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Oestrogen and vitamin D receptor (VDR) genotypes and the expression of ErbB-2 and EGF receptor in human rectal cancers

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Abstract

Oestrogen/oestrogen receptor (ER) and vitamin D/vitamin D receptor (VDR) systems have been implicated in the pathogenesis of colorectal cancers. The expression of erbB-2 and epidermal growth factor receptor (EGFR) in colorectal cancers has been suggested to have diagnostic and prognostic significance. In our study, *XbaI* and *PvuII* polymorphisms of the *ER* gene and the *BsmI* polymorphism of the *VDR* gene were studied in 56 Caucasian patients with rectal cancer. The relationship between the *ER* and *VDR* genotypes and the expression of oncogenes was also investigated. The presence of the x allele of *ER* gene significantly correlated with the overexpression of the *erbB-2* and *EGFR* oncogenes. Significantly increased erbB-2 expression was observed in patients with the *VDR* B allele. The XXbb allelic combination of the *ER*/*VDR* genes was associated with a significantly lower erbB-2 expression, whereas in the other genotypes significantly higher oncogene expression was seen. Our data raise the possibility that *ER*/*VDR* gene polymorphisms accompanied by variable oncogene expression might influence the pathogenetic processes of colorectal cancers. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Oestrogen receptor; Vitamin D receptor; erbB-2/HER-2; EGF receptor; Rectal cancer

1. Introduction

Oestrogen replacement therapy has been shown to decrease the incidence of colorectal cancer in postmenopausal women by 30–50% [1]. The oestrogen receptor (ER) is a member of the steroid/thyroid receptor family. ER has been demonstrated in colon tumour cell lines, as well as in human colorectal cancers [2,3]. The *XbaI* and *PvuII* restriction site polymorphisms of the *ER* influence the early onset of breast cancer [4] and they may also have an impact on the incidence of osteoarthritis [5] and low bone mineral density [6].

Oestrogens can upregulate vitamin D receptor (VDR) expression, thus influencing the effects of vitamin D_3 [7]. The VDR also belongs to the steroid/thyroid receptor family. Both oestrogen and vitamin D_3 regulate calcium metabolism, cellular proliferation and differentiation. Colorectal tumour cells and human colorectal epithelial cells also express VDR [8]. In some studies, *VDR BsmI* polymorphism has been found to be associated with low bone mineral density [9] and the susceptibility of type I diabetes mellitus [10]. *In vitro* and *in vivo* studies have shown decreased colorectal cancer incidence and epithelial cell proliferation after treatment with vitamin D_3 [11,12].

Some clinical data support the concept of an interaction between ER and VDR [13]. Certain allele combinations of *ER* and *VDR* appear to be accompanied by a further decreased bone mineral density than with *ER* or *VDR* alone [14]. Some reports have raised the possibility that the ligands of the steroid/thyroid receptor family (such as 17β-oestradiol, vitamin D_3, progesterone and retinoic acid) may regulate cellular proliferation by increasing the transcription rate of *erbB-2* (also called HER-2/neu) and epidermal growth factor receptor (*EGFR*) [15,16]. ErbB-2 and EGFR belong to the tyrosine kinase receptor family and regulate the expression of genes involved in cellular proliferation and differentiation leading to the development of tumours such as colorectal cancer. The diagnostic and prognostic significance of the erbB-2 and EGFR expression has been repeatedly proposed in colorectal cancers [17,18]. The *erbB-2* gene has an oestrogen-responsive element (ERE)

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[19], while the *EGFR* gene contains a vitamin D-responsive element (VDRE) [20].

In the present study, we investigated the relationship between the *ER* and *VDR* genotypes and the expression of erbB-2 and EGFR in human rectal cancers.

2. Patients and methods

2.1. Subjects and tumours

In our study, 56 patients with rectal cancer consecutively admitted to the Department of Surgery were studied. All patients underwent surgery. The mean follow-up period was 48 months (range 1–60 months). During the follow-up period, 16 patients died. The mean survival period from the time of diagnosis was 19 months (range 1–47 months). In the control group, *ER* and *VDR* genotypes of 112 age-matched healthy subjects (two controls for each patient) were determined. The enrolment criteria in the control group included the lack of gastrointestinal pain, family history for colorectal cancer and detection of blood in stool, as well as no pathological finding on abdominal ultrasound (US). Written informed consent was obtained from all subjects. Table 1 contains the clinical and pathological data of the patients and controls.

