Clinical relevance of the differential expression of the glycosyltransferase gene GCNT3 in colon cancer

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Received 7 May 2014; received in revised form 19 October 2014; accepted 23 October 2014
Available online 11 November 2014

KEYWORDS
Colon cancer
Metabolism
GCNT3
Glycosylation
Mucin biosynthesis
Prognostic marker
Response to chemotherapy

Abstract  Altered glycosylation is considered a universal cancer hallmark. Mucin-type core 2 1,6-N-acetylglucosaminyltransferase enzyme (C2GnT-M), encoded by the GCNT3 gene, has been reported to be altered in tumours and to possess tumour suppressor properties. In this work, we aimed to determine the possible role of GCNT3 gene expression as prognostic marker in colon cancer. We investigated the differential expression of GCNT3 gene among tumour samples from stage II colon cancer patients by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Univariate and Multivariate Cox regression analyses were used to determine the correlation between GCNT3 expression and disease-free survival. The risk of relapse in GCNT3 low-expressing cancer patients was significantly higher than that in GCNT3 high-expressing patients in both training (Hazard Ratio (HR) 4.26, \(p=0.002\)) and validation (HR 3.06, \(p=0.024\)) series of patients, and this association was independent of clinical factors. Additionally, qRT-PCR was used to explore the modulation of GCNT3 expression by different antitumour drugs. Three chemotherapeutic agents with different mechanism of action (5-fluorouracil, bortezomib and paclitaxel) significantly induced GCNT3 expression in several cancer cells, being observed the correlation between antitumour action and GCNT3 modulation, whereas this gene was not modulated in cells that do not respond.

http://dx.doi.org/10.1016/j.ejca.2014.10.021
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1. Introduction

Tumour cells usually show alteration of cellular glycosylation, which constitutes a universal cancer hallmark [1,2]. Glycoconjugates participate in adhesion and motility of non-malignant cells, as well as in the interaction with immune cells [1], and it has been postulated that human tumours are able to exploit these functions to promote proliferation and survival of cancer cells [3].

Mucins are one of the main series of glycoconjugates that are structurally and quantitatively altered in cancer [3]. They are the major family of O-linked glycoproteins [4] and they are involved in inflammation and cancer, some of them being able to induce cellular transformation and tumour formation in vivo, thus constituting direct drug targets for antitumour therapy [3]. Furthermore, high expression of mucins is linked to aggressive phenotype and chemotherapy resistance in pancreatic cancer [5,6], and mucin production determines sensitivity to the chemotherapeutic drugs bortezomib and gemcitabine [7].

Importantly, the biological functions of mucins are mostly determined by their carbohydrate moieties [8], which are synthesised by several enzymes, including glycosyltransferases. During malignant transformation, the mRNA levels, activities and Golgi localisation of glycosyltransferases are modified [9], leading to less complex structures in the O-glycosylation of mucins in human tumours [10], including colon cancer [11]. These alterations have been postulated to importantly regulate tumour growth and progression [10].

One of the glycosyltransferases that are altered in cancer is mucin-type core 2 1,6-N-acetylglucosaminyltransferase enzyme (C2GnT-M), which is encoded by the GCNT3 gene, and whose expression is detected in colon, kidney, pancreas, small intestine [12], trachea and stomach [13]. It is responsible for catalysing the formation of core 2 O-glycan, core 4 O-glycan and I branches [13]. In normal colon tissue there is a high C2GnT-M activity, whereas it lacks in several human colon cancer cell lines [9]. Moreover, GCNT3 gene expression has been recently found to be down-regulated in colon cancer samples in comparison to normal colon tissue [10,14]. Furthermore, colon cancer cells transfected to up-regulate GCNT3 gene in vitro showed an inhibited proliferation, adhesion and invasion capacities and cell death induction, and in vivo showed a decrease in tumour growth [10].

Due to the well-documented importance of glycosylation in cancer development and the recently reported tumour suppressor properties of GCNT3, together with the suggested role of mucin production in chemotherapy sensitivity in pancreatic cancer, we aimed to determine the potential applicability of GCNT3 gene expression as a biomarker for colon cancer patients.

