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Original Paper

ERβ isoform expression in colorectal carcinoma: an *in vivo* and *in vitro* study of clinicopathological and molecular correlates

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Abstract

Colorectal carcinoma shows several sex-related differences with regard to incidence, response to chemotherapy and microsatellite instability. These differences may relate to differential expression of ERβ1 (wild-type) as well as the truncated ERβ2 and ERβ5 splice variant isoforms, which have recently been detected in normal and malignant colorectal epithelium. This hypothesis was tested through the study of ERβ isoform protein and/or mRNA expression amongst 91 primary colorectal carcinoma cases and 20 colorectal carcinoma cell lines. Study of the latter showed an absolute correlation between mRNA and protein expressions for ERβ1 and ERβ2. ERβ1 and ERβ2 protein expression was lost in 22% and 49%, respectively, of the primary colorectal carcinomas. By contrast, ERβ5 expression was found in all primary colorectal carcinomas and all colorectal carcinoma cell lines studied. Lower ERβ1 protein expression was associated with poorer differentiation, higher pT stage and absence of microsatellite instability. Higher ERβ2 protein expression was associated with right-sided location and presence of lymph node metastases. Protein expression of ERβ1 correlated positively with expression of the oestrogen-responsive protein trefoil factor 1 (TFF1). There was no correlation between ERβ protein isoform expression and response to 5-fluorouracil therapy, tumour proliferation, or thymidylate synthase expression. These data suggest that ERβ1 and/or ERβ2 isoform expression may have prognostic value and may explain sex-related differences in microsatellite instability and colorectal carcinoma. The opposing associations shown by ERβ1 and/or ERβ2 in relation to colorectal carcinoma are in keeping with differential activities shown by the two isoforms.
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Keywords: **oestrogen receptor β; colorectal carcinoma; microsatellite instability; prognosis**

Introduction

Colorectal carcinoma (CRC) is a common malignancy in both sexes [1]. However, CRC shows several sex-related differences in incidence, response to chemotherapy and certain molecular characteristics. It has therefore been suggested that exposure to oestrogen and/or oestrogenic compounds may underlie these differences.

CRC is commoner in men than women, the difference being more striking amongst premenopausal women and age-matched men [1]. The latter observation, together with the suggestion that hormone replacement therapy reduces the chance of developing CRC in postmenopausal women [2], implies that oestrogens may protect against CRC. There is some limited mechanistic data to support this hypothesis. Tamoxifen (which has pro- and anti-oestrogenic activities) and the phyto-oestrogen genistein have both been shown to inhibit growth of CRC cells [3]. However, the actual effect of oestrogens themselves on the proliferation of such cells *in vitro* remains controversial [3,4]. Furthermore, it is unclear whether there is a link between oestrogen exposure and CRC proliferation *in vivo*.

There are data suggesting that women are more likely to respond to 5-fluorouracil (5-FU) based chemotherapy than men [5]. Again, this may relate to a relationship between oestrogens and cell proliferation, which in turn may determine sensitivity to chemotherapy [6]. A recognized determinant of CRC sensitivity to 5-FU therapy is the cellular level of thymidylate synthase (TS) [7,8]. Higher levels of TS, which is targeted and inhibited by 5-FU, correlate with increased resistance to the drug [7,8]. TS nuclear protein expression appears to be lower in women

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than men [9] and Nakayama and colleagues have recently shown that oestrogenic compounds reduce TS expression and increase 5-FU sensitivity in cultured CRC cells [4]. However, we are unaware of any attempts to extrapolate these findings to CRC *in vivo*. A well-described molecular sex-related difference in CRC involves microsatellite instability. Overall, microsatellite unstable CRCs are more commonly found in women than men [10,11]. However, if only younger patients with this subtype of CRC are considered, males far outnumber females [10,11]. There are no published data explaining these sex-related molecular differences in CRC.

