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Utility of G protein-coupled receptor 35 expression for predicting outcome in colon cancer

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Abstract

The utility of mRNA and protein determinations of G protein-coupled receptor 35, that is, GPR35a (GPR35 V1) and GPR35b (GPR35 V2/3), as indicators of outcome for colon cancer patients after curative surgery was investigated. Expression levels of V1 and V2/3 GPR35, carcinoembryonic antigen and CXCL17 mRNAs were assessed in primary tumours and regional lymph nodes of 121 colon cancer patients (stage I–IV), colon cancer cell lines and control colon epithelial cells using real-time quantitative reverse transcriptase-polymerase chain reaction. Expression of G protein-coupled receptor 35 was investigated by two-colour immunohistochemistry and immunomorphometry. GPR35 V2/3 mRNA, but not V1 mRNA, was expressed in colon cancer cell lines, primary colon tumours and control colon epithelial cells. Haematoxylin and eosin positive (H&E(+)), but not H&E(–), lymph nodes expressed high levels of GPR35 V2/3 mRNA ($P < 0.0001$). GPR35b and carcinoembryonic antigen proteins were simultaneously expressed in many colon cancer tumour cells. Kaplan–Meier and hazard ratio analysis revealed that patients with lymph nodes expressing high levels of GPR35 V2/3 mRNA and, in particular, in the group of patients with lymph nodes also expressing carcinoembryonic antigen mRNA, had a short disease-free survival time, 67 months versus 122 months at 12-year follow-up (difference: 55 months, $P = 0.001$; hazard ratio: 3.6, $P = 0.002$). In conclusion, high level expression of G protein-coupled receptor 35 V2/3 mRNA in regional lymph nodes of colon cancer patients is a sign of poor prognosis.

Keywords

GPR35b, GPR35 V1 mRNA, GPR35 V2/3 mRNA, CXCL17 mRNA, CEA, colon cancer, qRT-PCR, immunohistochemistry

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Introduction

Lymph node metastasis, as detected by haematoxylin–eosin staining, is still the most important prognostic characteristic of colorectal cancer (CRC) patients resected for cure.[1–3] Despite current treatment modalities, a large fraction of the patients (20%–50%) will recur.[4] It is therefore a very important task to identify biomarkers that detect all tumour cells in lymph nodes and ideally biomarkers that give information about the potential of the tumour cells to form distant metastasis.

G protein-coupled receptor 35 (GPR35) is member of a superfamily of transmembrane-spanning receptor

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proteins of which a large fraction serves as therapeutic targets.[5,6] No high affinity endogenous ligand for GPR35 is yet known. However, several low potency endogenous ligands, for example, kynurenic acid and the chemokine CXCL17 have been described.[5,7,8] Most recently, GPR35 was found to be a key promotor of sodium-potassium adenosine triphosphatase (Na/K-ATPase) ion pumping function and Src signalling activity without engaging a cognate ligand.[9] The GPR35 gene can be transcribed into three variants V1, V2 and V3, of which V1 encodes the shortest transcript, giving rise to the smallest isoform GPR35a. The other two variants (V2 and V3) both give rise to the slightly larger isoform GPR35b.[10] The difference between GPR35a and GPR35b is that the latter has a 32 amino acid residues extension at the N-terminal end, making the extracellular domain (EC1) longer. Both GPR35a and GPR35b are functionally active.[11,12] Studies at the mRNA level reveal that GPR35 is expressed at highest levels in small intestine followed by stomach; colon; rectum; and in some abdominal, myeloid and lymphoid cancer cell lines.[10,13–15]

In this study, we investigated whether determination of GRP35 levels in lymph nodes of colon cancer (CC) patients will be of value for predicting the formation of distant metastasis. The results show that mRNA analysis of the splice variant GPR35 V2/3 in lymph nodes of CC patients gives clinically useful information of patient survival, independently as well as in combination with carcinoembryonic antigen (CEA) mRNA.

Materials and methods

Clinical material

Primary tumour specimens were retrieved from 79 CC patients (median age: 74 years, range: 41–88 years; 45 women and 34 men). In total, 17 patients were in stage I (T1-2N0M0), 32 in stage II (T3-4N0M0), 23 in stage III (anyTN1-2M0) and 7 in stage IV (anyTanyNM1). Normal colon tissue specimens were obtained from 39 CC patients (18 women and 21 men; median age: 70 years, range: 57–85 years) and taken distant to any macroscopically detectable lesions. Tissue specimens were collected, processed and stored as described earlier.[16] Epithelial cells were isolated from resected normal colon tissues as previously described.[17,18]

Lymph nodes were dissected from surgically removed specimens and bisected. One half of each node was fixed for routine haematoxylin and eosin (H&E) staining and processed in clinical routine by independent hospital pathologists, and the other half was subjected to RNA extraction or processed for immunohistochemistry. They were collected from 131 CC patients (median age: 70 years, range: 41–88 years; 71 women and 60 men): 74 lymph nodes were from 24 stage I patients, 193 nodes from 54 stage II patients, 99 nodes from 44 stage III patients and 31 nodes from 9 stage IV patients. A total of 31 lymph nodes were judged positive by routine histopathology (H&E(+)) and 367 lymph nodes were H&E(–). Control nodes (n = 78) were from 13 patients (median age: 23 years, range: 9–32 years; 3 women and 10 men): 11 had ulcerative colitis, 1 had Crohn's disease and 1 had lipoma.

Cell lines

The following human cell lines were used: the CC cell lines LS174T, HT29, T84, HCT8 and Caco2; the T cell line Jurkat; the B cell lines CNB6 and KR4; the monocyte cell line U937; the endothelial cell line human umbilical vein endothelial cell (HUVEC); and primary foreskin fibroblast cells FSU. Culture conditions were as described.[17,19]

