An insight into the genetic pathway of adenocarcinoma of the small intestine


**Background:** Although the adenoma to carcinoma pathway in colorectal cancer is well described, the mechanisms of carcinogenesis in the small intestine remain unclear.

**Aims:** The aim of this study was to investigate candidate genes in the genetic pathway of adenocarcinoma of the small intestine.

**Subjects and methods:** A total of 21 non-familial, non-ampullary adenocarcinomas of the small intestine were analysed. DNA was extracted from formalin fixed paraffin wax embedded tissue using standard techniques. The replication error (RER) status was determined by amplification of BAT26. The mutation cluster region (MCR) of the adenomatous polyposis coli (APC) gene was screened using polymerase chain reaction single strand conformational polymorphism and direct sequencing. Immunohistochemistry was performed on formalin fixed paraffin wax embedded tissue using monoclonal antibodies for hMLH1, hMSH2, β-catenin, E-cadherin, and p53.

**Results:** Fourteen male and seven female patients with a median age of 64 years (range 21–85) presented with adenocarcinoma of the duodenum (10), jejunum (7), and ileum (4). One cancer (5%) was found to be RER+, and all tumours stained positive for hMLH1 and hMSH2. No mutations were detected in the MCR of the APC gene. β-Catenin showed increased nuclear expression with loss of membranous staining in 10 cancers (48%). Absent or decreased membrane expression of E-cadherin was found in eight cancers (38%). Strong staining of p53 was found in the nucleus of five cancers (24%).

**Conclusion:** We did not detect mutations in the MCR of the APC gene, and this suggests that adenocarcinoma of the small intestine may follow a different genetic pathway to colorectal cancer. Abnormal expression of E-cadherin and β-catenin was common and reflects an early alternative to APC in this pathway in which mutations may be found in adenocarcinoma of the small intestine.

Cancer of the small intestine is rare and accounts for only 1% of gastrointestinal malignancies, and this is despite the fact that the small intestine contains approximately 90% of the mucosal surface of the gastrointestinal tract. The incidence of colorectal cancer is 50 times that of small bowel cancer, yet the histopathological features of the two cancers are similar. Both cancers arise from adenomatous polyps and patients with one cancer are at increased risk of developing the other.

Adenocarcinoma together with carcinoid tumours are the most common histological types of cancer of the small intestine but patients may present with other primary malignant tumours, including lymphoma and leiomysarcoma. Familial adenomatous polyposis (FAP) and Crohn’s disease are recognised risk factors for small bowel cancer, and although adenocarcinomas cluster in the duodenum, the increased risk in Crohn’s disease is mainly localised to the terminal ileum. Other risk factors include Peutz-Jeghers syndrome, coeliac disease, and hereditary non-polyposis colorectal cancer (HNPCC).

It has been suggested that the rapid turnover of the small intestine mucosa, the relative absence of bacteria, and decreased transit time are all reasons why the incidence of cancers of the small intestine is less than that of the large intestine. Other suggestions include the alkaline pH within the small bowel, the liquid nature of small bowel contents, and a well developed local IgA mediated immune system.

The diagnosis of adenocarcinoma of the small bowel is often delayed due to the patient having non-specific symptoms, and the subsequent difficulties that this produces in performing the relevant investigation. This undoubtedly contributes to the poor prognosis despite surgery and adjuvant chemotherapy and radiotherapy. Approximately 50% of adenocarcinomas of the small intestine are poorly differentiated, and the majority have infiltrated through the bowel wall at presentation.

The histological progression to invasive colorectal cancer is termed “the adenoma-carcinoma sequence” and is accompanied by a series of well described genetic changes that involve activation of oncogenes and inactivation of tumour suppressor genes. Although the prevalence of cancer of the small intestine is low compared with colorectal cancer, the similarities in the two cancers suggest that they may share many of the genetic changes of carcinogenesis. If the two cancers differ in either the type of genetic changes or the frequency of these changes, then it may be hypothesised that the small bowel is resistant to the genetic events that occur in colorectal cancer. Furthermore, if a mechanism for any potential differences could be identified, this may have clinical applications in the future.