Oestrogen receptors (ER) represent the major pathway via which oestrogens and oestrogenic compounds act. It seems sensible, therefore, that attempts to explain sex differences in CRC should consider ER. Until recently, only one *ER* gene was thought to exist, viz *ERα*. Most studies have failed to demonstrate substantial expression of ERα protein and/or mRNA amongst CRCs [12–14]. Further, ERα expression was not found to explain gut-related expression of trefoil factor 1 (TFF1), which is an oestrogen-inducible protein expressed in at least two-thirds of CRCs [15,16]. The presence of a second *ER* gene, commonly known as *ERβ*, was first demonstrated in 1996 [17]. Since then, at least five splice variant isoforms of the ERβ gene product (ERβ1–ERβ5) have been described [18]. ERβ1, 2 and 5 have been demonstrated in normal colorectal mucosa and at much higher levels than ERα [19,20]. Of these three isoforms, only the full-length ERβ protein, equivalent to the ERβ1 isoform described by Moore and colleagues [18], can activate oestrogen-responsive elements (ERE) in reporter assays [21]. Therefore, many of the ways in which 'ERβ' has thus far been reported to mediate oestrogenic activity are likely to represent ERβ1–based activation. Less is known of the functions of the other ERβ isoforms. However, ERβ2 (also known as ERβcx) does show differential and potentially opposing activities to ERβ1, regarding binding of ligand and of ERE and non-ERE containing promoters [21]. There is little available data regarding the function of ERβ5. While there are only a few published studies of ERβ in CRC, these data already suggest possible roles for ERβ in explaining sex-differences in CRC. For example, ERβ appears to mediate both the inhibitory effects of tamoxifen on CRC cell proliferation and TS expression [4]. Nonetheless, more work is required to define fully the relations between ERβ and various aspects of CRC. Only two studies have explored differential expression of ERβ isoforms in CRC *in vivo* [20,22]. These studies only analysed isoform mRNA expression and a small number of clinicopathological features, and did not study the relationship with tumour proliferation, response to 5-FU therapy or microsatellite stability.

To determine whether ERβ1, 2 and/or 5 expression(s) may explain sex-related differences in clinicopathological and molecular features of CRC, we investigated mRNA and/or protein expression of these isoforms in primary CRC tissue and CRC cell lines. We were particularly interested in comparing expression of these isoforms with tumour proliferation, TS expression and response to 5-FU therapy, microsatellite stability and TFF1 expression.

Methods

Cases and cell line samples

Two cohorts of patients with CRC were studied. The first (cohort A) comprised 51 patients who had participated in a study of protracted venous infusional 5-FU for metastatic CRC [23]. The second (cohort B) comprised 40 cases of CRC that had previously been studied for evidence of microsatellite instability [24]. The following data were available for cohort A: response to chemotherapy, defined according to standard UICC guidelines [25] as complete response (CR), partial response (PR), no change (NC) or progressive disease (PD). Representative formalin-fixed, paraffin-embedded tissue blocks were collected for the 91 carcinomas. Tissue blocks of normal colorectum were taken from CRC resection specimens at a distance from the tumours. Study of the two patient cohorts was carried out in accordance with guidelines of the Lothian Local Research Ethics Committee. Cohort B contained a larger proportion of poorly differentiated and mucinous carcinomas than cohort A. Otherwise, there were no statistically significant differences between the two cohorts in the clinicopathological features collected.

cDNA samples were available for 20 CRC cell lines (established in the CRUK Cancer and Immunogenetics Laboratory or obtained from ATCC or ECACC): C10, C32, C70, C75, CC07, CC20, DLD1, HCA7, HCT116, HT29, HT55, HRA19, LIM1863, LOVO, LS174T, LS411, SKCO-1, SW48, SW480 and SW837. There were also protein lysates available for 10 of the cell lines (see below for details).

