LEF gene family and activates the expression of target
genes (Korinek et al, 1997).

We attempted to detect if any differential expression of β-cate-
nin was related to different morphological growth patterns in
colorectal carcinomas. Factors related to altered β-catenin expres-
sion including E-cadherin expression, mutations of the APC
tumour suppressor gene or the β-catenin gene itself were also
investigated. Materialising matrix metalloproteinase-7 (MMP-7)
expression, one of the downstream targets of β-catenin, was also
analysed in relation to β-catenin expression.

MATERIALS AND METHODS

Sample collection
We collected 51 primary colorectal carcinoma tissue samples from
sporadic colorectal cancer patients who underwent colectomies at
Chang Gung Memorial Hospital (CGMH). All samples were
collected immediately after resection and stored in a −80°C free-
zer. Normal mucosa samples were removed at the same time from
sites about 10 cm from each tumour. Whole tumour specimens
were then prepared for routine histopathological examination.
Formalin-fixed, paraffin-embedded colorectal carcinoma tissue
samples were preserved in the tissue archives of the Pathology
Department at CGMH.

Detailed morphological descriptions, histopathological data and
clinical data were obtained for each case from the Cancer Registry
of the Department of Colorectal Surgery at CGMH.

Tumours with exophytic cauliflower-like appearances with or
without a very shallow ulcer only, and with a height exceeding half
their diameter, were classified as polypoid (Figure 1A). Tumours
within depressed ulcers with or without very low elevated edges,
and showing endophytic growth, were classified as ulcerative
(Figure 1B).

Immunohistochemistry
Standard immunohistochemical detection with minor modifica-
tions was performed on sections from the archival, paraffin-
embedded tissue to detect E-cadherin, β-catenin and matrilysin
proteins.

Five-micron sections mounted onto slides were deparaffinised
and rehydrated in graded alcohols and distilled water. Endogenous
peroxidase activity was inhibited by incubation with 3% hydrogen
peroxide in methanol for 20 min. Antigen retrieval was done by
microwaving at high power for two cycles of 5 min each, with a
10-min break between cycles in citrate buffer at pH 6.0. Non-spe-
cific binding of secondary antibodies was blocked by incubation in
10% normal rabbit serum.

Incubations with primary antibodies were done at 37°C for
120 min for anti E-cadherin (Transduction Laboratories, Lexing-
ton, KY, USA) at 100 × dilution, 60 min at 37°C for anti-β-
catenin (Transduction Laboratories) at 200 × dilution and
1:300 for MMP-7 (Chemicon, Hofheim, Germany). After three
washes with phosphate buffered saline (PBS), the slides were
incubated with biotinylated antimouse immunoglobulin and
and rehydrated in graded alcohols and distilled water. Endogenous
proteins.

In areas of well-preserved tissue, the staining intensity
was as strong as that of normal glands, and the proportion of
positive cancer cells in each section was more than 90%. If there
was identifiable positive staining in less than 10% of the cancer
was considered preserved when staining of cancer cells
was as strong as that of normal glands, and the proportion of
positive cancer cells in each section was more than 90%. If there
was identifiable positive staining in less than 10% of the cancer
cells, the tumours were recorded as having a loss of E-cadherin
or β-catenin membrane expression. Nuclear staining of positive
cells was defined as an intense brown colour in the nucleus
(Figure 2). The pattern of nuclear staining was defined as follows:
negative group, no less than 5% scattered positive cells without
any clusters; focal group, positive cells clustered in focal areas,
when >5%, but <50% of the nuclei were stained; diffuse,
over-expressed group, positive cells distributed diffusely, homoge-
neously or heterogeneously, when ≥50% of the nuclei were
stained.