2. Materials and methods

2.1. Tumour samples

Formalin-fixed, paraffin-embedded (FFPE) samples were obtained from curative resection of colon cancer patients in La Paz University Hospital (Madrid, Spain) with the authorisation of the local ethics committee and the informed consent of the patients. Inclusion criteria comprised patient age ≥18, stage II primary colon cancer (according to the AJCC/IUAC classification), follow up ≥36 months and high quality of the RNA obtained from the sample. Exclusion criteria included incomplete tumour excision, patient death within 30 days after surgery, mixed histological features and other cancers in the previous 5 years, inflammatory bowel disease or specific gene-related tumours (i.e. familial adenomatous polyposis syndrome and hereditary non-polyposis colorectal cancer). An initial sample series (training series) consisted of tumour samples from 77 patients who underwent surgery from 2000 to 2004 in La Paz University Hospital. The results obtained in the training series were confirmed in the validation series, which comprised samples from 119 patients who experienced curative resection from 2005 to 2008. Clinical and histopathological parameters were prospectively collected after surgery and confirmed by the oncologists of the Medical Oncology Division at La Paz University Hospital (Table 1). Additionally, samples of apparently healthy tissues adjacent to tumours were taken from 18 patients to obtain RNA from human healthy colonic mucosa. High risk of relapse was assessed according to the American Society of Clinical Oncology (ASCO) criteria, which include: T4 stage, number of lymph nodes examined ≤12, poor histologic grade, emergency presentation with obstruction or perforation, and perineural or lymphovascular invasion.
Table 1
Clinicopathological parameters of the two series (training and validation series) of stage II colon cancer patients analysed in the study.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Training series</th>
<th>Validation series</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Patients (n)</td>
<td>77</td>
<td>119</td>
</tr>
<tr>
<td>Age (years)</td>
<td>Range 32–86</td>
<td>26–91</td>
</tr>
<tr>
<td></td>
<td>&lt;70 years 42 (54.55)</td>
<td>73 (61.34)</td>
</tr>
<tr>
<td></td>
<td>≥70 years 35 (45.45)</td>
<td>46 (38.66)</td>
</tr>
<tr>
<td>Mean</td>
<td>68.22</td>
<td>66.08</td>
</tr>
<tr>
<td>Median</td>
<td>69</td>
<td>66</td>
</tr>
<tr>
<td>Localisation</td>
<td>Right 37 (48.05)</td>
<td>48 (40.34)</td>
</tr>
<tr>
<td></td>
<td>Left 40 (51.95)</td>
<td>71 (59.66)</td>
</tr>
<tr>
<td>Grade</td>
<td>Low 5 (6.49)</td>
<td>10 (8.40)</td>
</tr>
<tr>
<td></td>
<td>Moderate 66 (85.71)</td>
<td>95 (79.80)</td>
</tr>
<tr>
<td></td>
<td>High 5 (6.49)</td>
<td>10 (8.40)</td>
</tr>
<tr>
<td></td>
<td>Unknown 1 (1.30)</td>
<td>4 (3.40)</td>
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<tr>
<td>Sex</td>
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<td>54 (45.38)</td>
</tr>
<tr>
<td></td>
<td>Men 44 (57.14)</td>
<td>65 (54.62)</td>
</tr>
<tr>
<td>No. lymph nodes examined</td>
<td>Mean 12.1</td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td>Range 1–29</td>
<td>0–43</td>
</tr>
<tr>
<td></td>
<td>≤12 lymph nodes 46 (59.70)</td>
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<td>&gt;12 lymph nodes 30 (39.00)</td>
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<td></td>
<td>Unknown 1 (1.30)</td>
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<tr>
<td>T1</td>
<td>T3 56 (72.73)</td>
<td>70 (58.82)</td>
</tr>
<tr>
<td></td>
<td>T4 21 (27.27)</td>
<td>49 (41.18)</td>
</tr>
<tr>
<td>Disease-free survival</td>
<td>Mean (months)</td>
<td>56.71</td>
</tr>
<tr>
<td></td>
<td>Median (months)</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Relapse (local, regional, distant)</td>
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</tr>
<tr>
<td></td>
<td>22 (28.57)</td>
<td>18 (15.13)</td>
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<tr>
<td>Survival time</td>
<td>Mean (months)</td>
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<tr>
<td></td>
<td>Median (months)</td>
<td>63</td>
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<tr>
<td></td>
<td>Death 13 (16.88)</td>
<td>11 (9.24)</td>
</tr>
<tr>
<td></td>
<td>47 (61.04)</td>
<td>76 (63.87)</td>
</tr>
<tr>
<td></td>
<td>No 30 (38.96)</td>
<td>24 (36.13)</td>
</tr>
</tbody>
</table>