Antibodies

Monoclonal antibodies specific for C-terminal peptides within ERβ1 (GenBank Accession No AB006590) and ERβ2 (GenBank Accession No AB006589) were prepared and characterized using standard methods described in detail elsewhere [26]. A monoclonal antibody directed against the C-terminus of ERβ5 (GenBank Accession No AF061055) was prepared by immunizing mice with a specific peptide (LLSHVRHARYAP) conjugated to tuberculin, according to standard methods [27]. Hybridoma culture media were pooled and dialysed against 20 mM phosphate buffer, pH 7; immunoglobulins were purified by

affinity chromatography on Hitrap protein G columns (17–0404; Amersham, Chalfont St Giles, UK) according to the manufacturer's instructions. Pooled eluted IgGs were desalted on PD10 columns (Amersham), pre-equilibrated in phosphate-buffered saline (PBS) and stored at −20 °C in PBS containing 20% glycerol.

Immunohistochemistry

Sections (5 μm) were cut from the tissue blocks, dewaxed and rehydrated. Following antigen retrieval (Table 1), the sections were blocked with 3% H_2O_2 in methanol for 5 min followed by normal rabbit serum diluted 1 : 4 in TBS for 30 min. Individual details for ERβ1, ERβ2, ERβ5 and TFF1 immunohistochemistry are given in Table 1. Biotinylated secondary rabbit anti-mouse antibody (1/500 dilution, 30 min; Dako, Ely, UK) was used for these four primary antibodies. After incubation with avidin–biotin horseradish peroxidase (HRP)-linked complex (30 min), the sections were visualized using diaminobenzidine tetrahydrochloride with Mayer's haematoxylin as a counterstain. Immunohistochemistry for thymidylate synthase and Ki67 (brief details shown in Table 1) had previously been performed for the cohort A cases, as is described fully elsewhere [9].

The ERβ1, ERβ2 and ERβ5 stained sections were assessed by two observers each using the 'category scoring' system, which has been well validated for assessing ERα immunohistochemistry [28] and takes into account intensity of staining (1 = weak; 2 = moderate; 3 = strong) and proportion of positively stained tumour cells (1 = 0–1%; 2 = 2–10%; 3 = 11–33%; 4 = 34–67%; 5 = 68–100%). Addition of the intensity and proportion scores provided the final category score used for further analysis. No cytoplasmic immunostaining was seen for any of the three isoforms and therefore only nuclear expression of each isoform was assessed. To allow comparisons with previous immunohistochemical studies of ERβ in CRC [29,30], absence of ERβ isoform expression was defined as staining of less than 10% of carcinoma cells. The TFF1-stained sections were assessed by a single observer using the same 10% cut-off to define absence of expression. A histoscore (0–300) was calculated for nuclear TS expression and positive TS expression defined as a histoscore of >70, as outlined in detail elsewhere [9]. Ki67 labelling indices were calculated by assessing the percentage of nuclei showing positive staining from counting 500 carcinoma cells [9].

Western blotting

Protein lysates (50 μg of each sample) were boiled for 5 min in Laemmli sample buffer before electrophoresis by 8% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes, which were then blocked with 5% skimmed milk in PBS for 1 h at room temperature. For both ERβ1 and ERβ2, membranes were incubated in 1/500 dilutions of primary antibody overnight at 4 °C. Actin was used as a loading control and blotted for by incubation in a 1/1000 dilution of primary antibody (Clone AC40; Sigma, Poole, UK) for 1 h at room temperature. After three washes, all blots were incubated in 1/1000 dilutions of HRP-linked rabbit anti-mouse antibody (Dako) for 1 h at room temperature. The blots were visualized using enhanced chemiluminescence (ECL plus system, Amersham).