Real-time quantitative reverse transcriptase-polymerase chain reaction

Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) assays for GPR35 V1 (NM_005301.3) and both GPR35 V2 (NM_00119 5381.1) and V3 (NM_001195382.1) (V2/3) mRNAs were constructed. Primers were placed in different exons and a reporter dye-labelled probe over the exon boundary. The 3′-primer was used as template for reverse transcription and Tth DNA polymerase (cat. no. 04991885001; Roche, Mannheim, Germany) was used for reverse transcription and amplification. The primer and probe sequences were as follows: 5′-primer 5′-GCCCTTCTCAGACAGCCACT-3′, 3′-primer 5′-CCACAGGTGTTGTAGGTGCC-3′, and probe 5′-TGCCTGCAGGACCAT-3′ for GPR35 V1 mRNA and 5′-primer 5′-GTCCCCACTCCACACCGT-3′, 3′-primer 5′-GAGGTCGCTGGAGCCACA-3′, and probe 5′-CTTGCGTCTCTCTGACCATGAATGG CA-3′ for GPR35 V2/3 mRNA. The qRT-PCR profiles were 63°C for 15 min, 95°C for 1 min followed by 45 cycles of 95°C for 15 s and 59°C for 1 min for GPR35 V1 and 63°C for 15 min, 95°C for 1 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min for GPR35 V2/3. Custom synthesised RNA oligonucleotides (Dharmacon, Lafayette, CO, USA) of the amplicon sequence in the respective qRT-PCR assay constituted RNA copy standards that were included at concentrations from 10^3 to 10^9 copies/μL in each qRT-PCR run. mRNA concentrations in unknown samples were determined from the standard curve. qRT-PCR assays for CEA and CXCL17 mRNAs were described earlier.[16,20] Concentrations of 18S ribosomal RNA (rRNA) were determined using real-time qRT-PCR (Applied Biosystems, Foster City, CA, USA) as

described.[21] mRNA concentrations were normalised to the 18S rRNA concentration in the same sample and expressed as mRNA copies/18S rRNA unit. All determinations were done in triplicate.

Antibodies and substrate

Anti-human GPR35 (rabbit IgG; ab188949, Abcam, Cambridge, UK); anti-CEA (mouse IgG1; Dako, Glostrup, Denmark); anti-mouse Ig ImmPress and anti-rabbit Ig ImmPress reagent kits (Vector Laboratories, Burlingame, CA, USA); mouse and rabbit IgG (Dako); 3,3′-diaminobenzidine (DAB; Vector Laboratories); fluorescein isothiocyanate (FITC)-conjugated anti-epithelial cell adhesion molecule (anti-EPCAM) mAb BerEP4 (F0860; Dako); Alexa Fluor 488-conjugated rabbit anti-Phospho-SRC (Tyr419) (44-660A1, Invitrogen, Carlsbad, CA, USA); Alexa Fluor 555-conjugated goat anti-rabbit IgG (ab150078, Abcam); Alexa Fluor 488-conjugated goat anti-rabbit IgG (A11008, Invitrogen) and FITC-conjugated anti-mouse (F0313; Dako).

Immunohistochemistry and immunomorphometry

As described earlier,[22,23] the sections were fixed with 4% paraformaldehyde, incubated with primary antibodies followed by incubation with ImmPress peroxidase-secondary antibody conjugate, and bound antibodies revealed by incubation with the peroxidase substrate DAB. Quantification of number positive cells was performed according to Weibel.[24]

Two-colour immunofluorescence

Tissue sections were incubated with anti-GPR35 antibody, followed by the Alexa Fluor 555-conjugated goat anti-rabbit Ig followed by FITC-conjugated BerEP4 or Alexa Fluor 488-conjugated anti-Phospho-SRC antibody. Images were analysed with NIS elements software using a Nikon fluorescence microscope.

Statistical analysis

Statistical significance of differences between primary tumour and normal colon tissue and between H&E(+) and H&E(–) lymph nodes was calculated using two-tailed Mann–Whitney rank sum test. Statistical significance of differences in mRNA levels between control lymph nodes and lymph nodes from different patient groups were analysed using Kruskal–Wallis one-way analysis of variance (ANOVA) test followed by the Dunn's multiple comparison post hoc test. Correlations between mRNA levels were analysed using the non-parametric two-tailed Spearman's rank correlation. The software utilised was Prism 6 (Graphpad Software, San Diego, CA, USA). The SPSS (IBM Corporation, Armonk, NY, USA) was used for statistical analyses of differences between patient groups in disease-free survival time and risk for recurrent disease after surgery according to Kaplan–Meier survival model in combination with the log-rank test and univariate Cox regression analysis. A P value <0.05 was considered to be statistically significant.

Results

GPR35 V2/3 mRNA but not GPR35 V1 mRNA is expressed in CC cell lines and normal colon epithelium

All five CC cell lines expressed high levels of GRP35 V2/3 mRNA, while GRP35 V1 mRNA was barely detected (Table 1). Similarly, isolated colon epithelial cells expressed high levels of GPR35 V2/3 mRNA, while the V1 transcript was not detected. The difference between the two splice variants was $>10^5$ times. Among the non-CC cell lines, only the T cell line Jurkat and the monocyte cell line U937 expressed V2/3 mRNA. Here, the expression level was about 100 times lower than in the CC cell lines. With the exception of the monocyte cell line, only very low levels of the V1 mRNA variant were detected.

GPR35 V2/3 mRNA is expressed in primary tumours from resected CC patients

The median value for GPR35 V2/3 mRNA in the primary CC tumours was 114.2 copies/18S rRNA unit compared to 40.7 copies/18S rRNA unit in the normal colon tissues. The difference in expression levels was statistically significant ($P < 0.0001$; Figure 1(a)). GPR35 V1 was barely detected in CC tumour tissues (Figure 1(b)).

GPR35 V2/3 mRNA is expressed at high levels in H&E(+) lymph nodes of CC patients

Expression levels of GPR35 V2/3 in lymph nodes increased slightly with the TNM stage of the tumour (Figure 1(c)). The difference in the expression was only significant between stages I and IV and between II and IV (Figure 1(c)). A highly significant difference in levels of GPR35 V2/3 between H&E(+) and H&E(–) nodes was observed ($P < 0.0001$; Figure 1(d)). The GPR35 V1 transcript was also assessed. In total, 15 H&E(+) and 15 H&E(–) nodes and 10 control nodes were analysed with no significant difference between the groups ($P > 0.05$).

Table 1. Expression levels of GPR35 V1 and GPR35 V2/3 mRNAs in cell lines of colon cancer (CC), lymphocytes, monocytes, endothelial cells, fibroblasts and normal colon epithelial cells.

Cell line	Type	GPR35 V1[a]	GPR35 V2/3[a]
LS174T	CC cells	0.002	181.7
HT29	CC cells	0.002	339.5
T84	CC cells	0.002	102.9
HCT8	CC cells	0.007	1028.0
Caco2	CC cells	0.002	288.5
Jurkat	T cells	0.01	3.0
CNB6	B cells	0.13	0.5
KR4	B cells	<0.0001	0.1
U937	Monocytes	3.6	2.2
HUVEC	Endothelial cells	<0.0001	0.3
FSU	Fibroblasts	0.01	0.3
Normal colon epithelial cells[b]	Freshly isolated cells	<0.0001 (<0.0001–0.07)[c]	247.5 (110.6–532.3)[c]

HUVEC: human umbilical vein endothelial cell; rRNA: ribosomal RNA.
[a]mRNA copies/18S rRNA unit.
[b]Five patients, two samples per patient.
[c]Median and interquartile range (IQR) from 25th to 75th percentile.

