CDX-2 and MIB-1 expression in the colorectum: correlation with morphological features of adenomatous lesions

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ABSTRACT

Tumours of the gastrointestinal (GI) tract, of which 70% arise in the colorectum, are a major cause of morbidity and mortality worldwide. Transformation from normal to malignant mucosa is a multistep process involving specific gene mutations and is called the adenoma-carcinoma sequence. Histologically, adenomas are of three types (tubular, tubulovillous and villous) and the extent of mucosal cellular abnormality of three grades (mild, moderate and severe). Cellular proliferation is a marker of malignant potential in many tissues. In the colon, cellular proliferation is partly controlled by the *CDX*-2 gene, a homeobox gene expressed in differentiated cells of the intestine that has proto-oncogenic potential in murine models. In the stomach, CDX-2 is expressed in intestinal metaplasia and decreasing expression through tumourogenesis shows its tumour suppressor potential. Down-regulation in colorectal cancer cell lines is also observed. This is a retrospective study of colorectal adenomas, and haematoxylin and eosin (H&E) and immunocytochemical staining for CDX-2 and MIB-1 (a cell proliferation marker) are performed on each case. Comment is made on the morphological features (adenoma type and dysplasia severity) and the grade of CDX-2 and MIB-1 expression. This study showed that dysplasia severity is linked to cellular proliferation ($P=0.011$) but adenoma type was not ($P=0.54$). CDX-2 was not linked to the morphological features discussed ($P=0.11$ and $P=0.16$) and CDX-2 and MIB-1 expression showed no correlation. Increased cell proliferation (MIB-1 expression) was seen in increasingly dysplastic adenomatous lesions of the colorectum. CDX-2 had no link to morphological features or cell proliferation of the dysplastic mucosa.

KEY WORDS: Adenomatous polyps.
CDX-2.
Dysplasia.
Proliferation.

Introduction

Tumours of the gastrointestinal (GI) tract are common and are a major cause of morbidity and mortality worldwide.[1] However, malignancies of the GI tract are not distributed uniformly along its length. In the upper GI tract, oesophageal and gastric tumours are relatively common entities, with tumours of the stomach showing a slightly higher prevalence than those of the oesophagus.[2] The small intestine, comprising the duodenum, jejunum and ileum and accounting for approximately 75% of the entire length of the GI tract, is rarely a site for primary tumours.[1] The colon and rectum together account for about 70% of all GI malignant tumours and this site in fact develops more primary neoplasms than any other organ in the body.[1]

Colorectal carcinogenesis is a vastly researched area. The development of colorectal cancer is referred to as the adenoma-carcinoma sequence.[1] This is a multistep process involving the transition of normal mucosa to mucosa at risk, further transition to adenoma, then adenocarcinoma *in situ* and subsequently invasive adenocarcinoma.[1]

The transition through these steps can be matched to specific genetic alterations. It is known that inactivating mutations of one allele of the tumour suppressor adenomatous polyposis coli (*APC*) gene, a so-called gateway gene, is perhaps the earliest event in colorectal carcinogenesis,[3] which places previously normal mucosa at risk of adenoma formation due to further carcinogenic mutations. Alteration to the second allele will cause transition to adenoma.[1] APC is part of the Wnt signalling pathway and at present the ever increasing knowledge of this pathway is leading to potential therapeutic modalities.[4] Mutation in the *APC* gene underlies familial adenomatous polyposis (FAP), which is characterised by many hundreds and commonly thousands of adenomatous lesions in the colon[1].

Other genes can be subject to mutation at this stage (e.g., β-catenin[1]) and have the same effect. Dysregulation of the *K-ras* proto-oncogene is thought to be another step in tumourogenesis, causing further adenoma growth,[5] as is inactivation, probably due to loss of heterozygosity, of the *DCC* (deleted in colon cancer) tumour suppressor gene.[3] *p53* is another gene implicated in transition through the adenoma-carcinoma sequence and is responsible for the transition of adenoma into carcinoma.[1]

Adenomas can be typed into one of three groups – villous adenoma, tubular adenoma or tubulovillous adenoma –

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depending on histological appearance. For an adenoma to be typed as tubular or villous at least 80% of the architecture must be tubular or villous. If this is not the case then it is typed as a tubulovillous adenoma.[6]

Type of adenoma has a role in the malignant potential of the lesion, with villous adenomas showing a greater malignant potential than tubular adenomas, with tubulovillous adenomas showing an intermediate malignant potential.[7] The presence of high-grade (severe) dysplasia in the lesion is also an indicator of malignant potential,[7] with low-grade (mild) dysplasia showing the lowest malignant potential and moderate dysplasia an intermediate malignant potential.