* Tumour category according to the TNM staging system: T3, tumour cross the muscularis propria and invades the subserosa, or the non-peritonealised pericolic or perirectal tissues; T4, tumour perforates the visceral peritoneum and/or directly invades other organs or structures.

2.2. Cell culture

Colon (SW620 and DLD-1) and breast (MDA-MB-231 and SK-BR-3) cancer cell lines were obtained from American Type Culture Collection (ATCC). Cells were cultured under standard conditions of temperature (37 °C), humidity (95%), and carbon dioxide (5%) in DMEM (SW620 and MDA-MB-231) or RPMI (DLD-1 and SK-BR-3) supplemented with 10% FBS, 2 mM glutamine, and 1% of antibiotic–antimycotic solution (containing 10,000 units/mL penicillin base, 10,000 µg/mL streptomycin base, and 25,000 ng/mL amphotericin B) (Gibco). Cells were treated with vehicle (controls) or the drugs 5-fluorouracil (5-FU) (Sigma–Aldrich), bortezomib (Selleck chemicals) or paclitaxel (Sigma–Aldrich) at effective antitumour concentrations.

2.3. Generation of colon cancer cells resistant to 5-FU

Colon cancer SW620 cells were cultured during 15 months in continuous exposition to increasing concentrations of 5-FU until reaching 150 µM [15]. These resistant cells (referred to as SW620-5FUR) were maintained in culture media containing 150 µM 5-FU until the beginning of each experiment.

2.4. Cell viability assay

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) assay as previously described [15]. SW620 colon cancer cells (30,000 per well) were seeded in 24-well plates. After overnight attachment, the cells were treated with different concentrations of 5-FU (Sigma–Aldrich). Following 48 h treatment, cells were incubated with MTT solution (5 mg/mL in PBS) at 1:10 dilution for 3 h, and the MTT metabolic product (formazan) was dissolved in 200 µL DMSO, and its absorbance was measured at 560 nm. Cell viability inhibition was calculated in relation to control (vehicle-treated) cells.

2.5. Gene expression analysis

RNeasy FFPE Kit and RNeasy Mini Kit (Qiagen) were used according to manufacturer’s instructions to obtain total RNA from FFPE tumour samples previously deparaffinated or from cultured cancer cells, respectively. RNA integrity of FFPE tumour samples was tested in 25% of the samples by gel electrophoresis, without observing any noticeable difference among them. Gene expression analysis was performed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). To that end, the same quantity of total RNA (1 µg of FFPE samples or 400 ng of cultured cells) was reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and GCNT3 gene expression was analysed in each sample (using equal amounts of the product from the previous reaction) by utilising the specific TaqMan probe in the 7900HT Real-Time PCR System (Applied Biosystems), as directed by the manufacturer. As internal reference of RNA quantity (endogenous controls), the expression of 18S was used in cultured cell samples and the geometric mean of the expression of GAPDH and B2M was used in clinical samples. We used the Real time StatMiner software (Integromics® Inc.) to verify the quality of the mRNA obtained. As a result of this analysis, four clinical samples (3 out of 80 from the training series and 1 out of 120 from the validation series) were excluded from the qRT-PCR analysis due to the lack of expression of endogenous controls (either B2M or GAPDH).
2.6. Statistical analysis