Stain interpretation
Slides were independently examined by two experienced observers
(JM Chiang and KF Ng) who were blind to the clinicopathologi-
cal data of the tumour and to the initial results of the other
observer. In areas of well-preserved tissue, the staining intensity
of the cell membrane or cytoplasm was evaluated using the stain-
ing of adjacent non-involved normal mucosa as the internal
control for each section. Membrane expression of E-cadherin or
β-catenin was considered preserved when staining of cancer cells
was as strong as that of normal glands, and the proportion of
positive cancer cells in each section was more than 90%. If there
was identifiable positive staining in less than 10% of the cancer
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APC and β-catenin gene mutational analysis

Genomic DNA from each tumour sample and corresponding normal mucosa was extracted. SSCP analysis of β-catenin exon 3 was performed using the following primer pair: exon 3, 5’-GATTGGATGGACATGG-3’ and 5’-TGTTCTTGAGTGAAGGACTGAG-3’. Samples were amplified through 35 cycles on a thermocycler (Perkin-Elmer) at 95°C denaturation for 50 s, 57°C annealing for 30 s and 72°C extension for 10 min. Polymerase chain reaction (PCR) was performed in a volume of 25 μl with 20 ng of genomic DNA in a PCR buffer containing 1.5 mM MgCl2, 200 μM each deoxyribonucleoside triphosphate, 5 pmol of each primer and 0.5 units of Taq polymerase (Perkin-Elmer, Branchburg, NJ, USA).

For the investigation of the APC gene, exon 15 mutations were performed using the protein truncation test (PTT) on genomic DNA as described previously (Van der Luijt, 1994). Exons 1–14 were screened by SSCP using published oligonucleotides and PCR conditions (Van der Luijt, 1994; Li et al., 1999). After PCR amplification, products were loaded onto 12.5% polyacrylamide

Figure 2  Representative immunohistochemical staining of E-cadherin and β-catenin in resected colon specimens from sporadic CRC. (A) E-cadherin and (C) β-catenin are mainly localised at the membranes of the cell-to-cell borders (preserved, paraffin-embedded colorectal carcinoma); (B) loss of membrane staining for E-cadherin. Both (D) and (E) show a loss of membrane staining of β-catenin with reciprocal change in nuclear β-catenin expression. (D) Diffuse, widespread nuclear immunostaining for β-catenin is profound in colon carcinoma cells, while (E) shows focal, clustered nuclear β-catenin expression. A–E: x 400.
gels from a GeneGel Excel 12.5/2.4 kit (Pharmacia Biotech, AB Uppsala, Sweden) and underwent electrophoresis at 20°C. The single and double strands of the PCR products were visualised by silver staining as described previously.

**Statistics**

Comparative data were analysed using the Mann–Whitney U-test, the Pearson’s correlation coefficient and the chi-square test. A two-sided P<0.05 was determined as statistically significant.

**RESULTS**

We collected 51 colorectal carcinoma samples, including 25 ulcerative tumours and 26 polypoid tumours. The clinicopathological parameters, including age, gender, tumour stage, tumour differentiation and tumour size were comparable between the two groups, except for right colon predominance in the polypoid CRC group and significantly deeper invasion depth in the ulcerative CRC group (Table 1). The difference in depth of invasion between these two groups were mainly reflected in the smaller-sized (<4 cm) tumour group, while no difference in invasion depth was found for the larger-sized (>4 cm) tumour group (Table 2).

Regarding β-catenin and E-cadherin expression by immunohistochemical staining, loss of membrane staining was observed in 23 out of the 51 (45%) and 22 out of the 51 (43%) cases, 23 out of the 51 (45%) and 22 out of the 51 (43%) cases, respectively. Forty-nine per cent (25 out of 51) of cases showed nuclear β-catenin expression and always showed a reciprocal loss of membrane staining except for two preserved cases (Table 3).

