Differences in the role of Gper1 in colorectal cancer progression depending on sex

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Abstract. To evaluate the role of 17β-oestradiol (E2) in the sex-dependent progression of colorectal cancer (CRC), the present study focused on E2 signalling mediated via the nuclear receptors [oestrogen receptor (ESR)1 and ESR2] and the membrane G protein-coupled oestrogen receptor 1 (Gper1) in males and females diagnosed with CRC. This study also investigated Gper1 signalling in the CRC cell lines DLD1 and LoVo, which differ in the p53 pathway. In cancer tissue, Gper1 becomes by far the most abundant E2 receptor due to an increase in Gper1 and a decrease in ESR2 expression. These changes are more prominent in males than in females. More pronounced differences in *Gper1* expression between cancer and adjacent tissues were observed in males in lower stages compared with those in higher stages of disease and females. High expression of *Gper1* was associated with worse survival in males without nodal involvement but not in females. The expression of E2 receptors in the CRC cell lines DLD1 and LoVo resembles that of human cancer tissue. Silencing of Gper1 (siGper1) caused an increase in the rate of metabolism in LoVo cells with wild-type *tp53*. In DLD1 cells with the mutated form of *tp53*, siGper1 did not exert this effect. High levels of Gper1 were associated with worse survival and could contribute to sex-dependent changes in the CRC prognosis. Tumour suppressor effects of Gper1 were, at least to some extent, dependent on signalling downstream of p53, which was more frequently deficient in males than in females. Overall, this suggests that up-regulation of Gper1 (or administration of a Gper1 agonist) would be more beneficial for patients with wild-type *tp53*.

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Introduction

Despite significant improvements in colorectal cancer (CRC) treatment, especially in countries with long-standing screening programs (1), CRC is still the third most frequently diagnosed oncologic disease, ranking second among causes of cancer-related mortality (2,3). A further rise in CRC incidence is predicted. The burden of CRC is attributed to increasing early-onset CRC, ageing of the population and a lifestyle shift toward a Western diet combined with low physical activity (3,4).

Interestingly, the age-standardized incidence of CRC is consistently higher in males compared to females, including CRC cases with early onset (5). CRC is more often diagnosed in the proximal colon in women, whereas distal anatomical CRC sites are detected more often in males (6-8). The most pronounced difference is observed in the incidence of rectal cancer, which is in 75% of cases diagnosed in males (2,3,9).

Among the possible reasons for this phenomenon are an unfavourable diet, obesity, alcohol consumption, smoking and low physical activity (10). These factors influence CRC progression differently depending on the sex of the patient and tumour localisation (11-13).

Although sex hormones have been implicated in the generation of differences in CRC prevalence between males and females, their precise role has not been completely elucidated (7,8,14). In particular, it is assumed that the most potent oestrogen, 17β-oestradiol (E2), influences CRC progression (8,15,16).

E2 signalling is mediated predominantly by two types of nuclear receptors, ESR1 and ESR2, and the G protein-coupled membrane receptor Gper1 (previous symbols GPER, GPR30) (15). Among the ligands of ESR1, ESR2 and Gper1 are, in addition to oestrogens, tamoxifen, raloxifene, bisphenols, dioxins, phthalates, quercetin, genistein, resveratrol and others (17,18). The ligands mentioned above can usually activate both nuclear and membrane-bound receptors, although their affinity can differ (19). Gper1 has a lower affinity for E2 than ESR1 and ESR2 (20). Direct binding of E2 to Gper1 was even questioned recently (21,22). However, there is substantial *in vivo* and *in vitro* evidence that Gper1 is involved in E2-mediated regulation (23). Recently, the binding of E2 to Gper1 was confirmed, and aldosterone was also proposed as a Gper1 agonist (24).

Nuclear E2 receptors exert most of their effects via transcriptional gene regulation. ESR1 and ESR2 activate thousands of genes overlapping by 30% via a specific DNA sequence called oestrogen response element (ERE) (25).

Compared to nuclear E2 receptors, Gper1 affects intracellular processes much faster thanks to the prompt activation of several intracellular signalling pathways. Signalling mediated by Gper1 includes an increase in cAMP synthesis and consequent activation of protein kinase A, Src-like nonreceptor tyrosine kinase (Src) and sphingosine kinase (SphK). Src and SphK can contribute to the activation of epidermal growth factor receptors (EGFR). Gper1 signalling can also lead to activation of protein kinase C and calcium mobilisation (17). Among Gper1-driven regulatory pathways also belongs Gper1/HIFα activation of NOTCH and consequent induction of VEGF transcription (26) and some others (18). Tissue-specific distribution and/or changes in the tissue-specific distribution of E2 receptors in disease can influence E2 signalling and facilitate its oncostatic or tumour suppressor effects (17,18,25).

Epidemiological studies of patients using hormone response therapy (HRT) implicated protective role of E2 in respect to CRC incidence. It was shown that oestrogen/progestin HRT is negatively associated with the risk of CRC (27). Similarly, the incidence of CRC in postmenopausal women exposed to E2 HRT was lower compared to women of the same age without HRT (28). Other studies later strengthened the evidence of E2's protective role regarding CRC incidence (15,16,29,30). It is of interest that patients with high levels of endogenous circulating E2 (31) and those exposed to E2 therapy whilst diagnosed with CRC demonstrated a worse disease prognosis (32). On the other hand, Mori *et al* (33) did not confirm the association of high circulating E2 levels with increased incidence and/or prognosis of CRC in postmenopausal women.

Most of the beneficial effects of E2 for patients are attributed to ESR2 (15,34-37), which is far more abundant in the gastrointestinal tract compared to ESR1 (38-40). The beneficial E2 effect mediated via ESR2 receptors was proven with the use of several *in vivo* experimental models. $Apc^{min/+}$mice bearing a mutation in the *Apc* gene are prone to developing multiple intestinal tumours. Ovariectomy (OVX) significantly increased the number of adenomas detected in the gut compared to control, and this effect was reversed by E2 administration. E2 treatment was also accompanied by an increase in ESR2 expression in the intestine (41). ESR2 deficiency in $Apc^{min/+}$ mice was associated with a higher adenoma number compared to control (42). The protective role of E2 executed via ESR2 was also demonstrated in OVX mice where tumorigenesis was induced by azoxymethane (AOM) (43), by combined treatment of AOM and dextran sulphate sodium (44,45) and in immunodeficient mice implanted with SW480 cells overexpressing ESR2 (46).

Interestingly, the expression of Gper1 mRNA exceeds that of ESR2 in healthy gut tissue (40,47). Similarly, it is the predominant E2 receptor in several colorectal cell lines (48). Despite that, the role of the membrane receptor Gper1 in E2-mediated effects on cancer progression has not been completely elucidated.

The tumour suppressor as well as the oncogenic role of Gper1 has been demonstrated for several types of tissues. Perhaps the most promising results were achieved in melanoma treatment. However, in most cancer types, including CRC, the role of Gper1 remains inconclusive (17,49,50).

It was shown that G-1, an agonist of Gper1, inhibits the upregulation of JUN oncogene expression in HT29 cells (48). G-1 administered at a concentration of 1 μM significantly inhibited the proliferation of HCT-116 and SW480 cells, increased the number of cells in the G2/M phase and stimulated apoptosis. The decrease in cell viability induced by G-1 was prevented by the administration of the ROS scavenger NAC. Growth of tumour xenografts in nude mice was inhibited by G-1 administration (51).