Quantification of GCNT3 expression (Q) was calculated with the $2^{-\Delta Ct}$ method ($\Delta Ct = Ct_{GCNT3} - Ct_{endogenous\,control}$), and statistically significant differences between control and tumour samples were assessed by Student’s t test. We used the Kaplan–Meier method to estimate disease-free survival (DFS), and Log-rank test and Univariate Cox regression analysis to test the association between DFS and GCNT3 gene expression. Multivariate proportional hazards Cox regression modelling was used to adjust for potential confounding factors. Hazard ratios (HR) and 95% Confidence Intervals (95% CI) were calculated from the Cox regression model. The threshold for dichotomisation of the expression data into a low and a high GCNT3 expression was selected based on the cutoff point with largest predictive ability, evaluated by the c-index methodology [16] using 100 times 5-fold cross-validation. The non-parametric Mann–Whitney test was used to determine the differences in the distribution of GCNT3 gene expression data among the different series within each clinical variable. Statistical significance was defined as p value <0.05. IBM SPSS Statistics version 20 (SPSS Inc.) was used to performed Student’s t tests. The Kaplan–Meier and Cox regression analyses were done using the R statistical software version 2.15 (www.r-project.org).

3. Results

Firstly, we determined the GCNT3 expression in colon cancer samples in comparison with healthy human colon tissue, in order to verify a differential expression in cancer accordingly with its putative tumour suppressor gene. To that end, GCNT3 mRNA levels were determined by qRT-PCR in the 77 tumour samples of the training series of patients and in 18 samples of healthy tissues adjacent to tumours. Median value of GCNT3 expression (Q) of the tumour samples was 6.99 whereas it was 23.38 in healthy tissues (3.34-fold reduction). This result was verified in an independent collection of samples of 119 stage II colon cancer patients (validation series), finding a 2.76-fold reduction of GCNT3 expression. Thus, results consistently showed an expression of GCNT3 in tumour samples significantly lower than that in the human normal colonic tissue (Fig. 1), suggesting a potential association between decreased GCNT3 expression and cellular malignancy.

To determine the relationship between GCNT3 expression and the potential malignancy of the tumour, we investigated its pattern of expression among the samples of the 77 patients of the training series. Their median follow-up was 72 months. We identified local and/or distant recurrence in 22 patients, of which 13 patients died of colon cancer. Mean colon cancer-specific survival time and mean time to relapse were 63.57 and 56.71 months, respectively. GCNT3 expression was distributed differentially within these patients. We established a threshold for dichotomisation of the GCNT3 expression data into a low and a high value of 2.75 Q (quantification value), based on the largest prediction ability and evaluated by the c-index using 100 times 5-fold cross-validation (CV). Under these conditions, 59 out of the 77 stage II colon cancer patients (76.6%) were above this cutoff, and 18 patients (23.4%) were below the cutoff (increased risk of relapse) (Table 2). The patients whose tumours showed high expression of GCNT3 had better clinical outcome than those with lower GCNT3 expression. Thus, median DFS was 25 months (95% confidence interval 15–NR) in patients with GCNT3 expression under the cutoff whereas it was not reached at the time of assessment in those patients who showed a higher expression (Fig. 2a). The 3-year DFS in GCNT3 high- and low-expressing cancer patients was 81% and 41%, respectively. Accordingly, increased risk of relapse for GCNT3 low-expressing cancer patients was 4.26 (CI 95%: 1.80–10.06, $p = 0.002$) (Table 2), and we didn’t find statistical differences in the distribution of GCNT3 expression within each clinical variable in the patients, suggesting that the association between GCNT3 gene expression and DFS was independent of clinical factors. These results points to GCNT3 as a potential prognostic factor in early-stage colon cancer since its overexpression is significantly associated with better clinical outcome.