Many studies have investigated cell proliferation in colorectal neoplasms, both as a marker for colorectal cancer in normal-appearing mucosa[8,9] and correlating cell proliferation with morphological features of the lesion.[10,11] Cell proliferation visualised by immunocytochemical methods has also been postulated as a prognostic indicator,[12] with severely dysplastic lesions showing higher proliferative rates than moderately and mildly dysplastic lesions.

Cell proliferation has been shown to increase in line with carcinogenesis (i.e., low expression in mildly dysplastic adenomas increasing to higher expression in carcinoma *in situ* to highest expression in invasive carcinoma[13]), but not all studies have corroborated these findings.[14] It is believed that cell proliferation and apoptosis are important factors in carcinogenesis, and invasive carcinoma is the result of cell proliferation overwhelming cell death (apoptosis).[10,11,13,15,16] Cell proliferation is used as a prognostic marker in many other tissues and disease states, including myxoid liposarcoma,[16] meningioma[17] and cervical intraepithelial neoplasia (CIN).[18]

There are many ways to label cell proliferation. By far the easiest way on tissue sections is by immunocytochemistry. The most widely used antibody is Ki-67, which recognises a proliferation-specific nuclear antigen expressed in the G_1, S, G_2 and M phases of the cell cycle but not in G_0 (i.e., not in quiescent cells).[19] A specific clone of the Ki-67 antibody, MIB-1, is shown to be the most sensitive of all those available.[19]

Cell proliferation in the colon during development is shown to be regulated in part by a gene called *CDX-2*,[20] which is part of the homeobox that is a 60 amino acid encoding DNA sequence originally found in *Drosophila* and subsequently identified in all three kingdoms of multicellular animals.[21] Homeobox genes are master developmental control genes that regulate morphogenesis and cell differentiation in animals and are implicated in normal mammalian development, as well as in congenital malformations and tumourogenesis.

The homeobox genes have been studied extensively over the past few years. The proto-oncogenic potential of homeobox genes has been shown in many studies[22–24] and particularly the role of *CDX-1* and *CDX-2* in the GI tract. *CDX-1* and *CDX-2* are caudal-related homeobox genes, and, as in *Drosophila*, are involved in development of the posterior (caudal) segment of the animal.[22,25] CDX-1 is expressed predominantly in undifferentiated cells of the intestinal crypts, whereas CDX-2 is expressed mostly in differentiated cells of the intestine (e.g., villous cells).[25]

CDX-2 expression studies in mice show that in late embryogenesis at a time of major GI developmental transition the CDX-2 levels are markedly elevated.[26] In adult mice, CDX-2 is expressed at a markedly higher level in the caecum than in the rectum, suggesting that this homeobox gene has a positional role in the GI epithelium.[27,28]

Experiments on knockout mice shows that those that were *CDX-2* –/– died *in utero* and those that were *CDX-2* +/– developed hamartomatous polyps in the proximal colon that ceased to express the solitary *CDX-2* allele.[29] Hamartomas are excessive but focal overgrowths of cells that do not reproduce the architecture of the surrounding tissue, even though they develop from cells native to the organ in which they arise.[16] There is a tenuous line between hamartoma and neoplasm and the same lesion may be described as either by different observers.[16] Thus, the loss of one *CDX-2* allele affects developmental growth of the intestine in a way that some workers consider to be neoplastic.

In humans, CDX-2 is implicated in the gastric carcinogenesis model (correa hypothesis),[27,32,33] implicitly that CDX-2 is responsible for intestinal differentiation that may be present in a tumour.[25,31] CDX-2 is also shown to be aberrantly expressed in intestinal metaplasia, a precancerous lesion of the gastric mucosa.[25,30,31] The tumour suppression potential of CDX-2 is shown through the observation of decreased expression in the later stages of tumourogenesis[30,31] and in experiments involving forced expression in cell lines resulting in decreased tumourogenecity.[30]