At present, there have been very few studies (usually with small numbers) of the molecular genetics of cancer of the small intestine. The aim of this study was to investigate the following candidate genes or their protein expression in the genetic pathway of adenocarcinoma of the small intestine: the 

**Abbreviations:** FAP, familial adenomatous polyposis; HNPCC, hereditary non-polyposis colorectal cancer; APC gene, adenomatous polyposis coli gene; RER, replication error; MMR, mismatch repair; MCR, mutation cluster region; PCR, polymerase chain reaction; SSCP, single strand conformational polymorphism.
Clinicopathological features of 21 non-familial, non-ampullary small bowel adenocarcinomas

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<td>Yes</td>
<td>No</td>
<td>hMLH1, hMSH2</td>
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</table>

Mismatch repair genes hMLH1 and hMSH2, the adenomatous polyposis coli (APC) gene, β-catenin, E-cadherin, and p53. HNPCC, which is characterised by microsatellite instability (or replication error, RER+) is caused by germline mutations in one of the DNA mismatch repair (MMR) genes.14 To date, inactivating mutations have been described in five mismatch repair genes: hMSH2, hMLH1, hPMS1, hPMS2, and GTBP (hMSH6).15-22 Although adenocarcinoma of the small intestine is 25 times more common in HNPCC kindreds than in the normal population,23 the incidence of RER+ sporadic adenocarcinoma of the small intestine is not known.

The APC gene, which is responsible for FAP, acquires truncating mutations in up to 80% of sporadic colorectal cancers.24 Unlike the germline mutations of FAP, 60% of mutations in sporadic colorectal cancer cluster in a region around codons 1200–1600—the mutation cluster region (MCR).24 This results in functions of the APC protein beyond the MCR being lost. This includes downregulation of the levels and activity of β-catenin,25-28 a 92 kDa protein which is important in the functional activities of both APC and E-cadherin. The selective advantage of APC mutations may be loss of regulation of β-catenin activity (which then accumulates in the nucleus), and this is supported by the finding of gain of function mutations of the β-catenin gene in those colorectal cancers with wild-type APC.27,28 E-cadherin encodes a 120 kDa transmembrane glycoprotein which localises mainly to the zonula adherens junctions and serves as the prime mediator of epithelial cell to cell adhesion. APC competes directly with E-cadherin for binding to β-catenin and therefore inactivation of either gene may be an indirect regulator of E-cadherin mediated adhesion. An alteration in any of these genes may cause abnormal architectural development of epithelium, which results in the initial development of adenomas in colorectal cancer.29

p53 is a nuclear oncosuppressor protein which is involved in the maintenance of genomic integrity. Mutations in the p53 gene occur commonly in a wide range of human cancers and result in increased expression of p53. The selective effect of p53 mutations is primarily on the frequency of programmed cell death or apoptosis.30 p53 mutations occur commonly in the adenoma to carcinoma sequence of colorectal cancer and are probably a late event.31

**MATERIALS AND METHODS**

**Materials**

Formalin fixed paraffin wax embedded archival tissue was available from a total of 21 non-familial, non-ampullary small bowel adenocarcinomas of the small intestine (table 1) (John Radcliffe Hospital, Oxford; University Hospital of Wales, Cardiff; Gloucestershire Royal Hospital, Gloucester; Southampton General Hospital, Southampton). Twelve male and nine female patients with a median age of 64 years (range 21–85) presented with adenocarcinoma of the duodenum (n=10, 48%), jejunum (n=7, 33%), and ileum (n=4, 19%). Six of the duodenal cancers were not resected and therefore only biopsy material was available for study. Of the remaining 15 cancers, there were three stage T4, 11 stage T3, and one stage T2 cancers. Three of the five cancers resected with lymph nodes had lymph node adenocarcinoma deposits. Fifteen cancers were moderately or well differentiated, with six being poorly differentiated.

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DNA extraction
DNA was extracted from 15 µm paraffin sections using a standard Nucleon DNA extraction kit (SL-8502 Nucleon QC, Didsbury, Manchester, UK). Briefly, the paraffin sections were added to xylene at 37°C for 20 minutes, prior to rehydration with alcohol and water. Proteinase K solution was then added and left for 55°C for three hours. After chloroform extraction, genomic DNA was precipitated with ethanol.

Determination of the replication error (RER) status
In order to determine the RER status of these cancers, we amplified BAF26, a single poly (A) tract, previously shown to be highly sensitive and specific for microsatellite instability, using fluorescent labelled primers and similar polymerase chain reaction (PCR) conditions to those previously described. PCR products were loaded on a 37% prism sequencer (ABI, Warrington, Cheshire, UK). Results were analysed using GeneScan software (version 2.0.2). All PCRs and analyses were repeated at least in duplicate. Any tumours presenting ambiguous results were further investigated using BAF25.

Screening for mutations in the APC gene
Using previously described primers for exon 15E-1 of the APC gene, the MCR was amplified specifically from genomic DNA. All reactions contained approximately 100 ng of template DNA in a total volume of 50 µl with final reaction concentrations of 1x PCR buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 0.1% Triton, 3 mM MgCl2), 200 mM dNTPs, 0.2 mM of each primer, and 1 U of Taq polymerase. Amplification was performed using a protocol of 95°C for five minutes, 35 cycles of 95°C for one minute, 60°C for one minute (55°C for exons 15E and 15I), 72°C for one minute, and finally 72°C for 10 minutes. In cases of poor amplification, flanking primers for exons 15 were used to perform a nested PCR prior to using specific primers for the region of interest.