There was no significantly different expression of E-cadherin between polypoid and ulcerative groups. However, a significant difference in membrane β-catenin staining was found between ulcerative (17 out of 25, 68%) and polypoid (six out of 26, 23%) groups. A significant difference in nuclear expression of β-catenin (P=0.001) was also observed between the ulcerative (18 out of 25, 72%) and polypoid (seven out of 26) groups of CRC (Table 3) when we defined more than 25% of cells with nuclear β-catenin expression as positive, and negative was defined as expression in less than 25% of the cells. Nonetheless, nuclear β-catenin expression was typically heterogeneous; we, therefore, analysed the extent of nuclear β-catenin expression with relation to these two distinct morphological types of CRC. We found a significant difference between polypoid and ulcerative types of CRC. However, we did not find a significant difference related to the depth of invasion or the location of the tumour (Table 4).

The frequency of APC mutations was 39 out of 51 (76%) cases. Among the 39 mutations found, 34 were detected in exon 15 trans-cated proteins (Figure 3), two mutations were found in exon 12, one in exon 10 and one in exon 6. Nineteen out of 25 (76%) APC mutations were found in polypoid tumours and 20 out of 26 (77%) were found in ulcerative tumours (Table 3). No β-catenin exon 3 mutation was detected in this study.

Nuclear β-catenin expression was analysed in relation to its related factors. There were no correlations found among nuclear β-catenin expression and APC gene mutations (Table 5). In addi-

**Table 1** Comparisons of clinical and histopathological parameters between ulcerative and polypoid colorectal carcinomas

|                | Ulcerative (n=25) | Polypoid (n=26) | P value |
|----------------|-------------------|----------------|---------|
| Age range (years) | 41–83             | 29–80          | 0.752   |
| Average age (years) | 61.3              | 60.6           |         |
| Sex             | M/F 17/8          | 14/12          | 0.301   |
| Tumour location |                   |                |         |
| Right colon     | 1                  | 4              |         |
| Left colon      | 4                  | 12             |         |
| Rectum          | 20                 | 10             | 0.010   |
| Duke’s stage    |                   |                |         |
| A               | 0                  | 4              |         |
| B               | 14                 | 13             |         |
| C               | 9                  | 6              |         |
| D               | 2                  | 3              | 0.186   |
| Depth of invasion |                |                |         |
| T1              | 1                  | 8              |         |
| T2              | 19                 | 17             |         |
| T3              | 5                  | 1              | 0.017   |
| Tumour differentiation |        |                |         |
| Well            | 5                  | 9              |         |
| Moderate        | 20                 | 15             |         |
| Poorly          | 0                  | 2              | 0.147   |
| Tumour histology |                  |                |         |
| Adenocarcinoma  | 22                 | 21             |         |
| Mucinous adenocarcinoma | 3    | 5              | 0.457   |
| Tumour size     |                   |                |         |
| <3 cm           | 7                  | 5              |         |
| 3–5 cm          | 12                 | 16             |         |
| >5 cm           | 6                  | 5              | 0.614   |

**Table 2** Comparisons of invasion depth between polypoid and ulcerative colorectal cancer

| Tumour size (cm) | Polypoid | Ulcerative | P value |
|-----------------|----------|------------|---------|
| ≤4              | 6        | 1          |         |
| >4              | 2        | 0          | 0.018   |

**Table 3** Comparisons of APC mutation and expression of β-catenin and E-cadherin between ulcerative and polypoid colorectal carcinoma

|                | Ulcerative (n=25) | Polypoid (n=26) | P value |
|----------------|-------------------|----------------|---------|
| Nuclear β-catenin expression |        |               |         |
| +               | 18                 | 7             | 0.001   |
| -               | 7                  | 19            |         |
| Membranous β-catenin expression |    |              |         |
| +               | 8                  | 20            |         |
| -               | 17                 | 6             | 0.001   |
| Membranous E-cadherin expression |  |            |         |
| +               | 15                 | 14            |         |
| -               | 10                 | 12            | 0.626   |
| APC mutation    |                   |                |         |
| +               | 19                 | 20            |         |
| -               | 6                  | 6             | 0.843   |
| β-catenin exon 3 mutation |        |               |         |
| +               | 0                  | 0             |         |
| -               | 25                 | 26            |         |

*^aT1: Tumour invades submucosa; T2: Tumour invades muscularis propria; T3: Tumour invades through the muscularis propria into the subserosa.

