Growth differentiation factor 15: a prognostic marker for recurrence in colorectal cancer

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**BACKGROUND:** Growth differentiation factor 15 (GDF15) belongs to the transforming growth factor beta superfamily and has been associated with activation of the p53 pathway in human cancer. The aim of this study was to assess the prognostic value of GDF15 in patients with colorectal cancer (CRC).

**METHODS:** Immunohistochemistry and tissue microarrays were used to analyse GDF15 protein expression in 320 patients with CRC. In a subgroup of 60 patients, the level of GDF15 protein in plasma was also measured using a solid-phase proximity ligation assay.

**RESULTS:** Patients with CRC with moderate to high intensity of GDF15 immunostaining had a higher recurrence rate compared with patients with no or low intensity in all stages (stages I–III) (HR, 3.9; 95% CI, 1.16–13.15) and in stage III (HR, 10.32; 95% CI, 1.15–92.51). Patients with high plasma levels of GDF15 had statistically shorter time to recurrence (P = 0.041) and reduced overall survival (P = 0.002).

**CONCLUSION:** Growth differentiation factor 15 serves as a negative prognostic marker in CRC. High expression of GDF15 in tumour tissue and high plasma levels correlate with an increased risk of recurrence and reduced overall survival.

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Colorectal cancer (CRC) is the third most common cancer in the Western world. Globally, the age-standardised incidence rate for CRC is 20.1 per 100 000 males and 14.6 per 100 000 females (Parkin et al., 2005). Surgery is the most important treatment, but a substantial number of patients will get a recurrence, a risk being stage-dependent. The use of adjuvant treatment with 5-fluorouracil (5-FU) and folinic acid, after curative surgical resection for CRC, reduces the relative risk of recurrence by 30–35% and the addition of oxaliplatin reduces it even further (about 15–20%) (Ragnhammar et al., 2001; Andre et al., 2004). The relative risk reduction appears to be relatively independent of stage (Gill et al., 2004). In patients with node-positive disease (stage III), the gains translate into meaningful improvements in overall survival, but in patients with node-negative disease (stage II), the survival gain is only in the order of a few percent (Gray et al., 2007). It is not clear which all patients in stages II and III will benefit from adjuvant treatment and which in all patients, chemotherapeutic drug/s will be most effective. Consequently, there is a need for individualised therapy in patients curatively operated for CRC stages II and III.

Several prognostic and predictive markers have been identified, although very few of them are currently used in clinical practice.

Microsatellite instability (MSI) is one prognostic factor in use for recurrence and overall survival in patients with CRC stages II and III (Watanabe et al., 2001; Popat et al., 2005). Other identified prognostic markers in primary disease are thymidylate synthase and loss of heterozygosity of 18q, but these are currently not used in clinical practice. No predictive marker for response to adjuvant therapy has yet reached clinical use, although some studies show that MSI tumours do not adequately respond to 5-FU (Hemminki et al., 2000; Elsaleh et al., 2001; Jover et al., 2010). Mutations in KRAS are clinically used as a predictor for poor response to treatment with EGFR-directed antibodies in patients with metastatic CRC (Amado et al., 2008; Karapetis et al., 2008).

Growth differentiation factor 15 (GDF15) belongs to the transforming growth factor beta superfamily and has a role in regulating inflammatory and apoptotic pathways in injured tissues and during disease processes. Under normal conditions, the placenta is the only tissue that expresses GDF15 in significant amounts; however, small amounts of GDF15 mRNA can be detected in a few other tissues, including the kidneys, pancreas, prostate and colon (Fairlie et al., 1999). Growth differentiation factor 15, also known as PTGF-β, TGF-PL or MIC-1, has been hypothesised to serve as a secreted biomarker for activation of the p53 pathway in human cancer (Seetoo et al., 2003; Koopmann et al., 2004; Weber et al., 2005; de Wit et al., 2005; Brown et al., 2006), and
in CRC MIC-1, serum levels and genotype were associated with decreased overall survival and extent of disease (Brown et al., 2003; Xue et al., 2010). Immunohistochemical analysis of GDF15 expression in CRC has also been associated with lymph node metastasis (Xue et al., 2010).

Our aim was to further validate the prognostic value of immunohistochemical GDF15 expression and GDF15 levels in plasma in a defined cohort of patients operated for CRC.