CDX-2 is also involved in colorectal carcinogenesis. Down-regulation is observed in colon cancer cell lines[32] and is related to tumour grade in human colorectal cancers,[33] again suggesting tumour suppressor potential. In fact, an experiment on a colorectal cancer cell line has given a potential mechanism for the potential tumour suppressor effects of CDX-2. The restoration of the wild-type *APC* gene in a colorectal cancer cell line showed induction of CDX-2 expression, suggesting that CDX-2 contributes to *APC* tumour suppressor effects (as previously discussed).[34] Other researchers have shown that down-regulation or silencing of CDX-2 is present in colorectal carcinomas and that the down-regulation is greater in high-grade (i.e., less differentiated and/or highly pleomorphic) lesions.[35–37]

Materials and methods

This was a retrospective study using archival surgical pathology tissue blocks from the Royal Berkshire Hospital, Reading, Berkshire, UK. Ethical approval was sought and gained from the West Berkshire Local Research Ethics Committee in April 2004.

Tissue was taken at endoscopic investigation and consisted of hot biopsy specimens and polypectomy samples. They were fixed in neutral buffered formalin (Genta Medical, UK) for a minimum of six and a maximum of 24 hours prior to paraffin processing, which was performed overnight using a VIP automated tissue processor (Bayer, UK).

Following haematoxylin and eosin (H&E) staining, slides were assessed for adenoma type (villous, tubulovillous or tubular) and severity of dysplasia (mild, moderate or severe). The criterion for assessing adenoma type was that a diagnosis of villous or tubular adenoma required the villous or tubular component to be at least 80% of the lesion's architecture. If this was not the case then it was diagnosed as

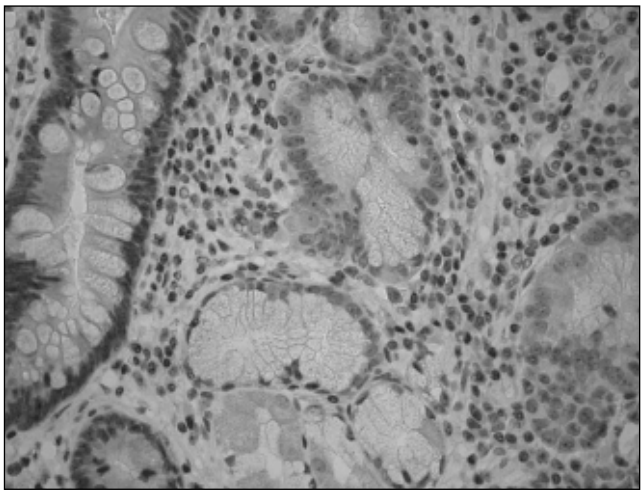

Fig 1. CDX-2 staining of intestinal metaplasia of the stomach. Nuclear staining of metaplastic cells should be seen (original magnification x100).

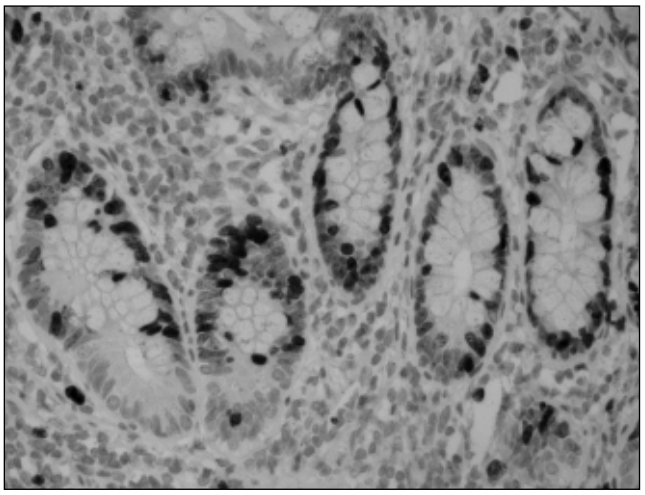

Fig 2. MIB-1 staining of terminal ileum. Nuclear staining of lower crypt nuclei should be seen (original magnification x200).

a tubulovillous adenoma. Dysplasia severity was assessed according to guidelines related to the degree of pleomorphism, loss of nuclear polarity, loss of goblet cell differentiation and architectural abnormality in the crypts.[38]

Immunocytochemistry for CDX-2 and MIB-1 was performed on a semi-automated NexES immunostainer (Ventana, France) in batches of 20 slides. A labelled avidin-biotin method was employed with DAB (3,3′-diaminobenzidine tetrahydrochloride) used as the chromogen. Pretreatment of the slides involved heat-mediated antigen retrieval (HMAR) in pH 6.0 citrate buffer (HD Supplies, UK) for two minutes for both antibodies.