Single strand conformational polymorphism (SSCP) analysis
SSCP was performed as previously described. Electrophoresis was performed in 10% non-denaturing polyacrylamide gels and the PCR products visualised with silver staining using standard methods.

Direct sequencing of the MCR of the APC gene
The nucleotide sequences of the PCR products showing an abnormal electrophoretic mobility on SSCP analysis were determined by direct sequencing of purified PCR product in a thermocycle sequencing reaction with the dRhodamine Sequencing kit on a 377 prism sequencer (ABI). The sequences obtained from our experiments were performed in duplicate and alongside samples with known wild-type genotypes.

Immunohistochemistry
Immunohistochemistry was performed on sections from formalin fixed paraffin wax embedded tumour tissue using the labelled streptavidin method. Fresh 4 µm thick sections were dewaxed in xylene and rehydrated in graded alcohols. Endogenous peroxidase activity was blocked by 15 minutes of incubation in 3% hydrogen peroxide in distilled water. Antigen retrieval was achieved by microwaving in 0.01 M citrate buffer, pH 6, and the sections were incubated with mouse monoclonal antibodies under the conditions shown in table 2. A second layer of biotinylated rabbit antimonue antibody (1:300; Dako, Ely, Cambridge, UK) was applied for one hour followed by a final layer of horseradish peroxidase labelled streptavidin (1:500; Dako) for one hour. The bound antibody was detected using diaminobenzidine tetrahydrochloride as the chromogen. Negative controls (performed using phosphate buffered saline instead of primary antibody) and positive controls (consisting of tissue known to be positive for the relevant antibody) were included in every experiment.

Results

<table>
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<th>Monoclonal antibody</th>
<th>Microwaving duration (min)</th>
<th>Concentration of primary antibody, duration of incubation</th>
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<tr>
<td>hMSH2</td>
<td>30</td>
<td>1:100 (Pharminagen), 18 h</td>
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<tr>
<td>β-catenin</td>
<td>10</td>
<td>1:100 (Affiniti), 1 h</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>20</td>
<td>Supernatant (ICRF), 1 h</td>
</tr>
<tr>
<td>p53</td>
<td>10</td>
<td>1:1000 (ICRF), 1 h</td>
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</tbody>
</table>

One of 21 (5%) cancers was found to be RER+. This was a 58 year old male who underwent resection of a moderately differentiated T4 Nx adenocarcinoma of the ileum.

Screening for mutations in the APC gene
Despite screening of the whole MCR, no mutations were detected in the APC gene in this study. However, we detected two polymorphisms. These were silent changes at codon 1201 (TCA→TCG, Ser→Ser) and 1220 (ACA→ACG, Thr→Thr). Both polymorphisms were found in heterozygotes and have not been previously described.

Immunohistochemistry
hMLH1 and hMSH2
All cancers expressed hMLH1 and hMSH2 protein in the tumour cell nuclei (fig 1).

β-catenin
β-catenin staining was found along the intercellular borders of normal small bowel mucosa. Abnormal (reduced) expression of β-catenin was observed in 17 of 21 (81%) cancers (fig 2). Decreased membranous staining was seen in all 17 of these cancers, with increased cytoplasmic and nuclear staining observed in 14 (67%) and 10 (48%) cancers, respectively. Complete loss of membranous staining in association with nuclear staining was seen in only one cancer. Although all cancers with nuclear expression of β-catenin had reduced membrane staining, this was not statistically significant (Fisher’s exact value, p=0.055).

E-cadherin
Uniform membranous staining of E-cadherin was localised at the intercellular borders in normal small bowel mucosa. Those cancers which showed membrane staining in over 25% of tumour cells were regarded as positive for E-cadherin expression. Reduced membrane expression of E-cadherin (<25% of tumour cells) was found in eight cancers (38%) (fig 3) with loss of cell surface staining in two of these cancers. Cytoplasmic staining, in the absence of membrane staining, was
regarded as negative, as membrane localisation is essential for E-cadherin function. Although poorly differentiated cancers were more likely to have reduced membrane staining (four of six poorly differentiated cancers versus four of 15 moderately/well differentiated cancers), this was not statistically significant (Fisher’s exact value, p=0.1).

The p53 immunohistochemistry showed strong staining in the tumour cell nuclei. A cancer was regarded as positive for p53 overexpression if there was staining of over 10% of the tumour cells. Overexpression of p53 protein was detected in the nuclei of five of the 21 (24%) cancers (fig 4).
Ten (5%) of the cancers in this study had wild-type APC and in colorectal cancers with muta-
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