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Table 4  Relationship between nuclear expression of \(\beta\)-catenin and histopathology of colorectal cancers

| Gross morphology |  |  |  |  |  |
|------------------|---|---|---|---|
| Polypoid         | 7 | 12 | 7 | 0 |
| Ulcerative       | 2 | 5 | 11 | 7 | 0.001 |

| Depth of invasion |  |  |  |  |
|-------------------|---|---|---|
| T1                | 2 | 4 | 3 | 0 |
| T2                | 7 | 11 | 13 | 5 |
| T3                | 0 | 2 | 2 | 2 | 0.607 |

| Tumour location   |  |  |  |
|-------------------|---|---|---|
| Right colon       | 1 | 1 | 2 | 1 |
| Left colon        | 1 | 7 | 8 | 0 |
| Rectum            | 7 | 9 | 8 | 6 | 0.490 |

Table 5  Correlation between nuclear \(\beta\)-catenin expression and APC mutation, E-cadherin expression and MMP-7 expression

| Nuclear \(\beta\)-catenin expression |  |  |  |  |  |
|----------------------------------|---|---|---|---|
| Negative                         | 5–24% | 25–49% | \(\geq 50\)% |  |
| APC                              |  |  |  |  |
| +                                | 17 | 17 |  |  |
| –                                | 8 | 9 |  | 0.483 |
| E-cadherin expression            |  |  |  |  |
| +                                | 15 | 14 |  |  |
| –                                | 11 | 11 |  | 0.848 |
| MMP-7 expression                 |  |  |  |  |
| +                                | 3 | 8 |  |  |
| –                                | 22 | 18 |  | 0.103 |

DISCUSSION

We clearly demonstrated that nuclear \(\beta\)-catenin expression was significantly related to ulcerative growth of CRC. This result is comparable to results in a recent report (Aust et al., 2001) describing altered distribution of \(\beta\)-catenin in ulcerative colitis-related colorectal cancer.

Morphologically, the gross appearance of advanced sporadic CRC is quite variable. It is usually difficult to categorise all tumours into either the polypoid or ulcerative groups because intermediate or mixed types showing variable amounts of both components can be found. Other less frequently encountered types are flat or plateau tumours or the rare pipe-like shaped (linitis plastica) tumours. This striking diversity in the gross appearance of CRC may reflect the underlying chaotic genetic instability. In our study, we investigated purely polypoid and purely ulcerative tumours. The implications of our findings are further discussed below.

Contradictory to previous studies showing that K-ras codon 12 mutation is selectively related to polypoidal growth of CRC (Yamagata et al., 1994; Chiang et al., 1998), our study indicated that there were different combinations of genetic alterations occurring in morphologically different tumours during colorectal carcinogenesis. Therefore, reports trying to define the sequence or the specific sites of genetic alterations during multi-step colorectal carcinogenesis should be very carefully considered because the same combinations of genetic alterations may not always be accumulated among the morphological types of CRC. Our findings highlight that future development of responsible genes for gene therapy or genetic diagnosis for CRC may need to be individualised.