On the other hand, Gper1 silencing prevented chromosomal instability induced by diethylstilbestrol and the occurrence of supernumerary centrosomes in HCT116 and control CCD 841 CoN cell lines. In these cell lines, Gper1 activation led to the presence of multipolar mitotic spindles, an increased number of cells with lagging chromosomes and aneuploidy. Interestingly, manipulation of Gper1 levels did not influence the rate of proliferation and cell cycle distribution under *in vitro* conditions (52). The oncogenic role of Gper1 was confirmed in HT-29 cells as Gper1 silencing prevented an E2-induced decrease in ATM. Under normoxic conditions, Gper1 activation caused down regulation of VEGF while the opposite effect was observed in a hypoxic microenvironment and prevented by Gper1 silencing. Under hypoxic conditions, Gper1 mediated an E2-induced increase in HT-29 and DLD1 cell migration (53). E2 and its agonist G-1, via Gper1, induced the expression of fatty acid synthase (FASN), which can promote CRC progression. FASN upregulation is executed by the epidermal growth factor receptor EGFR/ERK/AP1 pathway (54). Oncogenic properties of Gper1 were demonstrated in the CRC cell lines COLO205 and SW480 as the Gper1 antagonist G15 was able to reverse the effect of the E2-related endocrine disruptor nonylphenol, which induced proliferation, the expression of cyclin D1, c-myc and ERK1/2. Administration of G15 also inhibited the growth of colon carcinoma xenografts in mice (55). Disagreement in the results of performed studies can be partly related to different concentrations of G-1 used in experiments. In addition to experimental designs, the biological context seems to be of special importance in effects mediated by Gper1 (17,34,48,52,53).

In human studies, the results are also inconclusive. Up- as well as down-regulated Gper1 expression in CRC tissue was indicated (17), e.g. a strong trend toward increased Gper1 levels in CRC cancer tissue compared to adjacent tissue was reported by Gilligan *et al* (56) while down-regulated expression of Gper1 was reported by Liu *et al* (51). High expression of Gper1 was associated with better survival compared to low expression (51); by contrast, low expression of Gper1 was associated with better survival compared to high expression (56). High expression of Gper1 was also associated with worse survival by Bustos *et al* (53) and Abancens *et al* (48) but only in females in stages 3-4 of CRC. Worse survival of CRC patients exhibiting high expression of Gper1 is indicated by the Human Protein Atlas (47).

As there is evidence suggesting that manipulation of E2 signalling could be effective in clinical use, several agonists and antagonists of nuclear and Gper1 receptors have been developed, and some of them have been clinically tested (18,25,57). The most promising results were obtained in the clinical

study NCT04130516 evaluating the effects of Gper1 agonist LNS8801 that proved beneficial effects of Gper1 signalling in patients diagnosed with cutaneous (58) and uveal (59) melanoma. However, it was implied that G-1 can cause effects independent of Gper1 signalling (60-65). Interestingly, several clinically approved antagonists of nuclear E2 receptors also act as Gper1 agonists (18).

CRC is among the most frequently diagnosed oncologic diseases worldwide. To facilitate novel CRC treatment strategies, we focused on Gper1 functioning in CRC tumours to define the conditions under which Gper1 upregulation could be beneficial for patients.

Despite ongoing clinical trials aimed at the manipulation of Gper1 signalling in solid cancers, the involvement of Gper1 in the generation of sex-dependent CRC incidence is largely unknown. Similarly, the biological context that determines the effect of Gper1 has not been described sufficiently. Therefore, the present study is aimed to analyse the abundance of Gper1 in healthy and tumour tissues of males and females diagnosed with CRC according to disease stage and survival. Mutation in p53 has been identified in more than 50% of CRC patients and at a higher frequency in males compared to females (66). Therefore, using the CRC cell lines LoVo and DLD1, which differ in p53 functionality (67), we tested the hypothesis that the effects of Gper1 depend on the presence of wild-type p53.

Materials and methods

Tumour and adjacent tissues were obtained in cooperation with the First Surgery Department, University Hospital, Comenius University, Bratislava. The study included patients who had to undergo surgery for CRC treatment (for details see Table SI) and agreed to sign an informed consent. Patients were not subjected to any CRC treatment before surgery and during hospitalization were exposed to a standard hospital practice with lights on from 6:00 a.m. to 9:00 p.m. Surgery was performed in the morning hours (before noon). Adjacent tissues were collected ≥10 cm proximally and ≥2 cm distally from the tumour. After tissue excision, samples were promptly frozen in liquid nitrogen and stored at -70˚C until analysis. The experimental protocol was approved by the Ethics Committee of Comenius University in Bratislava (ECH 19001).

To extract mRNA from tissues and cells, RNAzol RT (Molecular Research Centre) was used as described previously (40). One microgram of RNA template from tissues and 0.02 μg from cells was used to synthesize cDNA with the ImProm-II Reverse Transcription System and random hexamers (Promega), according to the manufacturer's instructions.

To analyse gene expression, the QuantiTect SYBR Green kit (Qiagen, Germany) was used. Real-time PCR was performed with the StepOnePlus™ Real-Time PCR System (Applied Biosystems). The PCR conditions were activation of hot start polymerase at 95˚C for 15 min followed by 40 cycles at 94˚C for 15 sec, 53˚C for 30 sec and 72˚C for 30 sec. As a last step of PCR, samples were subjected to melting curve analysis. The sequences of primers for the amplification of particular genes are provided in Table SII. The expression of nuclear *u6* mRNA was used for normalisation.

The human colorectal carcinoma cell lines DLD1 and LoVo obtained from the American Type Culture Collection (ATCC) were used to reveal how *gper1* silencing influences cell migration and metabolism. DLD1 cells were cultured in RPMI 1640 GlutaMax medium (Gibco; Thermo Fisher Scientific, Inc.), and LoVo cells were incubated in F-12K medium (Bioconcept). Both cell lines were supplemented with 2% or 10% foetal bovine serum (FBS, Biosera) depending on the experiment; 2% FBS supplementation was used for the scratch assay while 10% FBS was used for the MTS test. The culture medium also contained penicillin (50 U/ml) (Gibco), streptomycin (50 μl/ml) (Gibco, USA) and ampicillin (50 μg/ml) (Oasis-lab). All experiments were performed in a biological Celculture® Incubator CCL-050B-8 (Esco Medical) with a humidified atmosphere containing 5% CO_2 at 37˚C. The cells were cultured in 96-well plates coated with 1% sterile gelatine.

To test the effect of *gper1* silencing, DharmaFECT 1 reagent was used to transfect cells with siGENOME Human Gper1siRNA-SMART pool or siGENOME non-targeting siRNA Pool #2 (Horizon) at a concentration of 100 nM.

The effect of siGper1 on wound healing was determined by scratch assay performed when the cell culture reached a confluence of 80-90% with the use of a 10-ul sterile tip. Images were taken immediately after scratch and later as indicated in the figure legends with the use of an inverted fluorescence microscope NIB-100F (Nanjing Jiangnan Novel Optics Co., Ltd.) and BEL Capture 3.2 software (BEL Engineering s.r.l.). Wound closure was evaluated with ImageJ software (68).

The rate of metabolism was measured by MTS test according to the manufacturer's instructions (CellTiter 96 AQueous Cell Proliferation Assay, Promega) employing a modified tetrazolium compound to produce water-soluble formazan. The absorbance was measured at 490 nm using a UV spectrophotometer (Epoch, Agilent Technologies, Inc.).