GPR35 V2/3 mRNA levels correlate with CEA and CXCL17 mRNA levels in lymph nodes from CC patients

mRNA levels for CEA and CXCL17 have previously been determined for 385 of the lymph nodes analysed in this study.[16,20] Figure 1(e) shows the GPR35 V2/3 mRNA values in these nodes grouped according to CEA mRNA levels, that is, CEA(–), CEA (int) and CEA (+) (<0.013, 0.013–3.67 and >3.67 copies/18S rRNA unit, respectively).[25,26] Median values of GPR35 V2/3 mRNA were 1.3, 1.6 and 78.3 mRNA copies/unit. The difference between the CEA(+) group and the other two groups was highly significant ($P < 0.0001$). In the following analysis, we used the lymph node with the highest GPR35 V2/3 mRNA value to represent each patient and compared GPR35 V2/3 mRNA with CEA mRNA and CXCL17 mRNA. Results for the entire group of patients and for stage III patients are shown in Figure 2(a)–(d). Highly significant correlations with high r-values were seen for both comparisons, particularly in lymph nodes from stage III patients.

CEA mRNA adds prognostic information to GPR35 V2/3 mRNA in lymph node metastasis of CC patients

The clinical utility of determining GPR35 V2/3 mRNA expression levels in metastatic lymph nodes of CC patients for predicting tumour recurrence was investigated. Hazard risk ratio was calculated using Cox regression analysis. Each patient was represented by the lymph node with the highest level. The patients were divided into five groups according to the 20th, 40th, 60th and 80th percentile of the GPR35 V2/3 mRNA values, corresponding to cut-off values of 1.0, 2.2, 3.5 and 11.7 mRNA copies/unit, respectively. Patients in the 80th percentile group showed a 2.4-fold increased recurrence rate compared to the 20th percentile group when followed for 5 years ($P = 0.07$) and a decreased mean survival time of 10 months ($P = 0.007$) according to Kaplan–Meier analysis (Figure 3(a)).

Adjusting the cut-off level between high and low GPR35 V2/3 mRNA levels, to 9.0 mRNA copies/unit, the difference in recurrence rate between the groups was 3.1-fold at 5 years and 2.6-fold at a follow-up time of 12 years ($P = 0.002$ and $P = 0.007$, respectively). The difference in mean survival time between the two groups was 11 months in 5 years ($P = 0.001$) and 46 months in 12 years ($P = 0.005$; Table 2, Figure 3(b)). These results indicate that high levels of GPR35 V2/3 mRNA are associated with poor prognosis.

Restricting the analysis to those CC patients that in their highest node had CEA mRNA values above the control level, that is, the CEA(int) plus the CEA(+) group, the difference in recurrence rate was found to be 3.5-fold at 5-year follow-up ($P = 0.003$) and 3.6-fold in 12 years ($P = 0.002$) between the two GPR35 V2/3 mRNA groups. The difference in mean survival time between the two groups was 12 months at 5 years ($P = 0.001$) and as large as 55 months at 12-year follow-up ($P = 0.001$; Table 2, Figure 3(c)). These results demonstrate that CEA mRNA analysis adds prognostic information to that of GPR35 V2/3 mRNA analysis.

We investigated whether CXCL17 mRNA determinations add prognostic value to GPR35 V2/3 mRNA analysis. No significant difference in the mean survival time or recurrence rate was found between the CXCL17(+)GPR35(+) and CXCL17(+)GPR35(–) groups (Table 2).