The resection specimens for immunohistochemistry were fixed in 4% formaldehyde and tissue samples were embedded in paraffin. Slides were obtained from tumour and oral resection margin (normal mucosa). For protein dot blotting, native tissue samples were used.

2.2. Analysis of the restriction site polymorphisms of the ER *and* VDR *genes*

Genomic DNA was isolated from the surgically removed rectal cancers and normal mucosa. One hundred mg of the tissue sample were mechanically homogenised in 1.2 ml DNA lysis solution (100 mM NaCl, 10 mM Tris–HCl pH 8.0, 25 mM ethylene diamine tetra acetic acid (EDTA) pH 8.0, 0.5% sodium dodecyl sulphate (SDS), 0.1 mg/ml proteinase K, final concentration) at 50°C. DNA was extracted by phenol-chloroform and precipitated by ethanol. The *XbaI* and *PvuII* polymorphic regions of the *ER* gene, as well as the *BsmI* polymorphic site of the *VDR* gene were amplified by polymerase chain reaction (PCR) technique.

Table 1
Clinical and pathological data of patients and controls

	Patients ($n=56$)	Controls ($n=112$)
Age (years)	64 (23–84)	63 (23–83)
Gender	*n* (%)	*n* (%)
Female	29 (52)	58 (52)
Male	27 (48)	54 (48)
UICC stage	*n* (%)	
I	12 (21)	
II	20 (36)	
III	19 (34)	
IV	5 (9)	
Grade of malignancy		
G1	3 (5)	
G2	48 (86)	
G3	5 (9)	

UICC, International Union Against Cancer.

For the *ER* gene, the following primers were used: primer S: 5′ CTG CCA CCC TAT CTG TAT CTT TTC CTA TTC TCC 3′ 34-mer, primer A: 5′ TCT TTC TCT GCC ACC CTG GCG TCG ATT ATC TGA 3′ 33-mer (10 μM final concentration). The PCR reaction was carried out in 50 μl of final volume using the following materials: 5 μl 10× (Mg-free) PCR reaction buffer, 1 μl deoxynucleotide triphosphate (dNTP) (10 mmol/l), 5 μl $MgCl_2$ (25 mmol/l), 10 μl of purified DNA, 1–1 μl primers A and B (10 μmol/l), 0.4 μl Taq (Promega, Madison, USA) and 26.6 μl 2D PCR water. The following reactions were applied: 95°C for 3 min, 35× (72°C for 90 s, 95°C for 45 s) and 72°C for 10 min. The PCR product was subsequently digested by using *XbaI* and *PvuII* restriction enzymes (Promega, Madison, USA, 10 U/μl) at 37°C overnight. The lack of the *XbaI*/*PvuII* restriction sites correspond to the X/P alleles and they are present in the x/p alleles.

For the *VDR* gene, the following primers were used: primer A: 5′ AAC CAG CGG GAA GAG GTC AAG GG 3′ 23-mer, primer B: 5′ CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA 3′ 30-mer (10 μmol/l final concentration). The PCR reaction conditions are the same as for *ER*. The amplified PCR product was digested by using *BsmI* restriction enzyme (Hybaid-AGS, Teddington, Middlessex, UK, 10 U/μl) for 90 min at 65°C. The *BsmI* restriction site is missing in the B allele and is present in the b allele.

For the PCR reactions, a Hybaid Touchdown thermocycler (Teddington, Middlesex, UK) was used. Electrophoretic separation was carried out in a 2% agarose gel containing 10 μg/ml ethidium bromide.