Statistical analysis. To evaluate Gper1 mRNA expression in human samples, the cohort was split into three groups according to TNM classification. Group 1 included distant metastasis-free patients without nodal involvement (T1-4N0M0), group 2 consisted of patients with nodal involvement and without distant metastases (T3-4N1-2M0) and the 3rd group was composed of patients with distant metastases (T3-4N0-2M1). Gene expression between three groups was compared by ANOVA followed by Tukey's post hoc test.

To compare E2 receptor expression between two groups, a Student's paired t-test was used. ANOVA followed by Tukey's post hoc multiple comparisons test was used to compare the expression of three types of oestrogen receptors (ESR1, ESR2 and Gper1) in human tissues and cells.

To analyse Gper1 mRNA expression with respect to overall survival, a Kaplan-Meier survival curve and a log-rank test were performed. Values were split according to the median; high expression > median, low expression ≤ median. The starting point for the log-rank test was the day of the surgery. The association of Gper1 and VEGFA mRNA expression was analysed by correlation analysis.

Data are provided as mean ± standard error of the mean (SEM) in relative units (r.u.). The threshold for significance

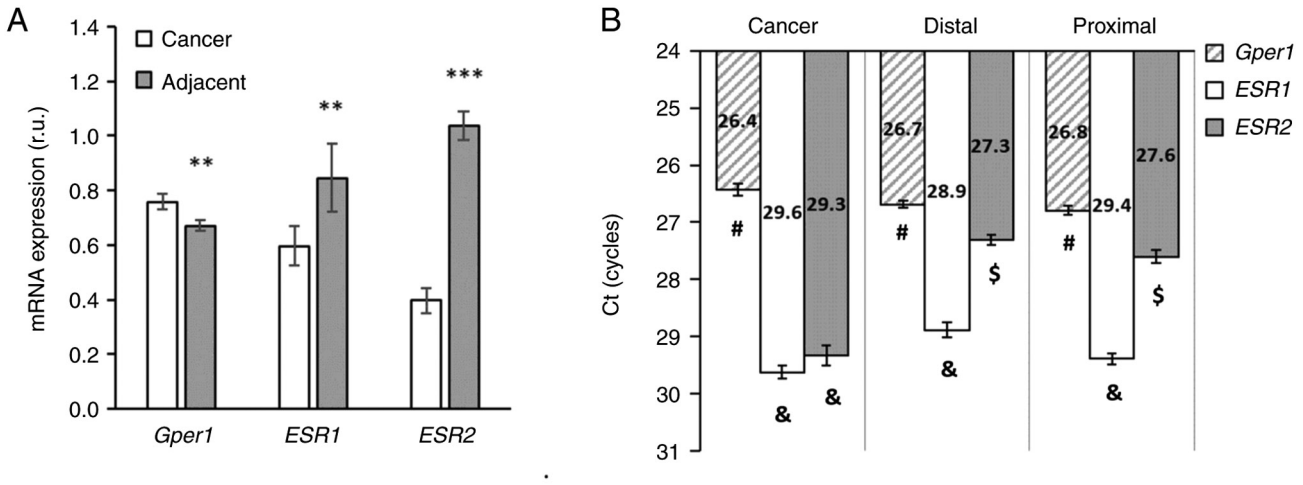

Figure 1. Relative expression of ESR1, ESR2 and Gper1 mRNA in the tumour and corresponding adjacent tissues of patients undergoing surgery for colorectal cancer. (A) Comparison of ESR1, ESR2 and Gper1 mRNA expression in the tumour (white columns) and averaged expression in the corresponding proximal and distal tissues (grey columns). Data are relativised; the averaged expression in samples was set to the same value for all receptors. (B) The abundance of E2 receptors in cancer and adjacent tissues is presented as threshold cycle (Cq-the cycle number of PCR at which the fluorescence reached the threshold in the amplification, the averaged number is shown in the middle of the columns). The threshold was set to 1 in all PCR assays. Data are shown as mean ± standard error of the mean. **P<0.01 and ***P<0.001 cancer tissue compared to adjacent tissue. Columns labelled with different labels are significantly different by ANOVA followed by Tukey's post hoc test at P<0.05. $^{\#}$P<0.05 compared with $ and &. $^{\$}$P<0.05 compared with # and &; $^{\&}$P<0.05 compared with # and $. r.u., relative units; ESR, oestrogen receptor; Gper1, G protein-coupled oestrogen receptor 1; E2, 17β-oestradiol.

for all statistical tests was set at P<0.05. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.).

Results

E2 receptors show robust differences in expression between colorectal tumours and adjacent tissues. In tumour tissue, the expression of *gper1* was higher compared to adjacent tissue, whereas the opposite pattern was observed in levels of nuclear E2 receptors. The expression of *esr1* and *esr2* was significantly lower in cancer tissue compared to adjacent tissues (Fig. 1A). The expression of *gper1* was higher in cancer tissue compared to adjacent distal and proximal tissues (Fig. 1B). Comparison of the absolute level of expression based on the threshold value of the PCR revealed that *gper1* is far most abundant in cancer as well as in the adjacent tissues compared to *esr1* and *esr2* mRNA levels (Fig. 1B).

The expression of *gper1* was higher in cancer tissue compared to adjacent tissue in males, but in females this pattern was observed only as a non-significant trend (Fig. 2). The expression of *esr1* was higher in the adjacent compared to cancer tissue and this difference, again, achieved a level of significance only in males (Fig. 2). The most robust difference in expression between cancer and adjacent tissue was observed in the expression of *esr2*. Unlike other receptors, this pattern was present in both sexes (Fig. 2).

In the whole cohort an increase in *gper1* expression in cancer tissue compared to adjacent tissue was observed in patients without nodal involvement and distal metastases (Fig. 3A). However, when the cohort was split into male and female patients, this difference was observed only in males (Fig. 3B and C). *gper1* expression did not differ between cancer and adjacent tissues in more advanced stages of disease (Fig. 3).

When the cohort was split according to nodal involvement only (without considering TNM staging as a whole) the sex-dependent difference in *gper1* expression became even more pronounced, and there was a highly significant increase in *gper1* expression in cancer tissue compared to adjacent tissue in males without nodal involvement (Fig. 4A). Similarly, male patients without distant metastases displayed a pronounced increase in *gper1* expression in cancer tissue compared to adjacent tissue that was not observed in females (Fig. 4B).

In accordance with the sex-dependent expression of *gper1* in CRC patients, the association of overall survival and *gper1* expression exerted a sex-dependent pattern. In the whole cohort, better survival was correlated with low *gper1* expression in patients without nodal involvement (Fig. 5A) but not in patients in higher stages of disease (Fig. 5B). When the cohort was split according to sex, a log-rank test revealed that *gper1* association with survival was generated by the male subcohort (Fig. 5C). In females the correlation between survival and *gper1* expression did not reach significance (Fig. 5D).

In human samples from our cohort, the expression of *gper1* significantly correlated with the mRNA expression of VEGFA in cancer tissue. This relationship was not observed in proximal and distal adjacent tissues (Fig. S1).

Transfection of siRNA targeting *gper1* expression successfully inhibited Gper1 mRNA expression in DLD1 as well as LoVo cells compared to control (Fig. S2A and B, respectively).

The distribution of E2 receptors in CRC cell lines LoVo and DLD1 resembled that of human cancer tissue. The expression of the membrane *gper1* receptor was much higher compared to mRNA levels of nuclear E2 receptors, and the expression of ESR1 mRNA was nearly undetectable (Fig. 6).

Silencing of *gper1* expression significantly stimulated metabolism in LoVo cells in a time-dependent manner that implicates the tumour-suppressor capacity of Gper1. In DLD1 cells we did not observe this effect (Fig. 7).