Reverse transcriptase PCR

Reverse transcriptase (RT)-PCRs for the three ERβ isoforms used a common forward primer (5′-AGTATG TACCCTCTGGTCACAG-3′) and reverse primers specific to each isoform: ERβ1 (5′-CATTCAGCATCT CCAGCAGCAG-3′); ERβ2 (5′-CGTTGCTCAGGC AAAAGAGTC-3′); and ERβ5 (5′-AGCACAATCCC ATCCCAAGC-3′). β-Actin RT-PCR served as an endogenous control and used the forward primer 5′-ACACCTTCTACAATGAGC-3′ and the reverse primer 5′-ACGTCACACTTCATGATG-3′. For all the

Table 1. Specific details for immunohistochemical protocols used for detecting ERβ1, 2 and 5 isoforms, Ki67, TFF1 and thymidylate synthase

Antigen	Antibody clone	Source	Primary antibody concentration (duration/temperature)	Antigen retrieval (duration)
ERβ1	PPG5/10	Serotec (MCA 1974S), Kidlington, UK	1 : 50 (overnight/4 °C)	0.1 M citrate buffer (pH 6); pressure cooking (7 min)
ERβ2	57/3	Serotec (MCA 2279S), Kidlington, UK	1 : 50 (overnight/4 °C)	Glycine/EDTA (pH 8); pressure cooking (7 min)
ERβ5	5/25	In house	1 : 50 (overnight/4 °C)	0.1 M citrate buffer (pH 6); pressure cooking (7 min)
Ki67	MM1	Novocastra, Newcastle upon Tyne, UK	1 : 100 (60 min/room temperature)	0.1 M citrate buffer (pH 6); microwave (10 min)
TFF1	pS2 (28AA)	Cancer Research UK	1 : 100 (30 min/room temperature)	Dako TRS fluid (Dako); microwave (15 min)
Thymidylate synthase	TS106	Gift from Professor PG Johnston, Belfast, UK	1 : 200 (60 min/room temperature)	Vector antigen retrieval fluid (Vector Laboratories, Peterborough, UK); microwave (10 min)

PCRs a 50 μl reaction volume was used comprising final quantities/concentrations of 1000 ng (ERβ isoforms) or 100 ng (β-actin) cDNA, 0.2 μM each primer, 1 U AmpliTaq Gold polymerase (Perkin-Elmer, Cambridge, UK), 1.5 mM $MgCl_2$, and 200 μM each dNTP. The cycling conditions were a denaturation step of 94 °C for 10 min, 45 (ERβ isoforms) or 25 (β-actin) cycles of 94 °C for 45 s, 55 °C for 60 s, and 72 °C for 45 s, and a final extension step of 72 °C for 10 min.

Microsatellite instability studies

Forty of the primary CRCs studied (cohort B) had already been typed as either microsatellite-stable (20 carcinomas) or microsatellite-unstable (20 carcinomas) in previous studies from our laboratories [24]. Of the 20 CRC lines, 10 (DLD1, HCA7, HCT116, HRA19, LOVO, LS411, LS174T, SW48, SW480 and SW837) had previously been typed as microsatellite-unstable and the remaining 10 as microsatellite-stable [31].

Statistics

2×2 Table statistical analyses were performed using the χ^2 test (with Yates' correction) or, where more appropriate, a two-tailed Fisher's exact test. As the ERβ isoform category scores, TS histoscores and Ki67 labelling indices did not follow Normal distributions (data not shown), relations between these variables were analysed using the Mann–Whitney U test and/or Spearman rank correlation test.

Results

Patient/case details

The median age of the 91 patients studied was 64 years (range 38–92 years). All other clinicopathological details collated are shown in Table 2.

ERβ1 in normal colorectum and primary CRCs

In normal large bowel, nuclear expression of ERβ1 protein was seen in epithelial, endothelial and smooth muscle cells, and in stromal fibroblasts, macrophages and occasional lymphocytes. There was a gradient of expression along the crypt axis, with loss of expression towards the upper crypt and into the surface epithelium (Figure 1). Of the 91 primary CRCs, 20 (22%) lacked ERβ1 protein expression (Figure 1). There was no correlation between ERβ1 score and age (Spearman rank correlation coefficient $(r) = 0.13$, $p = 0.21$). Higher ERβ1 expression was found in carcinomas of lower grade, lower pT stage and mucinous phenotype (Table 2). There were no significant associations between ERβ1 expression and Ki67 labelling index ($r = 0.16$, $p = 0.24$), TS histoscore ($r = 0.15$, $p = 0.29$) or response to 5-FU (Table 2). However, higher ERβ1 expression was seen in microsatellite-unstable carcinomas (Table 2). Finally, none of the 21 CRCs expressing TFF1 compared with 13/60 CRCs lacking TFF1 expression showed an ERβ1 score of less than 4 (0% vs 27%, respectively; two tailed Fisher's exact test, $p = 0.034$).