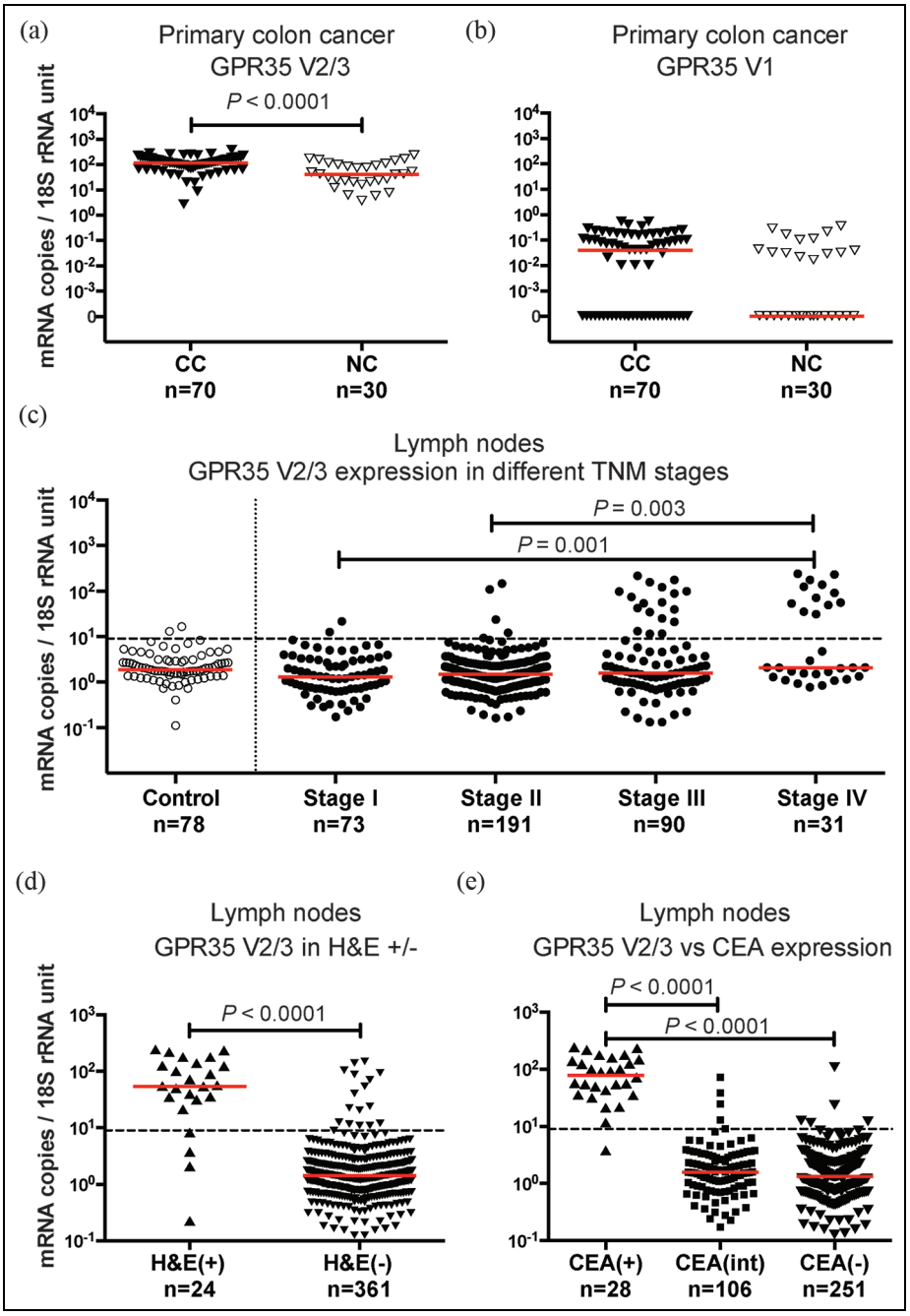

Figure 1. mRNA expression levels of GPR35 V2/3 and GPR35 V1 in primary colon cancer and normal colon tissues and of GPR35 V2/3 in lymph nodes. (a) GPR35 V2/3 and (b) GPR35 V1 mRNA levels in primary colon cancer (CC) and resected normal colon tissue (NC). (c) GPR35 V2/3 mRNA levels in lymph nodes from non-cancerous disease patients (control) and colon cancer patients in different TNM stages (stages I–IV). (d) GPR35 V2/3 mRNA levels in metastatic (H&E(+)) and non-metastatic (H&E(–)) lymph nodes. (e) GPR35 V2/3 mRNA levels in relation to CEA mRNA levels. In (e) all lymph nodes (n = 385) from 121 CC patients were divided into three groups according to their CEA mRNA content; CEA(+) = CEA mRNA levels $>$ 3.67 copies/18S rRNA unit, CEA(int) = intermediate CEA mRNA levels, that is, 0.013−3.67 copies/18S rRNA unit, and CEA(−) = CEA mRNA levels\0.013 copies/18S rRNA unit.

P values were calculated by two-tailed Mann–Whitney U test for comparison between expression levels in (a), (b) and (d) and by Kruskal–Wallis non-parametric ANOVA followed by a post hoc Dunn's test for multiple comparisons in (c) and (e). Statistically significant differences are given. Red horizontal lines indicate medians. The dashed line in (c)–(e) indicates the clinical cut-off for GPR35 V2/3 (9 mRNA copies/18S rRNA unit). (a)–(e) n; number of samples.

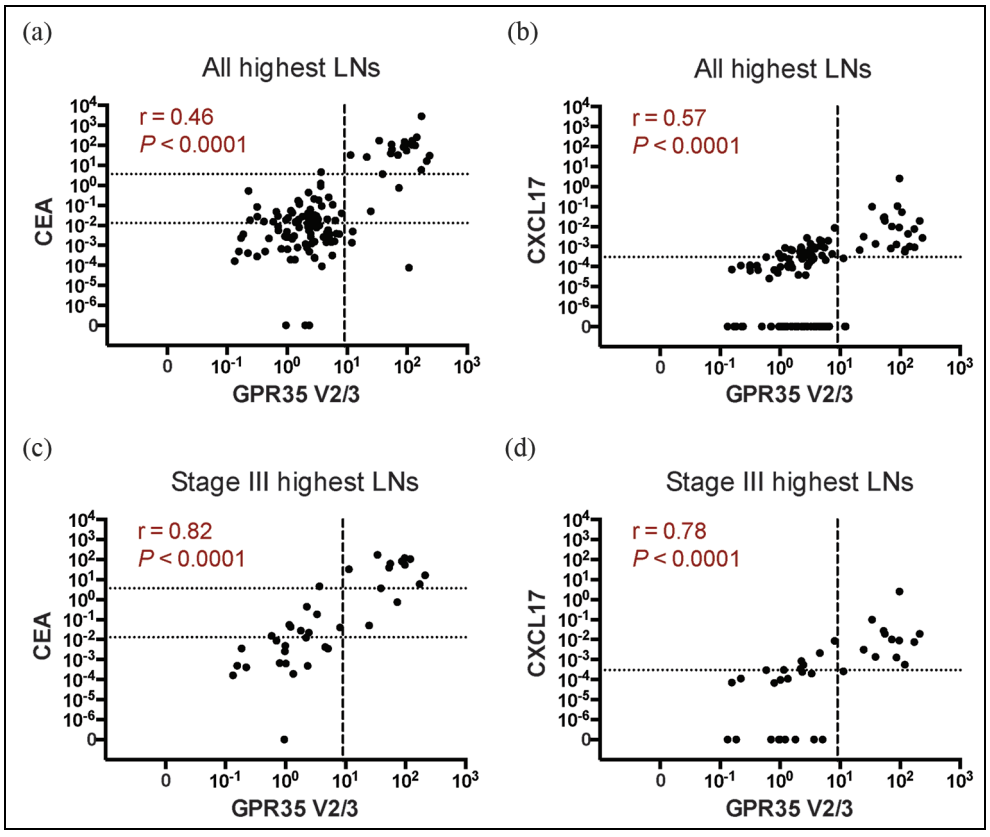

Figure 2. Correlation between mRNA levels of GPR35 V2/3 and mRNA levels of CEA and CXCL17 in the highest lymph node (LN) of each of the CC patients. (a) Correlation between GPR35 V2/3 and CEA mRNA levels in all CC patients. (b) Correlation between GPR35 V2/3 and CXCL17 mRNA levels in all CC patients. (c) Correlation between GPR35 V2/3 and CEA mRNA levels in stage III CC patients. (d) Correlation between GPR35 V2/3 and CXCL17 mRNA levels in stage III CC patients.
mRNA levels are given as mRNA copies/18S rRNA unit. The correlation coefficients (r) and the *P* values were calculated by two-tailed Spearman's rank order correlation test. The dashed line indicates a clinical cut-off value of 9 mRNA copies/18S rRNA unit of GPR35 V2/3. The dotted lines in (a) and (c) indicate a CEA mRNA level of 0.013 mRNA copies/18S rRNA unit, that is, the border between CEA(–) and CEA(int) and the border between CEA(int) and CEA(+), that is, a CEA mRNA level of 3.67 mRNA copies/18S rRNA unit. The dotted line in (b) and (d) indicates the border between CXCL17(–) and CXCL17(+), that is, a CXCL17 mRNA level of 0.0003 mRNA copies/18S rRNA unit.

Risk of recurrence and survival time after surgery are not correlated to GPR35V2/3 mRNA levels in primary tumours

No difference in recurrence risk or survival time was seen in CC patients divided into GPR35(+) and GPR35(–) patients using the median mRNA of CC tumours as cut-off (median: 114 mRNA copies/18S rRNA unit).