Although many in vitro studies using cell lines have proven that mutations in the APC tumour suppressor gene occurs in most colorectal cancer and leads to the activation of \(\beta\)-catenin (Munemitsu et al., 1995; Morin et al., 1997), this probably is not always the case in vivo. In our study, we showed that nuclear \(\beta\)-catenin expression was independent of APC tumour suppressor gene mutation, as was reported previously (Kobayashi et al., 2000). Furthermore, we did not find any \(\beta\)-catenin mutation itself. The question follows, therefore, what regulates or forces the nuclear translocation of \(\beta\)-catenin? Other genetic or epigenetic events may be present for modulating nuclear translocation. Tyrosine phosphorylation of \(\beta\)-catenin by a biochemical molecule such as intestinal trefoil factor has been reported (Liu et al., 1997). While, retinoic acid (RA) has been shown to decrease the activity of the \(\beta\)-catenin- lymphoid enhancer binding factor/T-cell factor signalling pathway. RA activity was also independent of APC tumour suppression and ubiquitination-dependent degradation of cytoplasmic \(\beta\)-catenin (Easwaran et al., 1999).

Although a significant relationship exists between ulcerative CRC and nuclear \(\beta\)-catenin expression, we did not find a significant increase in the extent of nuclear \(\beta\)-catenin expression related to the depth of invasion (Table 4). These findings indicate that although nuclear \(\beta\)-catenin expression may determine the ulcerative growth pattern of CRC, the depth of invasion may be determined by several other factors, and might be the result of a more complex process between tumour and stroma interaction. This finding also implies that higher amounts of nuclear \(\beta\)-catenin expression are probably necessary for the ulcerative growth from the early stage of tumour progression, while lower amounts of nuclear \(\beta\)-catenin expression may be sufficient to induce polypoid tumour growth (Brabletz et al., 2000). This finding supports a previous observation showing that \(\beta\)-catenin occurred in the highest concentrations in the invading line of endophytic growth of tumour cells. (Brabletz et al., 1998). Although we found a significant correlation between nuclear beta-catenin expression and ulcerative growth and also observed that most of the ulcerative tumours were rectal carcinomas (Table 1), we did not observe a significant relationship between tumour localisation and nuclear
on tumour localisation. Furthermore, the small number of cases in this study limited further analysis of whether there are different carcinogenic pathways in the colon and rectum.

Precisely how nuclear β-catenin expression confers ulcerative or endophytic growth to CRC remains poorly understood. Further analyses of several downstream factors of the nuclear β-catenin/TCF complex, including c-MYC (He et al., 1998), cyclin D1 (Tetsu and McCormick, 1999), gastrin (Koh et al., 2000), PPARG (He et al., 1999) MMP-7 (Brabletz et al., 1999; Crawford et al., 1999) are warranted. Some factors such as c-myc, cyclin D and gastrin reportedly relate to tumour proliferation, while others are related to tumour invasion. MMP-7 is regulated by β-catenin expression (Brabletz et al., 1999) and is a proteolytic enzyme related to tumour invasion. It may well be reasonable to relate proteolysis to ulcerative growth. However, in our study, we did not observe parallel expression between β-catenin and MMP-7, which is the case in cell culture studies. These negative findings may be explained similarly to other down-regulators such as tumour growth factor (TGF)-β, which is involved in a more complex process in vivo than in vitro (Gaire et al., 1994).

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Finally, the finding that nuclear β-catenin expression is closely related to ulcerative growth of CRC further supports a previous study that showed analogies between embryonic gastrulation and β-catenin expression (Kirchner and Brabletz, 2000). Strong, diffuse β-catenin nuclear expression was observed as necessary for mesenchymal transition of tumour cells that expressed invasion behaviour, while weak nuclear β-catenin expression was only enough for epithelial transitions responsible for cell proliferation (Kirchner and Brabletz, 2000). The connection of our data and the data from embryonic development, thus, supports the concept that tumorigenesis has the properties of a complex developmental disorder (Dean, 1998).

In summary, we observed that the different combinations of genetic alterations may selectively underlie different types of CRC. Nuclear β-catenin expression is related to the ulcerative growth patterns of CRC. Although the precise mechanism remains poorly understood, its expression is independent of APC mutation. These observations further highlight the heterogeneous nature of CRC, which should be kept in mind when developing a new gene therapy or a new genetic diagnosis for CRC.