ERβ2 in normal colorectum and primary CRCs

In normal large bowel, nuclear expression of ERβ2 protein was seen in epithelial and smooth muscle

Table 2. Clinicopathological and molecular features of 91 primary colorectal carcinomas and their relations to ERβ1 and 2 isoform protein expression, as assessed by category score

		ERβ1		ERβ2	
Clinicopathological/ molecular feature	**Subgroups (number of patients)**	**Median score**	***p* value***	**Median score**	***p* value***
Sex	Male (46)	6.0	0.22	3.0	0.25
	Female (45)	6.0		3.5	
Location of	Right (48)	6.0	0.76	2.25	0.013
Tumour	Left (43)	7.0		4.0	
Differentiation	Well/moderate (75)	7.0	0.043	3.0	0.46
	Poor (16)	5.5		3.5	
pT stage	1–3 (78)	7.0	0.031	3.0	0.98
	4 (13)	5.0		3.5	
pN stage	0 (30)	7.0	0.79	2.5	0.041
	⩾1 (61)	6.0		3.75	
Abundant extracellular	No (73)	6.0	<0.001	3.0	0.41
mucin[#]	Yes (18)	8.0		4.0	
Response to 5FU[†]	CR/PR (16)	7.0	0.19	3	0.47
	NC/PD (35)	6.0		2.5	
Microsatellite	Stable (20)	6.0	0.001	3.0	0.77
Stability[‡]	Unstable (20)	8.0		3.5	

* Two-tailed Mann–Whitney U test.
[#] Defined as extracellular mucin representing ⩾ 50% of the carcinoma.
[†] Data available for only 51 cases/patients.
[‡] Data available for only 40 cases/patients.