GPR35 is expressed in primary and secondary tumours of CC patients at the protein level

To establish that tumour cells indeed expressed GPR35 protein, we performed two-colour immunofluorescence experiments using anti-GPR35 (Figure 4(a)) and anti-EPCAM mAb BerEP4 (Figure 4(b)). The overlay picture (Figure 4(c)) demonstrated that almost all of the primary tumour cells expressed both markers. The consecutive-section-staining method was used to investigate GPR35 and CEA expression in lymph node metastasis and primary CC tumours. A large number of tumour cells were positive for CEA (Figure 5(b) and (f)). Staining with anti-GPR35 demonstrated that a number of the CEA-positive tumour cells also express GPR35 (Figure 5(a) and (e)). A large group of metastatic tumour cells in a H&E(+) lymph node expressed CEA (Figure 5(d)). Several of these cells also expressed GPR35 (Figure 5(c) and (g)).

Only few cells in the normal colon expressed GPR35 (Supplemental Figure S1A and S1B). In contrast, GPR35 expression in primary tumours was relatively high. The difference was statistically highly significant (Supplemental Figure S1A). Tumour stromal areas and lamina propria of normal colon contained only few positive cells (Supplemental Figure S1B). In total, 8% of the cells in H&E(+) lymph node metastasis of CC tumours were GPR35-positive, while 3% of the cells in

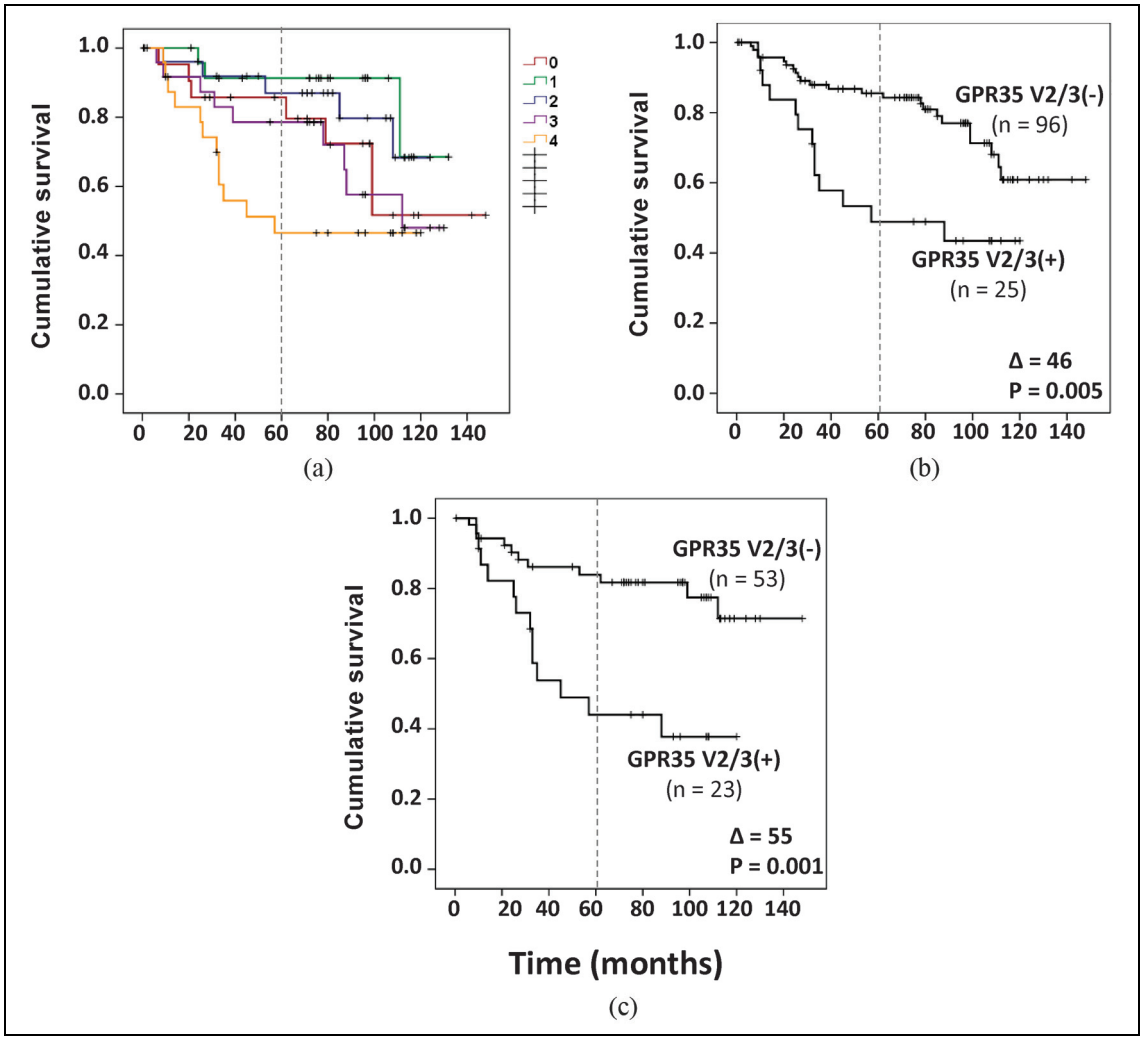

Figure 3. (a) Kaplan–Meier cumulative survival curves for CC patients divided into the five groups: 0%–20% (red), 21%–40% (green), 41%–60% (blue), 61%–80% (lilac) and 81%–100% (yellow) according to their GPR35 V2/3 mRNA expression values; (b) Kaplan–Meier cumulative survival curves for CC patients divided into the two groups, GPR35(+) and GPR35(–), according to the 70th percentile of the GPR35 V2/3 mRNA value (=9 mRNA copies/18S rRNA unit); (c) Kaplan–Meier cumulative survival curves of GPR35(+) and GPR35(–) patients in the CEA(+) plus CEA(int) subgroup of CC patients.
n: number of patients in the respective group.
Differences in postoperative survival time between groups are given as a Δ-value in months and statistical significance as a *P* value.

the surrounding area of the H&E(+) node were positive. The corresponding numbers for a H&E(–) node was 0.5% (Supplemental Figure S1C), which was significantly lower ($P = 0.002$ and $P = 0.05$, respectively).

GPR35-positive CC tumour cells express activated Src

To determine whether GPR35-positive tumour cells are engaged in the Src signalling, a two-colour immunofluorescence experiment using anti-GPR35 (Figure 4(e)) and anti-Phospho-SRC (Figure 4(f)) was performed. The overlay pictures (Figure 4(g)) demonstrated phosphorylated Src in a large fraction of the tumour cells.