PATIENTS AND METHODS

Patients

A population-based cohort of 320 patients with CRC, treated between August 2000 and December 2003 at the Central district Hospital in Västerås, were prospectively included to participate in the study after giving informed consent. The median follow-up time was 6 years (8–10) in surviving patients. Information about tumour size, grade, stage and location, neurovascular tumour infiltration, lymph node involvement and mucinous component were obtained from the pathology records, and had, thus, been assessed by different pathologists. Selection of tumour areas used for tissue microarrays (TMAs) was made by one single pathologist (KJ). Information about cancer recurrence, cause of death and the use of neo-adjuvant/adjuvant treatment was obtained from surgical and oncology records and by matching with the Clinical Database for Colorectal cancer held at the Regional Oncologic Center in Uppsala/Orebro region.

TMA construction

All cases were histopathologically re-evaluated on H&E stained tumour specimens by one pathologist (KJ), and areas representative of normal mucosa, invasive tumour, lymph node metastases and, when present, adenomatous tissue were selected. A manual arraying device (MTA-1, Beecher Instruments Inc., Sun Prairie, WI, USA) was used for extraction of five 1.0-mm cores from each case; two from the invasive tumour, one from normal mucosa, one from adenomatous mucosa and one from a selected lymph node metastasis.

Immunohistochemistry and annotation

Immunohistochemistry was performed on 4 μm TMA sections using HPA011191 (Atlas Antibodies, Stockholm, Sweden) as primary antibody to detect GDF15. Automated immunohistochemistry (Autostainer 480, Lab Vision, Fremont, CA, USA) was performed as previously described (Paavilainen et al., 2010). Immunohistochemically stained TMA sections were scanned in high-resolution scanners (ScanScope T2, Aperio Technologies, Vista, CA, USA) and separated into individual spot images representing different cores in the TMAs. The annotation process included estimation of the intensity of immunoreactivity for GDF15 (negative (0), weak (1), moderate (2), or strong, (3) and fraction (%) of GDF15-positive cells (<1% (0), 1–24% (1), 25–75% (2), or >75% (3)) (Figure 1). Tumours with no (0) or low (1) intensity and no (0) or low (1) fraction of GDF15 expression were allocated to one group and tumours with moderate (2) or high (3) intensity and moderate (2) and high (3) fraction of GDF15 expression were allocated into a second group.

Figure 1  These images represent the four levels of the intensity of immunoreactivity, resulting from immunostaining with GDF15 antibody on primary colorectal cancer tissues. Negative (A), weak (B), moderate (C) and strong intensity staining (D). Images with immunostaining present had 25–75% fraction of positive cells.
Pre-surgery EDTA plasma was analysed for GDF15 abundance. Patients initially included in the study were selected for GDF15 plasma analyses. The aim was to include 10 patients in each group after stratifying both for recurrence and stage. However, because of limitations in the number of cases in our cohort (i.e., recurrence in stage I) and limitations in availability of plasma samples, only nine patients with stage I (one recurrence), approximately 10 patients with and without recurrence from stages II and III, respectively, and 8 patients with stage IV were selected from the whole cohort. GDF15 was analysed by using SP-PLA (Darmanis et al, 2010). The protocol was modified to facilitate multiplex detection of various analytes, including GDF15, according to a method currently prepared for publication.

Statistical analyses

For categorical data, the $\chi^2$-test was performed. All P-values were two sided, and statistical significance was set at $P < 0.05$. Wilcoxon matched-paired sign test was used to compare GDF15 staining intensity and fraction between normal mucosa and tumour. Kruskal–Wallis ANOVA test was used to compare the median values of GDF15 plasma levels between different disease stages and the Mann–Whitney U-test was used to compare the median values of GDF15 plasma levels within each disease stage. The Kaplan–Meier method was used for survival analyses and comparison of the distribution of fraction of positive cells and intensity of immunoreactivity for GDF15 expression and only variables with a significance and independence of intensity and fraction of variate analyses were performed to evaluate the statistical significance and dependence of intensity and fraction of GDF15 expression and only variables with a $P$-value $<0.10$ in univariate analysis were included in the multivariate analyses. Patients with known metastases at the time of surgery and microscopically free resection margin (R0) were considered to be curatively operated. Overall survival was measured from the time of surgery to the time of death, irrespective of cause. Time to recurrence was calculated as time to any event related to the same cancer (Punt et al, 2007). Deaths from other cancers ($n = 4$), non-cancer-related deaths ($n = 65$), treatment-related deaths ($n = 6$) and loss to follow-up ($n = 1$) were censored. Second primary same cancers ($n = 4$) and other primary cancers ($n = 22$) were ignored. All observations were censored at the end of the study period.