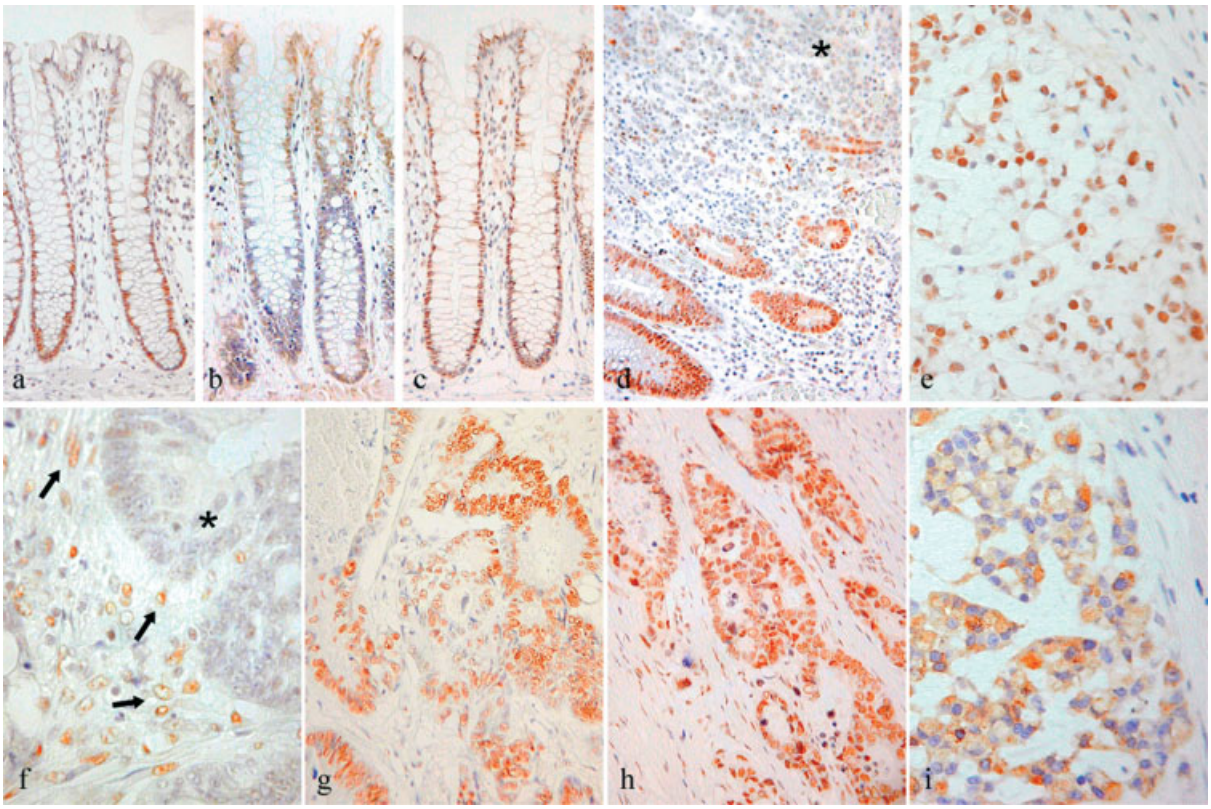

Figure 1. Examples of ERβ1 (a, d, e), ERβ2 (b, f, g), ERβ5 (c, h) and TFF1 (i) immunohistochemistry performed on normal colorectal epithelium (a–c) and colorectal carcinoma (d–i). The colonic crypt shows opposing expression patterns for ERβ1 (a) and ERβ2 (b) and more uniform expression throughout for ERβ5 (c). Examples of carcinomas showing absence and presence of ERβ1 (d and e, respectively) and ERβ2 (f and g, respectively) are shown. (d) The carcinoma lacking ERβ1 expression (*) is shown against immunoreactive background non-dysplastic epithelium. (f) The carcinoma lacking ERβ2 expression (*) is shown against immunoreactive stromal cells (arrowed). (h) Strong uniform expression of ERβ5 by CRC cells is seen. The pattern of ERβ1 expression (e) corresponds well with TFF1 expression (i)

cells, and stromal fibroblasts. There was a gradient of expression along the crypt axis with increasing expression towards the upper crypt and into the surface epithelium (Figure 1). Forty-five (49%) of 91 primary CRCs studied lacked ERβ2 protein expression (Figure 1). There was no correlation between ERβ2 score and age ($r = 0.13$, $p = 0.23$). Higher ERβ2 expression was found in carcinomas of right-sided location and those with lymph node metastasis (Table 2). There were no significant associations between ERβ2 expression and Ki67 labelling index ($r = 0.01$, $p = 0.97$), TS score ($r = -0.03$, $p = 0.85$), 5-FU response (Table 2), microsatellite instability (Table 2) or TFF1 expression (data not shown).

ERβ5 in normal colorectum and primary CRCs

In normal large bowel, nuclear expression of ERβ5 protein was seen in epithelial, endothelial and smooth muscle cells, and in stromal fibroblasts, macrophages and occasional lymphocytes. There was strong expression along the entire crypt axis (Figure 1). ERβ5 was expressed by all the primary CRCs studied (Figure 1). Further, all these CRCs showed category scores of >7. Correlation with clinicopathological or molecular features was therefore uninformative.

ERβ isoform expression in colorectal carcinoma cell lines

Amongst the 10 CRC cell lines for which protein lysates were available, there was absolute correlation between protein and mRNA expression for both ERβ1 and ERβ2 (Figure 2). Of the 20 CRC cell lines studied, 4 (20%) and 8 (40%), respectively, showed no ERβ1 and ERβ2 mRNA expression, whereas all 20 (100%) expressed ERβ5 mRNA. All of the microsatellite-unstable cell lines compared with 6/10 microsatellite-stable cell lines expressed ERβ1 mRNA, although this difference failed to reach statistical significance at the 95% level (100% vs 60%, respectively, two-tailed Fisher exact test, $p = 0.087$). In contrast, the proportions of microsatellite unstable and stable cell lines showing ERβ2 mRNA expression were identical (40% vs 40%, respectively).