2.3. Immunohistochemistry

The expression of erbB-2 protein was detected. Immunostaining was performed by using monoclonal mouse antibodies to (30 min, room temperature) human erbB-2 (BioGenex, Mainz, Germany) on paraffin-embedded sections. The working dilution was 1:180. The slide was pretreated with a 0.21% citric acid solution (pH 6.0) in the microwave oven for 3×5 min. The reaction was detected by the supersensitive immunodetection system distributed by BioGenex (Mainz, Germany). The staining was graded semiquantitatively: no reaction in the cells (grade 0), positive reactions in less than 20% of tumour cells (grade 1), positive reactions in

20–50% of the cells (grade 2) and positive reactions in more than 50% of the cells (grade 3).

2.4. Protein blotting

The expression of erbB-2 and EGFR external and internal domain was examined. Tissue samples for protein blotting were solubilised mechanically in a solution containing 0.05 M Tris–HCl (pH 8.0), 0.1% Triton X 100 and 0.5% SDS. Insoluble material was removed by centrifugation (10 000 rpm, for 5 min). Samples were adjusted to a protein concentration of 7 mg/ml with 0.02 M Tris–HCl (pH 7.5) containing 0.5 M NaCl. After blocking with 1% bovine serum albumin (BSA), 200 μl of samples were transblotted to nitrocellulose membranes (0.45 μm pore size, Serva) using a Bio-Dot microfiltration apparatus (BioRad, Hercules, USA) according to the instruction manual. The following working dilutions of the monoclonal antibodies were applied: for EGFR external and internal domain (Boehringer, Mannheim, Germany) 1:20, for erbB-2 (BioGenex, Mainz, Germany) 1:40. Incubation was carried out overnight at room temperature. After washing three times with TTBS, antimouse Ig-Fab conjugated with peroxidase (Boehringer, Mannheim, Germany) was used as second antibody in a dilution of 1:1000 for 1 h at room temperature. After washing three times with TTBS, the peroxidase activity was made visible with a solution containing 4 chloro-naphtol, peroxide, TBS and methanol. Semiquantitative evaluation was performed as follows: no visible reaction (grade 0), weak staining (grade 1), moderate staining (grade 2) and strong reaction (grade 3). In the case of erbB-2, tumour samples with identical scoring in both methods were included in the study.

2.5. Statistical analysis

Analysis of the relationships among clinical data, *ER* and *VDR* polymorphisms, oncoprotein expression and coexpression was performed by using Chi-square and Yates-corrected Chi-square test where appropriate.

3. Results

The *XbaI* allelic frequency and genotype distribution of the *ER* gene was not significantly different in controls (X = 47%, x = 53%, genotypes: XX = 14%, Xx = 64%, xx = 22%) and in patients with rectal cancer (X = 48%, x = 52%, genotypes: XX = 29%, Xx = 39%, xx = 32%). In the presence of the x allele of the *ER* gene, the expression of erbB-2 tyrosine kinase receptor was significantly higher (Table 2). In the genotypes xx or Xx, significantly increased erbB-2 (by immunohistochemistry and protein blotting) and the EGFR external domain (by protein blotting) expression was found in contrast to patients with the XX alleles (Table 3).

The *PvuII* restriction site P allele was found in 70% of our patients, while the frequency of the p allele was only 30%. In controls, the frequency of these two alleles was statistically different from patients, 50% for P and 50% for p, respectively ($P < 0.0015$). Genotype distribution of the *PvuII* polymorphism of the *ER* gene was significantly different in the two study groups (patients: PP = 50%, Pp = 39%, pp = 11%, controls: PP = 22%, Pp = 56%, pp = 22%; $P = 0.0013$; $Chi^2 = 13.34$). The different *PvuII* alleles and genotypes were not directly associated with the expression of oncogenes. No significant difference was seen in the combined genotype distribution of the *PvuII* and *XbaI* polymorphisms of the two groups.

The *BsmI* allele and genotype frequencies of the *VDR* gene in patients with rectal cancer and in controls were not statistically different (patients: B = 42%, b = 58%, and BB = 23%, Bb = 38%, bb = 39%; controls: B = 43%, b = 57%, and BB = 19%, Bb = 48%, bb = 33%). The overexpression of erbB-2 (by immunohistochemistry and protein blotting) was significantly more frequent in the presence of the B allele of the *VDR* gene (Table 4).