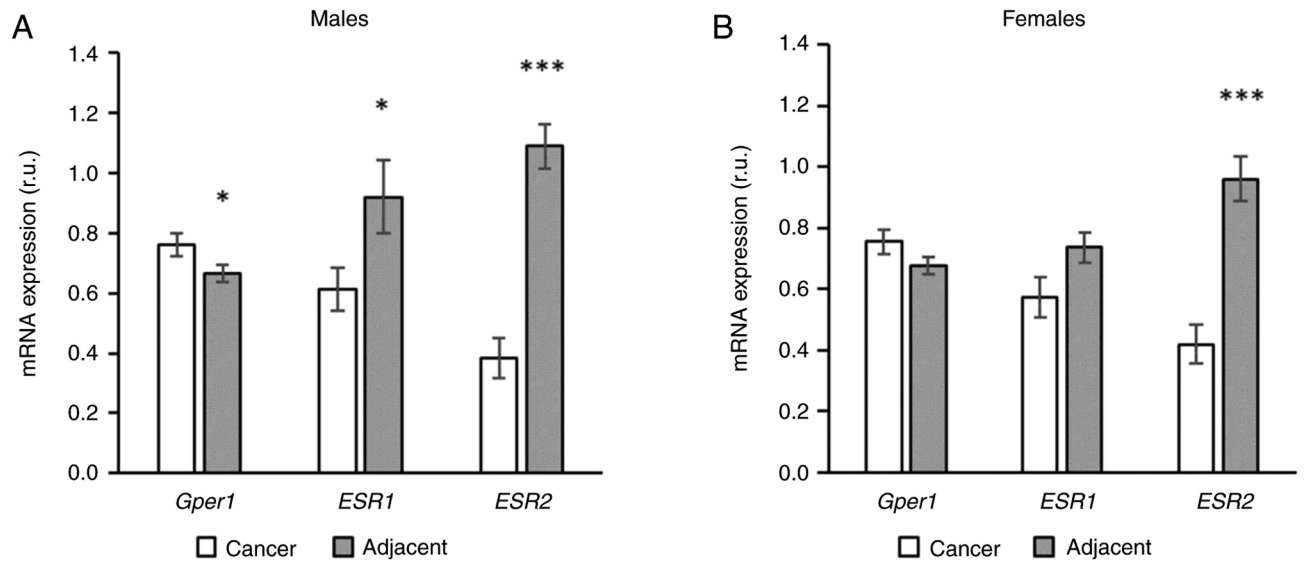

Figure 2. Gper1, ESR1 and ESR2 mRNA expression in cancer and adjacent tissues of (A) males and (B) females. Data are relativised to the same averaged expression in all three datasets (Gper1, ESR1 and ESR2). White columns show expression in cancer and grey columns display mRNA levels in the corresponding proximal and distal tissues. Data are shown as mean ± standard error of the mean. *P<0.05 and ***P<0.001 cancer tissue compared to adjacent tissue. r.u., relative units; ESR, oestrogen receptor; Gper1, G protein-coupled oestrogen receptor 1.

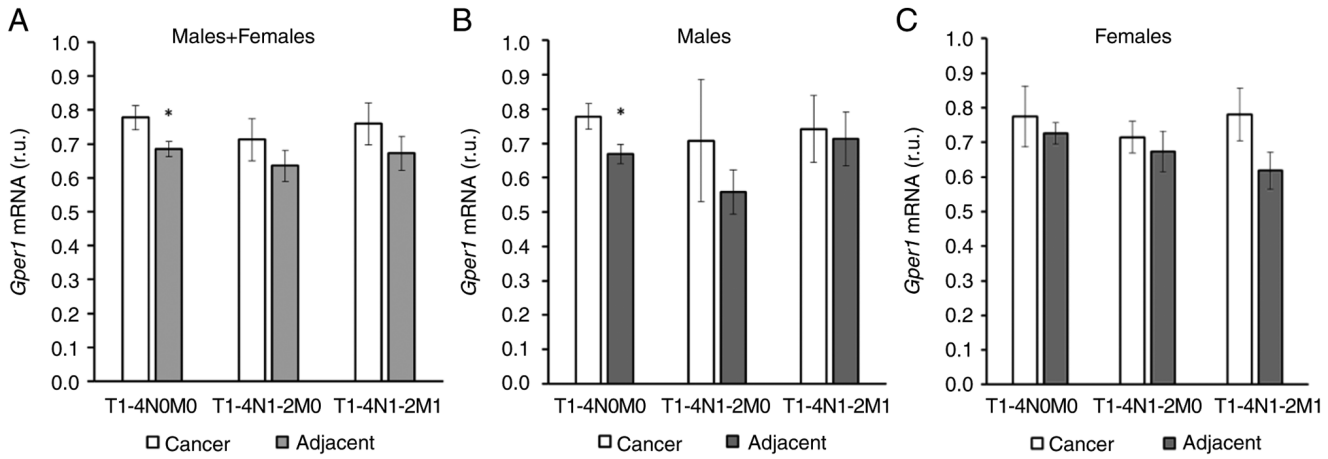

Figure 3. Comparison of Gper1 mRNA expression in colorectal cancer tissue. Expression of Gper1 compared with adjacent tissues in (A) the whole cohort, (B) males and (C) females according to the TNM classification. White columns show expression in cancer, and grey columns display averaged mRNA levels in the corresponding proximal and distal tissues. Data are shown as mean ± standard error of the mean. *P<0.05 cancer tissue compared to adjacent tissue. T, tumour invasion; N, nodal status; M, distant metastasis; r.u., relative units; Gper1, G protein-coupled oestrogen receptor 1.

Evaluation by the scratch test demonstrated a time-dependent decrease in wound closure in DLD1 cells with silenced Gper1 expression compared to control. Whereas 24h after Gper1 silencing there was no effect on the rate of wound closure, 48 h after transfection the width of the closed area was different between control and transfected cells with P=0.057 (Fig. 8A). Inhibition of wound healing by Gper1 silencing implicates the oncogenic potential of Gper1 in DLD1 cells.

Unlike in DLD1 cells, the administration of siRNA interfering with Gper1 expression did not influence the rate of wound closure in LoVo cells (Fig. 8B).

The expression of p53 mRNA did not differ significantly between DLD1 and LoVo cells. However, as DLD1 cells generate a mutated form of the p53 protein, expression of p53-inducible gene *p21* was significantly lover in DLD1 cells compared to LoVo cells (Fig. S3).

The expression of *gper1* and *tp53* showed a significant correlation in males (A) but not in females (B) undergoing surgery for CRC treatment (Fig. S4) (TCGA, Colorectal Adenocarcinoma, Nature 2012).