Discussion

There have been few previous studies of ERβ isoform expression in primary CRCs and its potential clinicopathological relevance [20,22]. These studies have been restricted to studying mRNA extracted from

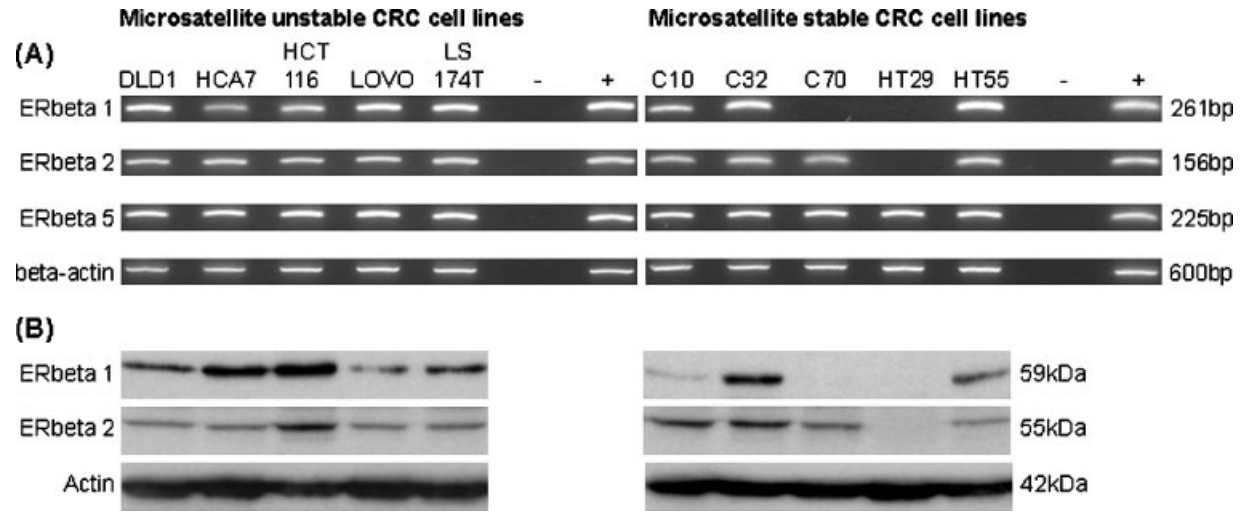

Figure 2. (A) ERβ1, 2 and 5 mRNA expression (assessed by RT-PCR) and (B) ERβ1 and 2 protein expression (assessed by western blotting) by 10 colorectal carcinoma cell lines. The carcinomas are divided into those that are microsatellite-unstable and those that are microsatellite-stable. Actin loading controls for both mRNA and protein are shown. The expected sizes of amplified products are shown beside each gel and the expected sizes of detected ERβ isoform protein are shown beside each blot. Positive (+), ie cDNA derived from normal testis, and water blank (−) controls are shown for the RT-PCR gels