| Expression of GDF15 in normal tissue adjacent to an invasive tumour (a) and invasive tumours (b) |

| GDF15 intensity of immunoreactivity staining | 0 | 1 | 2 | 3 | Total |
|---------------------------------------------|---|---|---|---|------|
| (a) Normal tissue                           |   |   |   |   |      |
| 0                                           | 70| 10| 14| 3 | 97   |
| 1                                           | 0 | 1 | 2 | 4 |  7  |
| 2                                           | 0 | 4 | 11| 1 | 16   |
| 3                                           | 0 | 2 | 8 | 0 |  10  |
| Total                                       | 70| 17| 35| 8 | 130  |
| (b) Tumour tissue                           |   |   |   |   |      |
| 0                                           | 29| 19| 81| 35| 163  |
| 1                                           | 0 | 0 | 45| 54| 103  |
| 2                                           | 0 | 0 | 31| 17|  48  |
| 3                                           | 0 | 0 | 3 | 0 |   3   |
| Total                                       | 29| 22|160|106|317   |

Abbreviation: GDF15 = growth differentiation factor 15. The intensity of immunoreactivity for GDF15 (negative (0), weak (1), moderate (2), or strong (3)) and fraction of positive cells (< 1% (0), 1–25% (1), 25–75% (2), or > 75% (3)) are presented in the tables.
a weak to moderate cytoplasmic expression in other normal cell types, including the colon and prostate. A differential expression pattern was found in cancer tissues representing several forms of cancer, within CRC ranging from negative tumours to tumours with strong GDF15 expression.

Immunohistochemical analyses

The intensity of immunoreactivity ($P < 0.001$) was higher in tumour tissue than in normal colon mucosa, but no difference in the fraction of positive cells was observed ($P = 0.19$) (Table 1). In addition, no difference in fraction ($P = 0.36$) or intensity ($P = 0.56$) of GDF15 expression between adenoma and normal tissue was observed, however, the number of patients with adenomas in the cohort was low ($n = 9$). Tumours expressing moderate or high intensity of GDF15 were less likely to have vascular invasion ($P = 0.036$). No other histopathological or clinical parameters were correlated with the intensity or fraction of GDF15 in the tumour (Table 2).

Survival analyses

Patients curatively treated for CRC in stages I–III or in stage III with moderate to high intensity of immunoreactivity for GDF15 had a higher recurrence rate and shorter time to recurrence compared with patients with no or low intensity for GDF15 (Figures 2A and B). These differences were statistically significant in multivariate analysis (stages I–III, (HR, 3.9; 95% CI,

![Figure 2](image-url)
Comparison with CEA

Low intensity of immunoreactivity in the primary tumour was associated with low levels of CEA (Figure 3A). When analysing patients with stage III disease, in which serum CEA was available for 91 of 100 patients, a decreased risk of recurrence was observed with low intensity of immunoreactivity for GDF15 in the primary tumour independent of CEA level. In patients with high intensity of immunoreactivity for GDF15 and CEA > 6 ng ml⁻¹, an increased risk of recurrence was observed (HR 2.33; 95% CI, 1.15–4.42). This remained statistically significant in the multivariate analysis (including gender, heredity for CRC, N stage and neural invasion) (Figure 3B).

Plasma analyses of GDF15

Plasma levels of GDF15 were available for 57 patients, 28 without and 21 with recurrent disease and 8 with stage IV disease. A nonsignificant trend of elevated plasma levels of GDF15 was observed in tumours with increasing intensity (P = 0.148) and fraction (P = 0.326) of GDF15 expression as assessed by immunohistochemistry (Figures 4A and B). Patients with high plasma levels of GDF15 had a shorter time to recurrence (P = 0.041) and a shorter overall survival (P = 0.002) in the univariate analysis, and this remained significant for overall survival in multivariate analysis (HR 2.11; 95% CI, 1.04–4.28) (Figure 4C).

There was no significant difference in median GDF15 plasma levels between patients with or without recurrence when comparing all stages; however, in patients with stage III, a trend of a higher median plasma level of GDF15 was observed compared with patients in stage III without recurrence (P = 0.072) (Table 4).