Discussion

To the best of our knowledge, this is the first study investigating GPR35 expression in CC primary tumours and lymph node metastases at both mRNA and protein levels. We developed two qRT-PCR assays for detecting GPR35 mRNA transcripts using copy standards. A very high expression level of GPR35 V2/3 mRNA was seen in CC cell lines, while the V1 transcript was hardly detected. Isolated normal colon epithelial cells also expressed GPR35 V2/3 mRNA at high levels but no detectable GPR35 V1. Expression levels in CC cell lines and isolated normal colon epithelial cells were similar. A small, but statistically

Table 2. Comparative analysis of average survival time after surgery and risk for recurrence of disease of CC patients with GPR35(+) and GPR35(–) lymph nodes.

Patient group	Category	5-year follow-up after surgery					12-year follow-up after surgery				
		Disease-free survival			Risk for recurrence		Disease-free survival			Risk for recurrence	
		Average[a] (months)	Difference (months)	*P* value	Hazard ratio (95% CI)[b]	*P* value	Average[a] (months)	Difference (months)	*P* value	Hazard ratio (95% CI)[b]	*P* value
All CC patients	GPR35(–)	54					117				
	GPR35(+)	43	11	0.001	3.1 (1.5–6.2)	0.002	71	46	0.005	2.6 (1.3–5.1)	0.007
CEA(int) plus CEA(+) group[c]	GPR35(–)	54					122				
	GPR35(+)	42	12	0.001	3.5 (1.5–7.8)	0.003	67	55	0.001	3.6 (1.6–8.1)	0.002
CXCL17(+) group[d]	GPR35(–)	52					100				
	GPR35(+)	43	9	0.1	2.0 (0.9–4.5)	0.1	71	29	0.1	2.0 (0.9–4.3)	0.1

CC: colon cancer; CEA: carcinoembryonic antigen; GPR35: G protein-coupled receptor 35; rRNA: ribosomal RNA.
[a]Mean survival time after surgery for CC patients as calculated by cumulative survival analysis according to Kaplan–Meier.
[b]Hazard ratio with 95% confidence interval (CI) for CC patinets as calculated according to univariate COX regression analysis.
[c]CEA(int) plus CEA(+) group: CEA mRNA levels in the highest lymph node higher than the highest level of control lymph nodes, that is, 0.013 mRNA copies/18S rRNA unit.
[d]CXCL17(+) group: CXCL17 mRNA levels in the highest lymph node $\geqslant$ 0.0003 mRNA copies/18S rRNA unit.

significant, difference was seen between primary CC tumours and normal colon tissues, with CC tumours expressing higher levels. However, high levels of GPR35 V2/3 mRNA in primary tumours did not correlate with higher risk of recurrence. Therefore, it appears that GPR35 analysis at the mRNA level in primary tumours is not clinically useful to predict disease recurrence. At the protein level, GPR35-positive cells were more frequent in primary CC tumours than in controls. This apparent contradiction between mRNA and protein determinations of GPR35 cannot be explained at present. Possibly, the anti-GPR35 antibody, which is directed against an intracellular loop of the GPR35 protein, reacts with an epitope that normally is hidden by an interacting protein but at least partly accessible in CC tumours. Alternatively, GPR35b protein accumulates in tumour tissue due to defective cellular mechanisms.

Contrary to the findings in primary CC tumours, GPR35 V2/3 mRNA was an excellent biomarker to predict disease recurrence if used for analysis of lymph nodes, particularly when combined with CEA mRNA analysis. Expression of high levels of GPR35 V2/3 mRNA in lymph nodes is indeed a sign of poor prognosis. Hence, recurrence rate was increased 3.1-fold in CC patients with high levels of GPR35 V2/3 mRNA compared with patients with low levels of GPR35 V2/3 mRNA, and mean survival time decreased with 11 months when followed for 5 years. Even more striking results were obtained if GPR35 V2/3 mRNA analysis was combined with CEA mRNA analysis, in which case the recurrence rate was increased to 3.6-fold with 55 months reduction in the mean survival time at 12-year follow-up for the GPR35 V2/3(+) group. No such effect was seen by combining GPR35 V2/3 mRNA with CXCL17 mRNA.

GPR35 protein expression was significantly elevated in primary tumour tissue of non-small-cell lung cancer as compared to normal lung, and high expression levels correlated with poor prognosis.[27] In contrast, although GPR35 protein expression in breast cancer primary tumours was increased, it did not correlate with poor prognosis.[10] Our results in CC primary tumours are similar to those in breast cancer. However, the data also show that analysis of lymph nodes is more likely to give clinically useful information about patient survival than analysis of the primary tumour probably due to that secondary tumours are less heterogenous and selected for expressed genes that are relevant in the metastasis process.

The two-colour immunofluorescence staining with anti-GPR35 and anti-EPCAM mAb BerEP4 or anti-Phospho-SRC and consecutive-sections-staining procedure with anti-CEA and anti-GPR35 antibodies reveal that CC tumour cells are of epithelial origin, express CEA and GPR35 and are involved in Src signalling.