whole tissue fragments, a method with inherent limitations. First, subtle differences in epithelial expression of the different ERβ isoforms cannot be detected. Using immunohistochemical studies, we demonstrated that ERβ1 and ERβ2 have contrasting expression patterns along the crypt axis; this suggests opposite effects of the isoforms on cell proliferation and differentiation, at least in colorectal epithelium. This may relate to differential activities of ERβ1 and ERβ2, as outlined earlier [21]. Another limitation of previous RT-PCR studies relates to ERβ isoform expression in non-epithelial cells. We found ERβ1, 2 and 5 expression in smooth muscle cells, macrophages, lymphocytes, endothelial cells and/or fibroblasts. The varying presence of such cells in normal vs. malignant colorectal samples studied may explain a previous conclusion that ERβ isoform expression is increased in primary CRC [22]. In contrast, our immunohistochemical and *in vitro* data agree more with the previous findings of Campbell-Thompson and colleagues, that ERβ1 and 2 mRNA expression is reduced in CRC *in vivo* [20]. With regard to protein expression in primary CRCs, two groups — using antibodies that did not distinguish between ERβ1 and ERβ2 — reported that 33–38% of carcinomas lacked ERβ expression [29,30]. Employing the same 10% cut-off limit, we found 22% and 49% of primary CRCs to lack ERβ1 and ERβ2 protein expression, respectively. This was similar to our inability to demonstrate ERβ1 and ERβ2 mRNA expression in 20% and 40% of CRC cell lines, respectively. With regard to ERβ5, more knowledge of this isoform's physiological functions may help explain its uniform expression in both normal and malignant colorectal epithelium.

What is the clinicopathological relevance of down-regulation of ERβ1 and ERβ2 in a proportion of CRCs? In particular, how do these changes relate to the sex-related differences in CRC discussed earlier? Disappointingly, we found no differences between the sexes in ERβ1 and ERβ2 protein expression in primary CRCs. However, our preliminary data do suggest that loss of ERβ1 associates with more aggressive CRCs, whereas the opposite is true for ERβ2. Similar opposing prognostic correlates for ERβ1 vs. ERβ2 have recently been reported for prostatic carcinomas [32]. It is tempting to link these findings to differential activities of ERβ1 and ERβ2 and the opposing patterns of expression of the two isoforms in the normal colonic crypt. ERβ isoform expression was not found to predict response to 5-FU therapy in its own right, or to supplement the predictive information given by TS expression (data not shown). Therefore, CRC cell line-based data suggesting that ERβ-mediated oestrogenic reduction of TS expression causes increased 5-FU sensitivity [4] does not appear to translate to the *in vivo* setting.

An association between ERβ1 and mucin expression was unexpected. However, this may simply reflect the association between ERβ1 expression and microsatellite instability, as it is well established that microsatellite-unstable carcinomas are more likely to show a mucinous phenotype than their microsatellite-stable counterparts [33]. We are unaware of genes directly involved with mucin production that are oestrogen-responsive. However, it is interesting that ERβ1 expression was shown to associate positively with expression of TFF1, which in turn is known to be co-expressed with certain mucin sub-types, eg mucin M1 [34]. Finally, the fact that TFF1 expression in CRC is associated with ERβ1 expression further confirms the functional importance of this isoform relative to ERα in the colorectum.

Our tissue protein and cell line mRNA analyses both demonstrated a positive association between ERβ1 expression and microsatellite instability. The opposing predispositions to developing microsatellite-unstable

CRCs shown by pre- and postmenopausal women may be directly attributed to the effects of oestrogen exposure. Such exposure may protect against microsatellite instability-related colorectal carcinogenesis, but its loss with the menopause may cause a rebound increased predisposition to this process. Can this hypothesis be supported by observed changes in ERβ isoforms? There are limited data on the effects of varying oestrogenic exposure on expression of these isoforms in human intestine. However, Weyant and colleagues recently reported that ovariectomy in MIN mice leads to both increased small intestinal adenomas and increased background enterocyte 'ERβ' expression (and decreased ERα expression) [35]. While not stated in the paper itself, the 'ERβ' antibody used was likely to have at least detected ERβ1. To tie in all the above observations, we propose that ERβ1 activation in the intestine predisposes to microsatellite instability-related colorectal carcinogenesis. Such activation is somehow suppressed before the menopause, whereas oestrogen withdrawal leads to rebound increased ERβ1 expression (and decreased ERα expression) and hence to a higher risk of microsatellite-unstable carcinoma formation. This hypothesis would then not only explain our observed positive association between ERβ1 expression and microsatellite instability but also the negative association previously shown between ERα expression and microsatellite instability (using a sensitive immunoassay on primary CRC tissues) [36].

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