Comparison between plasma levels of GDF15 and CEA

The GDF15 plasma levels gradually increased with disease stage, whereas the CEA levels were low in stages I–III and markedly increased in stage IV (Figure 5). There was a weak correlation between the GDF15 plasma levels and CEA in the whole cohort (P < 0.001; r = 0.49), however, a stronger correlation was observed in stage IV (P = 0.045; r = 0.72).

Table 3  Results from the univariate and multivariate Cox regression analyses estimating the effect of the intensity, fraction of GDF15 expression on time to recurrence in patients curatively operated for CRC in stages I–III (n = 277) and in stage II (n = 131) and stage III (n = 100) separately

|                | Univariate analyses                      | Multivariate analyses                      |
|----------------|------------------------------------------|--------------------------------------------|
| Time to        | P-value (95% CI)                          | HR (95% CI) P-value (95% CI)               |
| Recurrence     |                                          |                                            |
| Intensity      |                                          |                                            |
| moderate and   |                                          |                                            |
| strong vs no   |                                          |                                            |
| and weak      | 0.08 (0.27 – 5.09)                        | 0.002 10.32 (1.15 – 92.51)                 |
| staining      |                                          |                                            |
| Stage II       |                                          |                                            |
| 0.003 11.02 (1.32 – 92.35) | 0.003 10.32 (1.15 – 92.51)                 |
| Stage III      |                                          |                                            |
| 0.012 3.83 (1.17 – 12.5) | 0.012 3.9 (1.16 – 13.15)                  |
| Stages I–III   |                                          |                                            |
| Fraction 25–75% and > 75% vs < 1% and 1–25% |                                          |
| Stage II       |                                          |                                            |
| 0.6 0.7 (0.16 – 3.03) |                                          |
| Stage III      | 0.28 1.51 (0.73 – 3.11)                  | 1.59 – 38.03 (95% CI)                      |
| Stages I–III   |                                          |                                            |
| Abbreviations: CI = confidence interval; CRC = colorectal cancer; GDF15 = growth differentiation factor 15; HR = hazards ratio. In the multivariate analyses adjustment for CEA, sex, location, stage, vascular or neural invasion and adjuvant or neoadjuvant therapy was performed.

Figure 3  Curatively treated patients with colorectal cancer stages I–III (n = 277). Box plot revealing higher CEA levels in those with increased expression of GDF15 defined as intensity of immunoreactivity (I) compared with low expression (0) (A). Time to recurrence in curatively treated patients with CRC and stage III divided into subgroups according to intensity of immunoreactivity for GDF15 and preoperative CEA levels. Group 0: intensity low (0–1) and CEA < 6; Group 1 (n = 12): intensity low (0–1) and CEA > 6 (n = 2); Group 2: intensity high (2–3) and CEA < 6 (n = 55); Group 3: intensity high (2–3) and CEA > 6 (n = 22) (B).
DISCUSSION

In this study, we demonstrated a higher recurrence rate in patients curatively operated for CRC stages I–III with moderate or high intensity of GDF15 expression, compared with tumours with no or low intensity of GDF15 expression. This was also demonstrated separately for patients with stage III disease, but not for patients with stage II disease.

Our data are consistent with previous findings by Xue et al. (2010) who investigated the expression of GDF15 in 69 CRC cases by immunohistochemistry. They demonstrated not only an association between upregulation of GDF15 and development of metastases but also, different from our study, an increased immunohistochemical GDF15 expression in stages III and IV compared with stages I and II. However, unlike our study, the study by Xue et al. (2010) used a combined score to quantify the intensity and fraction of GDF15 immunostaining, thus, limiting further comparisons between the two studies.

Brown et al. (2003) demonstrated an association between high GDF15 blood levels, presence of metastatic disease and an elevated risk of death. In our study, we observed that the risk of death was more than two times higher (HR 2.2; 95% CI, 1.3–3.7) in patients with elevated GDF15 plasma levels (>116 pg/ml), which can be compared with the results documented on patients with elevated GDF15 serum levels (>1150 pg/ml) by Brown et al. (2003) (HR 2.11; 95% CI, 1.04–4.28). In our study, the plasma levels of GDF15 were not significantly different between patients with or without recurrence in stages I–III, even though there was a trend of a higher plasma levels in patients with recurrence in stage III.