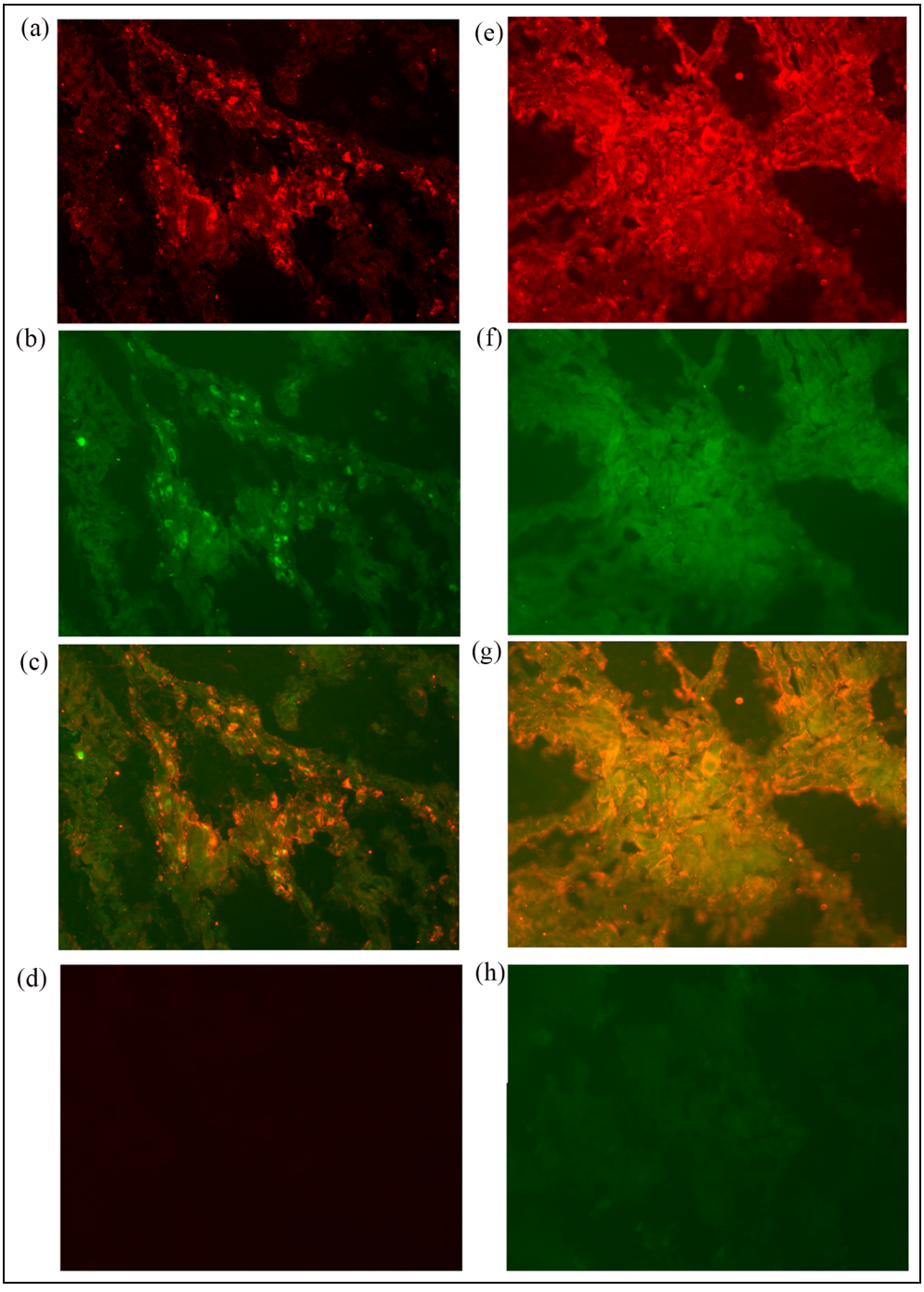

Figure 4. Two-colour immunofluorescence staining of primary colon cancer tissue with anti-GPR35 and BerEP4 or Phospho-SRC. Two different tissue sections are shown. Section one in (a), (b) and (c) and section two in (e), (f) and (g). (a), Anti-GPR35 = red colour. (b), BerEP4 mAb = green colour. (c), Overlays of (a) and (b) = yellow colour of double-stained areas. (e), Anti-GPR35 = red colour. (f), anti-Phospho-SRC = green colour. (g), Overlays of (e) and (f) = yellow colour of double-stained areas. (d) and (h), Negative controls for anti-GPR35 (d) and anti-Phospho-SRC (h) using normal rabbit IgG instead of the primary antibody. Original magnification: 200×.

Moreover, they reveal that a large fraction of the CC tumour cells exhibits these characteristics. Our findings are in line with those of Schneditz et al.[9] and indicate that GPR35 plays a central role in oncogenesis.

What is the nature of the CC tumour cells in lymph nodes that are particularly aggressive, that is, tumour cells that express high levels of GPR35? We hypothesise that they are relatively poorly differentiated tumour

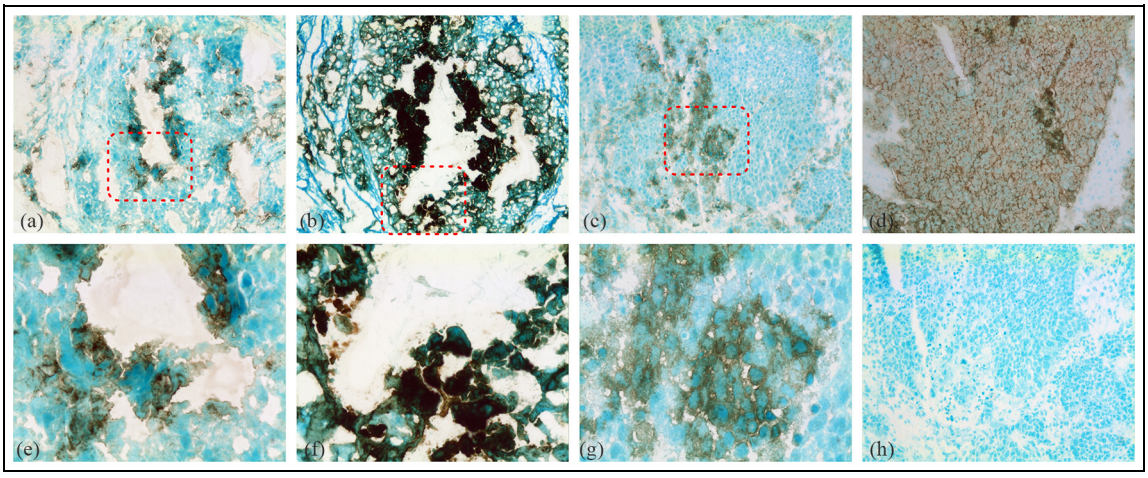

Figure 5. Immunoperoxidase staining of tissue sections of primary tumours and lymph node metastases of colon cancer. (a) Anti-GPR35 staining of primary CC tissue, original magnification 100×. (b) Anti-CEA staining of primary CC tissue, original magnification 100×. (c) Anti-GPR35 staining of an H&E(+) lymph node of a CC patient, original magnification 100×. (d) Anti-CEA staining of an H&E(+) lymph node of a CC patient, original magnification 100×. (e) Higher magnification of indicated area in (a), 400×. (f) Higher magnification of indicated area in (b), 400×. (g) Higher magnification of indicated area in (c), 400×. (h) Negative control of H&E(+) lymph node of a CC patient, original magnification 100×.
Positive cells stained brown to black. Methyl-green was used for counterstaining. Note that (a) and (b), (e) and (f), (c), (d), and (h) are consecutive sections.

cells with similar properties to the less differentiated CC cell lines HCT8 and HT29.[28]

In conclusion, this study demonstrates that GPR35 V2/3 mRNA is a marker of aggressive tumour cells, since it is highly expressed in primary and metastatic tumour cells. It can be considered as an independent marker of poor prognosis in CC, and its prognostic value is augmented if combined with CEA mRNA.