Discussion

The relative ratio of E2 receptors in CRC tissue undergoes remodelling. While the expression of nuclear E2 receptors decreases in cancer tissue compared to adjacent tissue, the opposite pattern is observed in Gper1 mRNA expression. Therefore, Gper1 is by far the most abundant E2 receptor in the CRC tumour followed by the ESR2 and very low

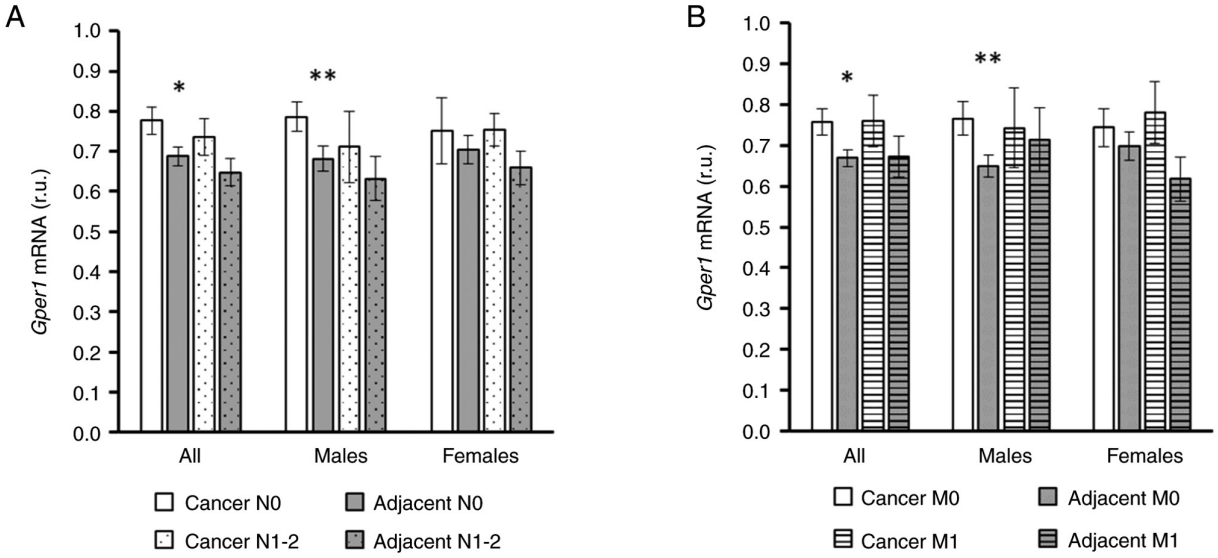

Figure 4. Comparison of Gper1 mRNA expression in colorectal cancer in relation to clinic-pathological characteristics of patients. Gper1 mRNA expression in patients with (A) nodal involvement (B) the presence of distant metastases. White columns show expression in cancer, and grey columns display averaged mRNA levels in the corresponding proximal and distal tissues. Dots are used to show the results for the sub-cohort with nodal involvement, and horizontal hatching indicates the presence of distant metastases. Data are shown as mean ± standard error of the mean. *P<0.05 and **P<0.01 cancer N0/M0 tissue compared to adjacent N0/M0 tissue. r.u., relative units; Gper1, G protein-coupled oestrogen receptor 1.

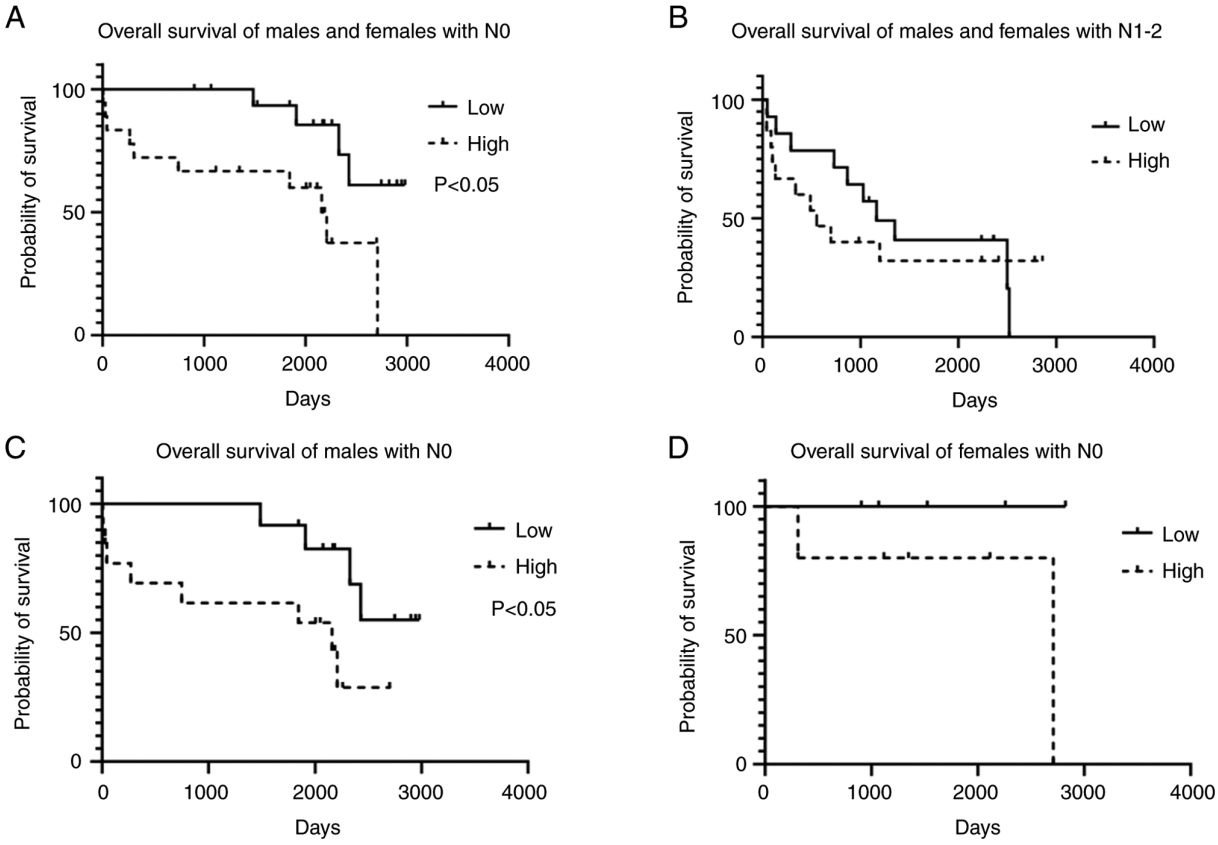

Figure 5. Association of overall survival and *Gper1* expression evaluated by Kaplan-Meier. Survival curve for the (A) whole cohort without lymph node involvement (N0); (B) whole cohort with lymph node metastases (N1-2); (C) male patients without lymph node involvement; and (D) females without lymph node involvement. The solid line indicates low Gper1 expression (≤ median), and the dotted line indicates high Gper1 expression (> median). Gper1, G protein-coupled oestrogen receptor 1.

ESR1 mRNA expression. The increase in *gper1* expression in tumours is most pronounced in males in the lower stages of disease. A higher expression of Gper1 mRNA was associated with worse survival in the whole cohort and in males without metastases in nodes. This dependency was not detected in males with nodal involvement and females.