The presence of vascular invasion is known to be an independent prognostic factor for both colon (Shepherd et al., 1989; Petersen et al., 2002) and rectal cancer (Talbot et al., 1980; Willett et al., 1999; Smith et al., 2008). A study by Petersen et al. (2002) even proposed that the presence of vascular invasion along with three other pathologically determined parameters could be used to make decisions regarding adjuvant therapy in stage II CRC. In our study, increased GDF15 expression was negatively associated with vascular invasion. This observation is supported by a previous report on the anti-angiogenic activity of GDF15 (Ferrari et al., 2005) in which GDF15 was demonstrated both in vivo and in vitro to inhibit angiogenesis in endothelial cells. This inconsistent finding, of decreased vascular invasion and higher risk for recurrences, could be a result of the increased likelihood of a significant outcome by chance because of multiple testing; nevertheless, it could also be explained by the divergent molecular mechanisms of GDF15. GDF15 has been implicated both as a promoter and inhibitor of tumour growth (Tan et al., 2009; Levy and Hill, 2006; Abd El-Aziz et al., 2007). The conflicting results between in vitro and in vivo studies regarding the role of GDF15 in tumourigenesis can probably be attributed to the interaction of the tumour with the microenvironment (Albertoni et al., 2002; Krieg et al., 2010). The current belief is that GDF15 has pleiotropic effects in cancer progression by functioning as a tumour suppressor inhibiting tumour growth, inducing apoptosis in early stages, although it promotes proliferation, migration, invasion and metastasis in more advanced disease stages (Mimeault and Batra, 2010). This belief of a dual and stage-dependent role of GDF15 in tumourigenesis could potentially, in our study, explain why a high intensity of GDF15 expression was associated with a shorter time to recurrence in stage III but not in stage II disease.

We confirmed a difference in the intensity of immunoreactivity of GDF15 between normal mucosa and invasive tumour tissue, but failed to demonstrate a difference in the fraction of GDF15-positive cells between normal tissue and invasive tumour tissue. This indicates that there might be a pathophysiological distinction between activity of GDF15 expression (measured as intensity) and actual number of cells expressing GDF15 (measured as fraction).
We demonstrated a gradual trend of increasing GDF15 plasma levels between invasive tumour tissue and normal mucosa, supporting that intensity of immunoreactivity rather than fraction of positive cells for GDF15 better serves as a prognostic marker in CRC.

Plasma levels of GDF15 have been studied as a biomarker in cardiovascular disease (Hochholzer et al, 2010) and elevated levels have been seen in metastatic CRC, breast and prostate carcinomas compared with normal controls (Welsh et al, 2003). The correlation between GDF15 plasma levels and CEA in the whole cohort including all disease stages was weak, but when analysing patients with stage IV disease, a stronger correlation was observed. We demonstrated a gradual trend of increasing GDF15 plasma levels from stage I to IV, whereas the CEA levels were low in patients with early disease stages, but significantly elevated in stage IV patients. The predictive value of CEA for detecting colorectal cancer in early stages is known to be low (Fletcher, 1986), although there is clear evidence that preoperative plasma CEA levels correlate with stage and serve as an independent prognostic factor of survival (Wanebo et al, 1978; Wolmark et al, 1984; Slentz et al, 1994). Consistent with a previous study by Brown et al (2003), GDF15 plasma levels in our study was an independent prognostic factor of survival supporting that measurement of GDF15 levels in plasma might add additional prognostic information in patients with CRC. The samples in our study were strategically selected to get a more reliable estimate the GDF15 plasma analyses but still limited by the small sample size and, therefore, decreased the power and the precision of the results.

Other issues regarding immunohistochemistry related to different fixation techniques and duration of fixation of the tumour tissue could potentially also have influence our results (Atkins et al, 2004; Leong, 2004; Paavilainen et al, 2010). However, in our study all tissue specimens were handled at the same pathology department. Consequently, in this prospectively collected material, we predict low variability of the actual handling of the tissue specimens. The antibody used in the immunohis-tochemistry staining had a high specificity on protein array and a single band corresponding to the predicted size in kDa on the western blot. Therefore, we believe that it is less likely that the specificity of the antibody influenced our results.

In conclusion, we have demonstrated that increased GDF15 expression may have a negative prognostic value in patients curatively operated for CRC stages I–III and III disease. However, the actual role of GDF15 in tumourigenesis is still unclear, and further research regarding both its pathophysiological role and clinical use as a prognostic marker for CRC is needed preferably in prospective clinical trials.

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