The combination of the *VDR* gene *BsmI* and *ER* gene *XbaI* polymorphisms revealed that patients with genotypes Xxbb, XxBb, XXBb and xxBb have significantly more frequent erbB-2 expression than in cases of the XXbb genotype (Table 5).

Significant differences were found in the oncoprotein expression pattern of normal and tumour tissues (Table 6). There was no significant correlation between the *ER* and *VDR* genotypes or their combinations and clinical parameters, age, gender, infiltration deepness of tumour, number and localisation of lymph node metastases, lymphatic or blood vessel infiltration, serum carcinoembryonic antigen (CEA) or CA-19.9 levels.

4. Discussion

In our present study, we have demonstrated an association between the *XbaI* polymorphism of the *ER* gene

Table 2
The expression of the erbB-2 in the presence of the *ER* gene x allele in the rectal cancers

	Presence of x allele (xx, Xx) *n* (%)	Presence of X allele (XX) *n* (%)
No erbB-2 expression	9 (23)	10 (62)
erbB-2 expression[a]	31 (77)[b]	6 (38)
Total ($n = 56$)	40	16

[a] Positive staining for oncogene: grades 1, 2, 3.
[b] $Chi^2 = 6.47$, $P = 0.011$.

Table 3
The expression of the erbB-2/EGFR external domain in the presence of ER *XbaI* genotypes in rectal cancer

	xx Genotype *n* (%)	Xx Genotype *n* (%)	XX Genotype *n* (%)
No erbB-2[a]/no EGFR[b] external Domain expression	5/6 (28/33)	4/4 (18/18)	10/9 (63/56)
erbB-2[a]/EGFR[b] external Domain expression[c]	13/12 (72/67)	18/18 (82/82)	6/7 (37/44)
Total ($n=56$)	18	22	16

[a] $Chi^2=8.5$, $P=0.014$ (for erbB-2).
[b] $Chi2=5.9$, $P=0.049$ (for the EGFR external domain).
[c] Positive staining for oncogenes: grades 1, 2, 3.

Table 4
The expression of the erbB-2 in the presence of the *VDR* gene B allele in rectal cancers

	Presence of B allele (BB, Bb) *n* (%)	Presence of b allele (bb) *n* (%)
No erbB-2 expression	8 (24)	11 (50)
erbB-2 expression[a]	26 (76)[b]	11 (50)
Total ($n=56$)	34	22

[a] Positive staining for oncogene: grades 1, 2, 3.
[b] $Chi^2=4.18$, $P=0.041$.

(the presence of the x allele) and the overexpression of two cooperative members of the tyrosine kinase receptor family, the erbB-2 and EGFR external domain in human colorectal cancers. The expression of erbB-2 was more pronounced also in the presence of the B allele of the *VDR* gene. Moreover, combinations of *ER*/*VDR* genotypes containing x and B alleles markedly influenced the expression of erbB-2. The cytoplasmatic overexpression of erbB-2 is considered to be a prognostic factor in colorectal cancers [18], while EGFR plays an important role in the development of the hyperproliferative state of colonic mucosa and colon carcinogenesis [21]. ErbB-2 and EGFR are frequently coexpressed in tumours, which may have an impact on the prognosis of patients. The signal transduction of the two receptors is increased by their heterodimerisation [22]. The external domain of EGFR contains the sequence necessary for heterodimerisation with erbB-2 [22]. The increased expression of erbB-2 and the EGFR external domain in the presence of the x and B alleles might provide an increased risk for colorectal cancer. The distribution of the *XbaI* and *BsmI* genotypes in cancer patients was not different from healthy controls, however, this fact does not contradict the former hypothesis, since additional factors may be required for x and B alleles in order to influence the expression of erbB-2 and EGFR. The number of deceased patients was small during the 5 years of our study, thus the effect of our findings on survival requires further investigation.