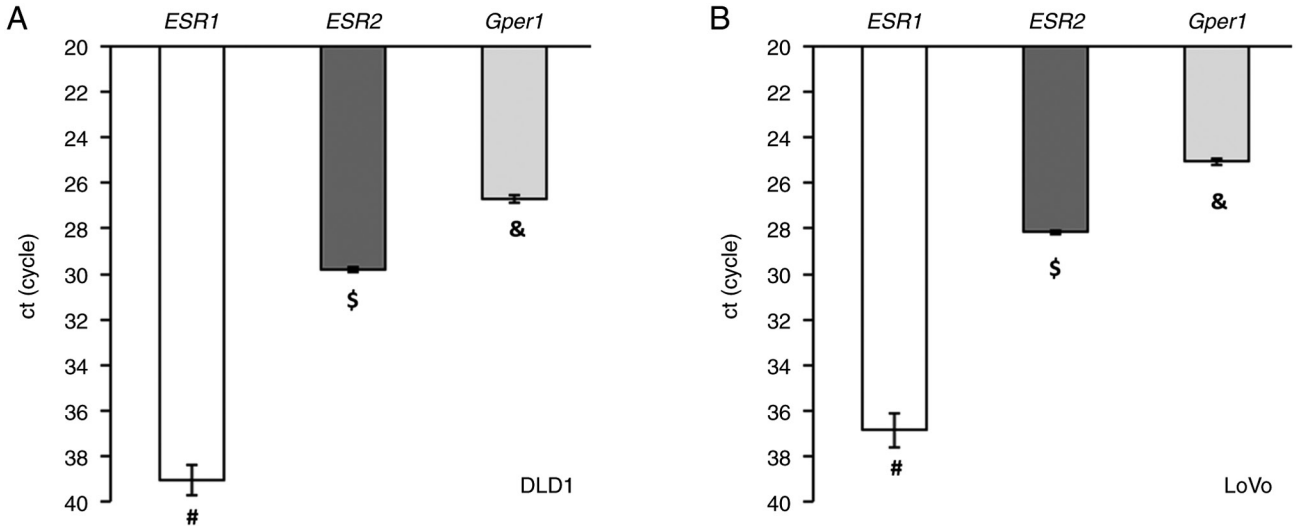

Figure 6. Relative expression of Gper1, ESR1 and ESR2 receptors. mRNA expression of E2 receptors was measured in (A) DLD1 and (B) LoVo colorectal cell lines. Ct=the PCR cycle number at which the fluorescence reached the threshold in the amplification. The threshold was set at 1 in all PCR assays. Columns labelled with different labels are significantly different by ANOVA followed by Tukey's post hoc test at $P<0.05$. $^{\#}P<0.05$ compared with \$ and &. $^{\$}P<0.05$ compared with # and &; $^{\&}P<0.05$ compared with # and \$. ESR, oestrogen receptor; Gper1, G protein-coupled oestrogen receptor 1; E2, 17β-oestradiol.

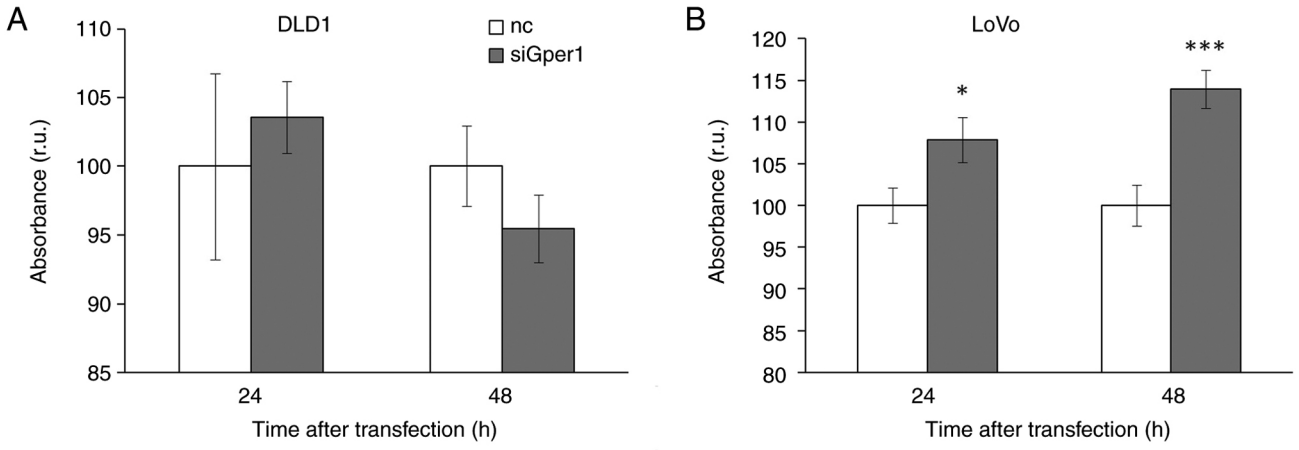

Figure 7. Effect of Gper1-silencing on the rate of metabolism. Metabolism was measured in (A) DLD1 and (B) LoVo cells by the MTS test. White columns display results from the control group, and grey columns show formazan production in Gper1-silenced cells. Results are presented as mean ± standard error of the mean. $^{*}P<0.05$ and $^{***}P<0.001$ compared with the nc group. r.u., relative units; nc, negative control; si, short interfering; Gper1, G protein-coupled oestrogen receptor 1.

We suppose that sex-dependent differences in E2 receptor expression can contribute to sex-dependent features of CRC progression.

In accordance with our data, Gilligan *et al* (56) showed an increase in Gper1 expression in CRC tissue compared to adjacent tissue. Several factors can induce an increase in Gper1 mRNA levels in cancer tissue. Firstly, Gper1 belongs to HIF-1 targeted genes that are expressed in response to hypoxia (69). The stimulatory effect of hypoxia on Gper1 mRNA expression was also demonstrated in the colon cancer cell line HT-29 and the rectal cancer cell line C80 (53). The oncogenic role of Gper1 was implicated as it was demonstrated that Gper1 cooperated with HIF-1α in the activation of VEGF expression in hypoxia (26,70). In line with this finding, we detected a positive correlation between Gper1 and VEGFA mRNA expression in cancer but not in the adjacent tissues.

We observed a decrease in ESR2 mRNA expression in CRC tumours compared to proximal and distal parts of the gut, which is consistent with previous studies (71-77) that reported lower ESR2 expression in CRC tissue compared to adjacent tissues at the protein and mRNA levels.

According to our results, the expression of ESR1 mRNA is lower in cancer tissue compared to adjacent tissues, and this difference is more pronounced in males than in females. Previously, a decrease was observed in ESR1 expression in colorectal cancer tissue, which was attributed to CpG island methylation (78). No significant differences between cancer and adjacent tissue have also been reported (38). According to Jiang *et al* (79), the expression of the dominant ESR1 isoform does not differ in its mRNA levels between tumour and matched normal tissues. However, the ER-α46 isoform that shares most of its sequence with the dominant isoform

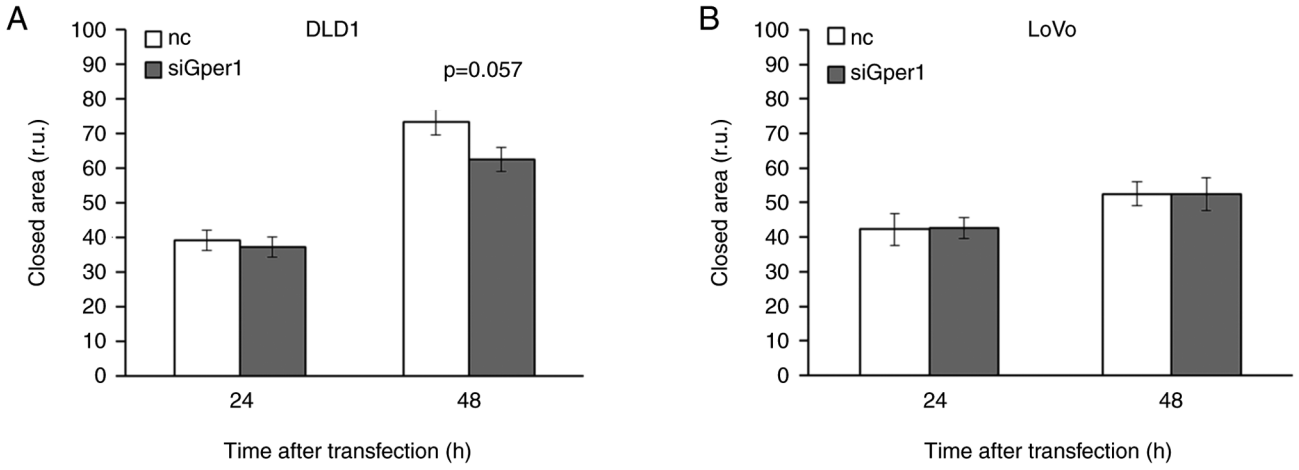

Figure 8. Effect of Gper1-silencing on the migration. Migration was measured in (A) DLD1 and (B) LoVo cells by the scratch assay. White columns show results from the control group, and grey columns display the rate of wound closure of Gper1-silenced cells. Results are presented as mean ± standard error of the mean. r.u., relative units; Gper1, G protein-coupled oestrogen receptor 1; si, short interfering; nc, negative control.