Control material used in this study was taken from surgical specimens from patients who had not refused consent for taking tissue for research/training purposes. A single control slide was included with every batch of slides stained. Gastric intestinal metaplasia was used as the CDX-2-positive control material and terminal ileum was used as the MIB-1-positive control material. Staining was observed in metaplastic nuclei (Fig. 1) and in intestinal crypt nuclei (MIB-1) (Fig. 2).

CDX-2 immunocytochemical staining was scored taking into account the proportion of dysplastic cells staining in the adenoma and the intensity of that staining (Table 1). Scoring of MIB-1 staining was by calculation of the percentage of the dysplastic cell population that expressed MIB-1 (Table 2).

Statistical significance of the results was evaluated using two-way ANOVA tests and correlation analysis.

Results

CDX-2 expression and adenoma type

The range of CDX-2 scores for all adenoma types was 0–8, with a mean value of 5.18 (Table 3). There was no statistically significant variation in CDX-2 score between adenoma types (P=0.16).

CDX-2 expression and dysplasia severity

The range of CDX-2 scores for all levels of dysplasia severity was 0–8, with a mean value of 4.93 (Table 4). There was no statistically significant variation in CDX-2 score between levels of dysplasia severity (P=0.11).

Table 1. System used for calculating CDX-2 scoring.

Score for proportion of staining
0 = No nuclei staining
1 = <1% nuclei staining
2 = 1–10% nuclei staining
3 = 11–33% nuclei staining
4 = 34–66% nuclei staining
5 = 67–100% nuclei staining
Score for staining intensity
0 = No staining
1 = Weak staining
2 = Moderate staining
3 = Strong staining
Total score was achieved by addition of intensity score and percentage score.

Table 2. System used for calculating MIB-1 score.

Score	Proportion of cells staining
0	No cells
1	<10% of cells
2	<20% but >10%
3	>20%

Table 3. Results for CDX-2 score by type of adenoma.

	Dysplasia	Total score	No	Mean
Tubular	All	143	31	4.612903
Tubulovillous	All	187	38	4.789474
Villous	All	43	7	6.142857
All	All	373	76	5.181745

MIB-1 expression and adenoma type

The range of MIB-1 scores for all adenoma types was 0–3, with a mean value of 1.61 (Table 5). There was no statistically significant variation in MIB-1 score between adenoma types (P=0.54).

MIB-1 expression and dysplasia severity

The range of MIB-1 scores for all levels of dysplasia severity was 0–3, with a mean value of 1.63 (Table 6). There was a statistically significant variation in MIB-1 score between levels of dysplasia severity (P=0.011).

CDX-2 and MIB-1 expression

Correlation analysis showed an r value of 0.29 (i.e., no convincing correlation). The r^2 value was 0.08 and the P value for the r and r^2 values was 0.01. There was no statistically significant correlation between CDX-2 and MIB-1 expression (Table 7).

Table 4. Results for CDX-2 score by severity of dysplasia.

	Total Score	No	Mean
Mild	43	10	4.3
Moderate	213	44	4.840909
Severe	124	22	5.636364
All	380	76	4.925758

Table 5. Results for MIB-1 score by type of adenoma.

	Dysplasia	Total Score	No	Mean
Tubular	All	47	31	1.516129
Tubulovillous	All	66	38	1.736842
Villous	All	11	7	1.571429
All	All	124	76	1.608133

Table 6. Results for MIB-1 score by severity of dysplasia.

	Total Score	No	Mean
Mild	17	10	1.7
Moderate	62	44	1.409091
Severe	45	22	2.045455
All	124	76	1.631579

Table 7. Correlation analysis of CDX-2 and MIB-1 expression.

r	r^2	*P* one-tailed	*P* two-tailed
0.2901	0.0842	0.005477	0.010953

Discussion

This study demonstrated that CDX-2 expression is not linked to either adenoma type or severity of dysplasia. From previously published literature it was expected that CDX-2 expression would reduce with increasing level of dysplasia severity (increasing malignant potential and loss of epithelial differentiation) and show lower expression in villous lesions than in tubular lesions (decreasing expression of CDX-2 with increasing malignant potential). However, the data suggest greater expression with increasing severity of dysplasia and increased expression (albeit marginally) in villous lesions. However, this proved to have no statistical significance in both instances. Hence, there is no statistically significant link between CDX-2 expression and either dysplasia severity or adenoma type.