Oestrogen-ER and vitamin D-VDR systems may play an important role in the pathogenetic processes leading to the development of colorectal cancers. Some epidemiological data demonstrated that a semi-vegetarian diet with high phyto-oestrogen content in Asia is protective against the development of colorectal cancer [23]. In animal experiments, phyto-oestrogen-containing food proved to be anticarcinogenic [23]. A recent meta-analysis shows that oestrogen replacement therapy decreases the risk of colorectal cancer in post-menopausal women [1]; however, some contradictory results also exist. Increased dietary calcium and vitamin D_3 intakes are correlated with a decreased epithelial cell proliferation in some tumours such as colorectal cancer [11]. In animal models, carcinogen-induced tumour size is decreased after dietary treatment with calcium and vitamin D_3 [12]. In addition, oestrogens upregulate VDR, thus influencing the effect of vitamin D_3 [7]. Short-term oestrogen therapy in rats after ovariectomy also corrects the level and activity of VDR, which might contribute to its protective effect against colorectal cancer. A possible site of interaction between oestrogen and vitamin D systems could be the *aromatase* gene possessing a VDRE in its promoter [24]. The aromatase enzyme directs the peripheral conversion of androgens to oestrogen.

The expression of ER subtypes in colonocytes and tumour cells is controversial. The presence of ER-α in normal colonic epithelium, several colon cancer cell lines and human colon cancer tissue has been confirmed by several authors [2,3]. However, some authors were not able to demonstrate the existence of ER in normal or malignant colonocytes [25], but in the stromal cells of the large intestine [26]. Recently, Foley and colleagues [27] have found a strong ER-β expression in malignant human colon tumours, while ER-α protein was present at a low level by western blot. Increased methylation of *ER-α,* a silencing mechanism for the expression of the gene, in colorectal cancer has also been described [28]. Polymorphisms examined in our study mostly relate to *ER-α*, however, the exact sequence of *ER-β* and thus, the presence of these polymorphic sites in *ER-β* have not yet been elucidated. Moreover, the effect of *XbaI*

Table 5
ErbB-2/EGFR external domain expression in the most frequent combined *ER* and *VDR* genotypes in rectal cancers

Genotype	XXbb	Xxbb	XxBb	XXBb	xxBb
No erbB-2[a]/no EGFR external Domain expression	5/3	0/1	1/0	1/3	1/2
erbB-2[a]/EGFR External domain expression[b]	2/4	7/6	4/5	4/2	4/3
Total	7	7	5	5	5

[a] $Chi^2 = 11.84$, $P = 0.018$ (for erbB-2). XXbb versus other genotypes: Chi2 = 7.682, $P = 0.005$ (for erbB-2).
[b] Positive staining for oncogenes: grades 1, 2, 3.

Table 6
Overexpression of erbB-2, EGFR external and internal domain in rectal cancers and normal mucosa cells

	Cancer with positive reaction[a] *n* (%)	Normal mucosa with positive reaction[a] *n* (%)
erbB-2	37/56 (66)[b]	3/56 (5)
EGFR external domain	37/56 (66)[b]	6/56 (11)
EGFR internal domain	40/56 (71)[b]	5/56 (9)

[a] Positive staining for oncogenes: grades 1, 2, 3.
[b] $P < 0.0001$ versus normal mucosa.

allelic variants might be mediated directly and/or via indirect pathways, such as the vitamin D/VDR system which is present in tumour cells.

Several indirect observations have already been made about the mutual interaction of the nuclear steroid/thyroid receptor family and the tyrosine kinase receptor superfamily [16]. 17β-oestradiol and vitamin D_3 may regulate cellular proliferation by increasing the transcription rate of *erbB-2* and *EGFR* [15,16]. Many other genes have response elements for both ER and VDR in their promoter regions. *ErbB-2* contains an ERE [19], while *EGFR* has a VDRE in the promoter region [20]. Polymorphisms of *ER* and *VDR* may be associated with altered receptor expression and function [29,30]. Polymorphisms of the *ER* and *VDR* genes have been suggested to be associated with the occurrence of several disorders [5,6,9,10], including breast cancer [4]. Our present findings may add to the list of disorders related to polymorphisms of the *ER* and *VDR* genes. Potential interactions between the *ER* and *VDR* genes have also been suggested by others [13,14].

In conclusion, our data raise the possibility that *ER* and *VDR* gene polymorphisms accompanied by variable oncogene expression might be part of the pathogenetic processes resulting in the development of colorectal cancers.

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