was down-regulated in cancer tissue compared to adjacent tissue. On the other hand, an increase in *esr1* mRNA in cancer compared to adjacent healthy tissue has been reported lately (80). Although data referring to ESR1 expression in CRC seem to be inconclusive, there is a consensus on the decrease in nuclear ESR2 and increase in Gper1 expression in cancer compared to adjacent tissue.

The expression of all three types of receptors in one set of samples has rarely been studied. There is a strong evidence that the expression of ESR2 in the gut is more abundant compared to that of ESR1 (71,76,81). The assumption that ESR2 is the predominant form of the E2 receptor in the gut can, to some extent, be caused by later identification of the Gper1 receptor compared to nuclear E2 receptors (82). Previously, it has been shown that the expression of Gper1 mRNA is more abundant compared to that of both ESR1 and ESR2 in the rat colon (40). According to datasets available from the Human Protein Atlas, the expression of E2 receptors is Gper1>ESR2>>ESR1 (47), which is in line with Harvey and Harvey (83).

The density of E2 receptors changes in CRC tumour tissue. In CRC the abundance of E2 receptors shows the pattern Gper1>>ESR2>ESR1. The order of E2 receptor density in the healthy colon is similar, but there is an abrupt decrease in ESR2 and an increase in Gper1 expression in the tumour compared to the healthy gut. Changes in the abundance of E2 receptors in CRC are more pronounced in males than in females.

As CRC occurs to a lesser extent in females compared to males (83), and sex-dependent differences in nuclear E2 receptor expression have been revealed (72,74-76,84), female sex hormones were suggested to be involved in the regulation of CRC progression (85). Beneficial effects of E2 mediated via ESR2 receptors have been convincingly demonstrated (76,86); however, contradictory reports are available concerning the role of *Gper1* in CRC progression (34,53,56,83). Similarly, little is known about sex-related differences in Gper1 expression, although they have been implied (70,83). According to our results, there are more pronounced differences in Gper1 expression in cancer tissue compared to adjacent tissue in males than in females. This difference is noticeable mainly in males without nodal metastases.

In our cohort poor survival correlated with high Gper1 mRNA expression more significantly in males without nodal involvement than in males in higher stages of disease or females. These results are in accordance with Gilligan *et al* (56), who reported an association of worse survival in patients with high Gper1 expression. Information about the correlation in males and females separately was not provided. Bustos *et al* (53) reported sex-dependent differences in relapse-free survival and Gper1 expression; however, as we do not have data allowing this type of analysis, a comparison was not possible. Our results are in accordance with the Human Protein Atlas (47), according to which there is a stronger association between Gper1 and survival in males compared to females and worse survival in patients with high Gper1 expression. The association reaches significance only in males in stage I-II and not in patients with higher stages of disease. Interestingly, when the survival of males and females together are correlated with Gper1 expression, the association does not reach significance, which also implicates a sex-specific dichotomy in regulation.

There are reports implicating Gper1 as a tumour suppressor in pancreatic cancer, melanoma and adrenocortical cancer and as a tumour promoter in glioblastoma and endometrial and ovarian cancers, whereas in the case of lung, prostate, breast and colorectal cancers, information about the regulatory impact of Gper1 is inconclusive (17,18,34,87,88). Gper1-mediated effects are dependent on the biological context. Our results imply that intracellular conditions are vital for the interpretation of Gper1 signalling, even at the level of one type of solid cancer.

Gper1 is known to induce signalisation mediated via the EGFR receptor with tyrosine kinase activity (17,89,90). A deregulated EGFR pathway has been associated with the progression of many types of cancer, including CRC (91,92). Therefore, several tyrosine kinase inhibitors and EFGR antibodies have been developed and introduced into clinical practice (92). Inhibition of EGFR signalling was shown to benefit patients diagnosed with CRC, and the EFGR monoclonal antibodies Cetuximab and Panitumumab are now routinely used to treat this type of cancer (93). *In vitro*

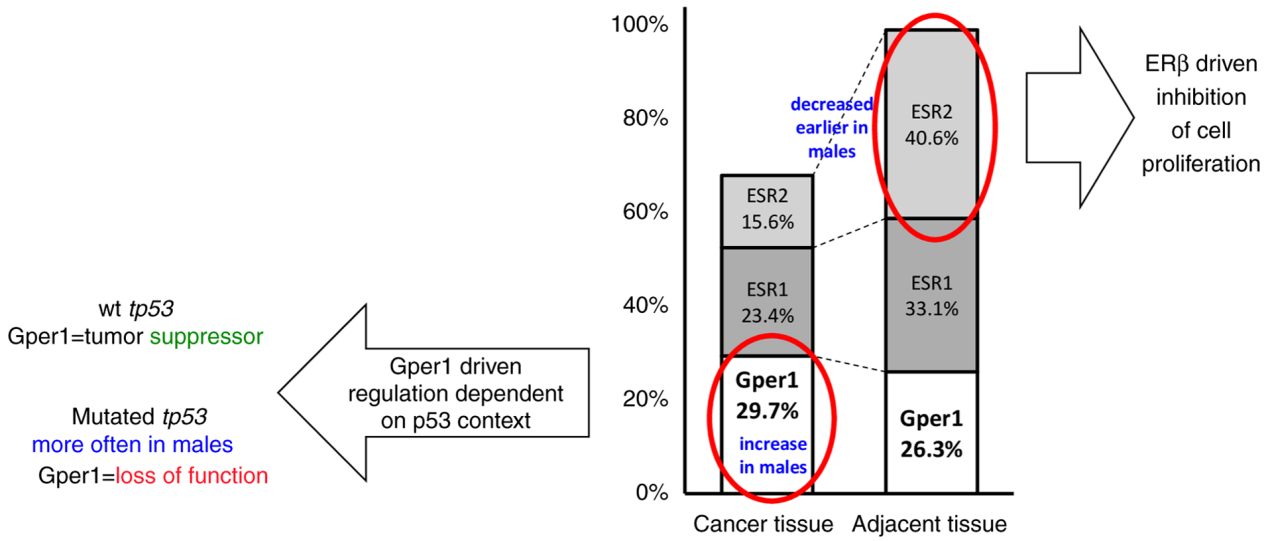

Figure 9. Possible mechanism of how Gper1 signalling contributes to sex-dependent differences in CRC. In cancer tissue, the expression of ESR2, which inhibits CRC progression, decreases in the earlier stages of disease in males compared to females (74). E2 signalling is further modulated by an increase in Gper1 expression in cancer tissue in males in the early stages of disease, which is not observed in females. Therefore, in the CRC tumour Gper1-mediated regulation strongly influences how E2 signalling will be interpreted by the cell. Gper1 demonstrates an oncostatic effect in the LoVo cell line carrying wild-type *tp53*, which is not observed in DLD1 cells with mutated *tp53*. It was concluded that Gper1 functioning is dependent on the p53 intracellular context. As the mutated form of the gene is more frequent in males compared to females, and the two most important gut receptors, Gper1 and ESR2, show a sex-dependent pattern of expression, we suppose that sex-dependent interpretation of E2 signalling and the availability of a functional p53 pathway contribute cooperatively to the differences in CRC progression observed between sexes. ESR, oestrogen receptor; Gper1, G protein-coupled oestrogen receptor 1; E2, 17β-oestradiol; CRC, colorectal cancer; wt, wild-type.