Author contributions

B.S., H.A., M.A., M.-L.H., S.H. and G.L. conceived and designed the experiments. G.L. collected the surgical specimens. H.A., M.A. and A.I. constructed the GPR35 qRT-PCR assays. M.A. and H.A. performed qRT-PCR determination of GPR35 mRNA levels. L.O. performed the Kaplan–Meier survival and univariate Cox regression statistical analysis. B.S., H.A. and M.A. performed two-colour immunofluorescence and immunohistochemistry. B.S., S.H., M.-L.H., H.A. and M.A. analysed the data and wrote the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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Informed consent

Samples were collected after patients' written informed consent. The study was approved by the Local Ethics Research Committee of the Medical Faculty, Umeå University, Umeå, Sweden (registration no. 03-503).

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Supplemental material

Supplemental material for this article is available online.

References

1. Chang GJ, Rodriguez-Bigas MA, Skibber JM, et al. Lymph node evaluation and survival after curative

resection of colon cancer: systematic review. *J Natl Cancer Inst* 2007; 99(6): 433–441.
2. Iddings D and Bilchik A. The biologic significance of micrometastatic disease and sentinel lymph node technology on colorectal cancer. *J Surg Oncol* 2007; 96(8): 671–677.
3. Nicastri DG, Doucette JT, Godfrey TE, et al. Is occult lymph node disease in colorectal cancer patients clinically significant? A review of the relevant literature. *J Mol Diagn* 2007; 9(5): 563–571.
4. Böckelman C, Engelmann BE, Kaprio T, et al. Risk of recurrence in patients with colon cancer stage II and III: a systematic review and meta-analysis of recent literature. *Acta Oncol* 2015; 54(1): 5–16.
5. Shore DM and Reggio PH. The therapeutic potential of orphan GPCRS, GPR35 and GPR55. *Front Pharmacol* 2015; 6: 69.
6. Divorty N, Mackenzie AE, Nicklin SA, et al. G protein-coupled receptor 35: an emerging target in inflammatory and cardiovascular disease. *Front Pharmacol* 2015; 6: 41.
7. Maravillas-Montero JL, Burkhardt AM, Hevezi PA, et al. Cutting edge: GPR35/CXCR8 is the receptor of the mucosal chemokine CXCL17. *J Immunol* 2015; 194(1): 29–33.
8. Agudelo LZ, Ferreira DMS, Cervenka I, et al. Kynurenic acid and GPR35 regulate adipose tissue energy homeostasis and inflammation. *Cell Metab* 2018; 27(2): 378–392.e5.
9. Schneditz G, Elias JE, Pagano E, et al. GPR35 promotes glycolysis, proliferation, and oncogenic signaling by engaging with the sodium potassium pump. *Sci Signal* 2019; 12(562): eaau9048.
10. Okumura S, Baba H, Kumada T, et al. Cloning of a G-protein-coupled receptor that shows an activity to transform NIH3T3 cells and is expressed in gastric cancer cells. *Cancer Sci* 2004; 95(2): 131–135.
11. MacKenzie AE, Caltabiano G, Kent TC, et al. The antiallergic mast cell stabilizers iodoxamide and bufrolin as the first high and equipotent agonists of human and rat GPR35. *Mol Pharmacol* 2014; 85: 91–104.
12. Guo YJ, Zhou YJ, Yang XL, et al. The role and clinical significance of the CXCL17-CXCR8 (GPR35) axis in breast cancer. *Biochem Biophys Res Commun* 2017; 493(3): 1159–1167.
13. Uhlen M, Ponten F, Hober S, et al. Human protein atlas, 2014, https://www.proteinatlas.org/ENSG00000178623-GPR35/tissue
14. Deng H, Hu H and Fang Y. Tyrphostin analogs are GPR35 agonists. *FEBS Lett* 2011; 585(12): 1957–1962.
15. Wang J, Simonavicius N, Wu X, et al. Kynurenic acid as a ligand for orphan G protein-coupled receptor GPR35. *J Biol Chem* 2006; 281(31): 22021–22028.
16. Rashad Y, Olsson L, Israelsson A, et al. Lymph node CXCL17 messenger RNA: a new prognostic biomarker for colon cancer. *Tumour Biol* 2018; 40(9): 1010428318799251.
17. Fahlgren A, Hammarström S, Danielsson A, et al. Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. *Clin Exp Immunol* 2003; 131(1): 90–101.
18. Lundqvist C, Hammarström ML, Athlin L, et al. Isolation of functionally active intraepithelial lymphocytes and enterocytes from human small and large intestine. *J Immunol Methods* 1992; 152(2): 253–263.
19. Ohlsson L, Hammarström ML, Israelsson A, et al. Biomarker selection for detection of occult tumour cells in lymph nodes of colorectal cancer patients using real-time quantitative RT-PCR. *Br J Cancer* 2006; 95(2): 218–225.
20. Öberg AN, Lindmark GE, Israelsson AC, et al. Detection of occult tumour cells in lymph nodes of colorectal cancer patients using real-time quantitative RT-PCR for CEA and CK20 mRNAS. *Int J Cancer* 2004; 111(1): 101–110.
21. Bas A, Forsberg G, Hammarström S, et al. Utility of the housekeeping genes 18S rRNA, beta-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. *Scand J Immunol* 2004; 59(6): 566–573.
22. Sitohy B, Hammarström S, Danielsson A, et al. Basal lymphoid aggregates in ulcerative colitis colon: a site for regulatory T cell action. *Clin Exp Immunol* 2008; 151(2): 326–333.
23. Ohlsson L, Hammarström ML, Lindmark G, et al. Ectopic expression of the chemokine CXCL17 in colon cancer cells. *Br J Cancer* 2016; 114(6): 697–703.
24. Weibel ER. Point counting methods. In: Weibel ER (ed.) *Sterological methods*, vol. 1. London: Academic Press, 1979, pp. 101–161.
25. Ohlsson L, Israelsson A, Öberg A, et al. Lymph node CEA and MUc2 mRNA as useful predictors of outcome in colorectal cancer. *Int J Cancer* 2012; 130(8): 1833–1843.
26. Ohlsson L, Lindmark G, Israelsson A, et al. Lymph node tissue kallikrein-related peptidase 6 mRNA: a progression marker for colorectal cancer. *Br J Cancer* 2012; 107(1): 150–157.
27. Wang W, Han T, Tong W, et al. Overexpression of GPR35 confers drug resistance in NSCLC cells by beta-arrestin/Akt signaling. *Onco Targets Ther* 2018; 11: 6249–6257.
28. Barbat A, Pandrea I, Cambier D, et al. Resistance of the human colon carcinoma cell line HCT-8 to methotrexate results in selection of cells with features of enterocytic differentiation. *Int J Cancer* 1998; 75(5): 731–737.