It was also shown that MIB-1 expression (cell proliferation) is not linked to adenoma type. It was thought that cell proliferation would be increased in lesions of greater malignant potential but the data do not show an upward trend with increasing malignant potential. Statistically, there is no link between MIB-1 expression and adenoma type.

It is confirmed that MIB-1 is linked to the severity of dysplasia in the lesion. This was strongly accepted at the 5% significance level. The data show no upward trend with increasing severity but statistically there was a significant difference. This lack of an apparent upward trend was probably due to the small sample size of mildly dysplastic lesions relative to the moderately and severely dysplastic lesions, which gave an incorrect impression of no trend.

Finally, the link between CDX-2 and MIB-1 expression was subjected to correlation analysis. This showed a weak and statistically insignificant correlation and therefore acceptance at the 5% significance level that CDX-2 and MIB-1 expression do not correlate. This conflicts with published research suggesting that cell proliferation is regulated in part by CDX-2.

The significance of the present findings is a further understanding of the role of CDX-2 in neoplastic disease processes. It is shown to be involved in premalignant diseases of the GI tract and contributed to cell proliferation in the neoplastic lesions in previous studies, and the present study has reinforced the link with premalignant lesions. However, this study has shown that CDX-2 expression in the neoplastic cells has no role in defining either adenoma type or severity of dysplasia, and fails to support a link between CDX-2 expression and cell proliferation demonstrated in other studies.

Cell proliferation, studied by MIB-1 expression, is related to the severity of dysplasia but not to adenoma type. There are levels of malignant potential in dysplasia severity and in adenoma type, but cell proliferation (disordered cell proliferation is a hallmark of neoplastic disease) is only related to one of the morphological features. This does not contradict the fact that villous lesions show greater malignant potential than do tubular lesions, but this greater malignant potential is not due to disordered or increased cell proliferation. The increasing severity of dysplasia, which infers greater malignant potential, can be linked to increasing cell proliferation, and is supported by the vast majority of previous research in this area.

It should be noted that the sample size (n=76) studied is not large and the limitations imposed by such a sample size is evident in the results obtained with MIB-1. An increase in MIB-1 expression was expected but this is not apparent in the data relating to adenoma type and dysplasia severity. Clearly, the small sample size limits the ability to draw firm conclusions from the data produced.

Further work to arise from this study should include a comparison of results by image analysis of immunocytochemistry staining. Visual analysis is subjective and shows both inter-observer and intra-observer error. Validation of visual techniques versus image analysis in general should be undertaken, as the latter is likely to be integrated in routine histopathology in the future and may have an impact on scoring of immunocytochemical staining in general.

It has been suggested that cell proliferation could be used as a prognostic marker in colorectal neoplasms to assess the degree of dysplasia. From the present study it is clear that there is a link between cell proliferation and dysplasia. Thus, further investigation into cell proliferation and disease recurrence (i.e., does high cell proliferation in adenomatous lesions indicate a likelihood of recurrent disease or progression to carcinoma?) and other prognostic factors would be advantageous in determining whether or not cell proliferation can be used as a prognostic marker in colorectal neoplasms.

A link between CDX-2 and cell proliferation has been highlighted in the literature; however, the present study failed to demonstrate such a link. The exact mechanism of any relationship remains unclear and clarification at the molecular level would aid the understanding of a possible link between CDX-2 expression and cell proliferation in colorectal neoplasms. It has been shown that *CDX-2* is a developmental gene expressed in differentiated cells of the colorectum, and a clear link between CDX-2 expression and cell proliferation, demonstrated at the molecular level, would add to the knowledge of colorectal carcinogenesis.

Colorectal carcinogenesis is a well-studied area with vast knowledge of the adenoma-carcinoma sequence and the mutations responsible. *CDX-2* is a relatively new addition to the genes known to play a role in carcinogenesis, and further investigation of its exact role and interaction in cell proliferation, its link with *CDX-1*, a closely related homeobox gene, and with adenomatous polyps and adenocarcinoma of the colorectum is required.

In conclusion, this study shows a statistically significant link between cell proliferation and dysplasia severity in adenomatous lesions of the colorectum. However, it fails to demonstrate a link between CDX-2 expression and adenoma type, CDX-2 expression and dysplasia severity, and cell proliferation (MIB-1 expression) and adenoma type. It also fails to show any correlation between CDX-2 expression and cell proliferation. Thus, further work should focus on CDX-2 and its role in colorectal carcinogenesis, and the exact interactions it has with CDX-1 and cell proliferation pathways. □

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