studies elucidated the molecular mechanisms of cancer inhibition caused by EFGR repression in several cancer cell lines, including DLD1 (94-97). Gper1 signalling mediated via the Gs-coupled cAMP/PKA/CREB pathway has also been shown to promote cell proliferation (98). Therefore, as Gper1 is known to induce both EGFR- and cAMP-mediated regulation and is reciprocally involved in VEGFA release, presumably, an oncogenic role of Gper1 might be expected in CRC.

However, signalling mediated by Gper1 is highly complex. The same group that discovered the connection between Gper1 and EGFR two years later revealed that Gper1 inhibits EGFR signalling via the cAMP/PKA pathway by Raf-1 inactivation (89). To further elucidate the ambiguous effects of Gper1 on CRC progression, we investigated the effect of Gper1 silencing in CRC cell lines LoVo and DLD1.

According to our results, the distribution of E2 receptors in the CRC cell lines DLD1 and LoVo resembles that observed in human tissues: the highest mRNA expression is that of *gper1*, and the expression of ESR1 is nearly undetectable. Gper1 silencing in the DLD1 cell line, which expresses mutated *tp53*, inhibited the wound closure, implicating the oncogenic potential of Gper1. By contrast, the decrease in Gper1 availability caused an increase in the metabolic rate in LoVo cells that preserved p53 functionality (99).

The p53 protein, encoded by the gene *tp53*, is a well-known tumour suppressor that inhibits cell cycle progression and initiates DNA repair and/or apoptosis and autophagy, in response to DNA damage, hypoxia, nutrition deficiency, oxidative stress and some hormones. Cell cycle inhibition is executed by induction of p21 expression. p21 inhibits the activity of several types of cyclin/CDK complexes that release repression of retinoblastoma protein (RB). As cyclin/CDK complexes are inactivated by p21 and cannot phosphorylate RB, hypophosphorylated RB inhibits E2F-induced transcription, which is necessary for cell proliferation (100,101).

The p53 protein is frequently mutated in many types of cancer, including CRC. According to TCGA, more than 50% of CRC patients carry a mutated form of *tp53*. The presence of a mutated form of p53 is accompanied by worse survival than that of wild-type *tp53*. Interestingly, *tp53* mutations are more frequent in males compared to females, which may also contribute to the higher CRC incidence in males compared to females (66).

We hypothesize that the signalling pathway downstream of p53 influences the outcome of Gper1 signalling. Previously, it was shown that Gper1 expression in the triple-negative breast cancer cell lines MDA-MB-231 and MDA-MB-468, which express a mutated form of *tp53*, had been induced by γ-radiation, whereas in MCF-7 cells expressing wild-type *tp53*, the opposite pattern was observed. Therefore, a tumour suppressor role dependent on p53 was attributed to Gper1 (102). Our results agree with this statement as Gper1 was associated with oncostatic functions in LoVo but not in DLD1 cell lines. Previously, the oncogenic potential of Gper1 has been described in the CRC cell lines HT-29, DLD1, COLO205 and SW480 (53,55) carrying mutations in the *tp53* gene (66,103-106).

Although there are reports implicating a functional relationship between p53 and oestradiol, the exact mechanism has either not been completely elucidated or, more likely, comprises several ways by which E2 and p53 signalling interfere (107). These interferences can differ in a tissue-dependent manner, e.g. E2 administration decreases p53 expression in lung cancer cells by induction of methyltransferase 1 expression, which increases methylation of the *tp53* promoter (108). On the other hand, a protective role of E2 in non-malignant colonocytes executed via p53-mediated regulation has been implicated (109).

The effects of E2 on p53 expression have been previously investigated mainly with respect to the effects of ERα on breast cancer progression. ERα interacts with *tp53* and influences its expression. Most of studies report an increase in *tp53* expression in response to ERα binding (107,110-113). On the other hand, there are also results implicating ERα-induced suppression of p53-regulated gene expression (114,115). However, in DLD1 cells, ESR1 expression is nearly undetectable; therefore, we do not suppose that a substantial increase in p53 expression can be induced via ERα. The results are inconclusive with respect to ERβ signalling and p53 expression. Although induction of p53 activity in response to ERβ activation has been reported (107,113), a stimulatory effect of ERβ interacting with the *tp53* promoter on *tp53* expression has not been detected (111).

In silico analysis showed a positive correlation between Gper1 and *tp53* expression in CRC tissues of males (TCGA) (116). The E2 receptor ligands bisphenol A (117) and G-1 (102,118,119) have also been shown to induce *tp53* expression. Silencing of Gper1 caused a decrease in *tp53* expression in uveal melanoma cell lines (88). The exact mechanism of how Gper1 executes its effect on p53 transcription is unknown. A cAMP response element (CREB) was identified in the human *tp53* gene (120,121) and CREB binding protein plays a key role in p53 activation (122). However, whether Gper1 executes its stimulatory effect on *tp53* expression via this region remains to be elucidated.

To conclude, the expression of E2 receptors in healthy tissue follows the descending order Gper1>ESR2>>ESR1 with nearly undetectable expression of ESR1 mRNA. In CRC patients the ratio of receptors changes more in males than in females; the expression of Gper1 mRNA increases while the expression of ESR2 and ESR1 decreases, resulting in the descending order Gper1>>ESR2>ESR1. Therefore, under conditions of cancer progression, most E2 signalling is mediated via the membrane E2 receptor Gper1. High expression of Gper1 is associated with worse survival in males without nodal involvement in comparison with other subcohorts (Fig. 9).

The relative expression of E2 receptors in DLD1 and LoVo cells is similar to that observed in human CRC tumours. In LoVo cells with wild-type *tp53*, a tumour suppressor effect of Gper1 was observed. This effect was not detected in DLD1 cells with the mutated form of *tp53*. We suppose that Gper1 activates the EFGR/RAS oncogenic pathway as well as the p53 pathway. In case the p53 pathway is not functional, the oncogenic potential of Gper1 overwhelms its tumour suppressor effects. As the frequency of *tp53* mutations as well as changes in Gper1 expression are more robust in males compared to females, we suppose that these effects can contribute to sex-dependent differences in CRC incidence.

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Availability of data and materials

The datasets are available from the corresponding author on reasonable request.

Authors' contributions

IH and RR designed and administered the human study. IH and RR confirm the authenticity of all the raw data. RR obtained samples and organized their transport to the laboratory. IH performed analysis of human samples. IH and DV performed cell culture experiments. IH performed *in silico* analysis, interpreted results, wrote the manuscript and prepared the figures. IH, RR and DV reviewed and revised the manuscript for the scientific content. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The design of the study describing the sampling of human tissues was approved by the Ethics Committee of the Comenius University in Bratislava (approval no. ECH 19001). All patients included in the study agreed to sign an informed consent. The manuscript does not contain experiments using animals or embryonic cells.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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