To verify this hypothesis, we assessed GCNT3 expression in an independent series of 119 patients (validation series), in which median follow-up was 42 months, median DFS was 39 months, and 18 patients relapsed. Similar to the training series, 95 out of the 119 tumours analysed showed GCNT3 expression over the cutoff point (Table 2). Kaplan–Meier plots exhibited worse survival in those 24 patients with low GCNT3 expression than in patients with GCNT3 expression over the cutoff ($p = 0.01$) (Fig. 2b). Thus, low GCNT3 expression was associated with a median DFS of 26 months whereas it was not reached in patients with higher concentrations of the enzyme. In this series of patients, the 3-year DFS was 89% and 75% in high- and low-expressing GCNT3 cancer patients, respectively. Univariate analysis of the prognostic significance of GCNT3 showed that lower expression of this gene was significantly associated with an increased risk of colon cancer relapse compared to higher expression [HR 3.06 (CI 95%: 1.21–7.78, $p = 0.024$)]. Analysis of the association of clinicopathological parameters and GCNT3 expression using the Univariate analysis pointed as potential confounding clinical factors ($p < 0.05$) to T stage ($p = 0.016$), vascular invasion ($p = 0.020$) and perineural invasion ($p = 0.025$). Therefore, Multivariate analysis included these factors as well as age >70 as main non-modifiable
factor. Under these conditions, the statistical strength of the association between GCNT3 expression and risk of colon cancer relapse was increased [HR 3.63 (CI 95%: 1.22–10.83, \( p = 0.018 \)] (Table 2).

Results shown above suggest that expression of the metabolic GCNT3 gene might be used as a molecular prognostic biomarker in early-stage colon cancer. Moreover, previous studies showed that alterations in mucin metabolism are related to chemotherapeutic sensitivity, and GCNT3 overexpression can suppress tumour growth. These results suggest that GCNT3 down-regulation may be achieved to contribute to maintain the tumour characteristics, and GCNT3 up-regulation might be a general event in anticancer therapy. Thus, the modulation of GCNT3 expression by the pyrimidine analogue 5-FU, the proteasome inhibitor bortezomib, and the mitotic inhibitor paclitaxel were analysed, finding that the three chemotherapeutic drugs significantly induced the expression of GCNT3 in tumour cells at effective concentrations (Fig. 3A). GCNT3 expression was also tested in colon cancer cells resistant to 5-FU (SW620-5FUR), where the concentration to inhibit 50% of tumour cell proliferation at 72 h is >5000 \( \mu \text{M} \) whereas in the parental SW620 cells is close to 25 \( \mu \text{M} \) [15]. In these cells, by contrast, similar treatment with 5-FU that did not inhibit tumour cell growth, did not modulate GCNT3 expression either. Moreover, the up-regulation of GCNT3 expression induced by increasing concentrations of 5-FU correlates with the antitumour effect (cell viability inhibition) induced by the drug at those different concentrations (Fig. 3B), which further confirms the association between antitumour effect and GCNT3 up-regulation.

These results suggest not only a relevant role of GCNT3 expression in the inhibition of tumour progression, but also a potential use of the expression of this metabolic-related gene as a marker of response to chemotherapeutic treatment.

4. Discussion

Colorectal cancer is the third most commonly diagnosed cancer type in males and the second in females worldwide, and its incidence is still increasing in many countries [17]. In resectable colon cancer, there is a considerable risk for cancer recurrence after surgical intervention in stage III and high-risk stage II colon cancer patients [18]. Adjuvant chemotherapy substantially decreases the risk of relapse. However, the treatment decision in stage II colon cancer remains especially challenging, due to surgery alone results in an overall survival at 5 years of approximately 80%, and adjuvant chemotherapy only improves the survival in less than 5% of the patients [19]. Therefore, the challenge is to clearly differentiate the stage II colon cancer patients that are more likely to relapse, from those that will not relapse even without receiving chemotherapy.

Currently, the most common protocol to stratify colon cancer patients is the TNM staging system (T: tumour size or depth, N: lymph nodes examined, M: metastasis) [20,21]. Although several molecular (DNA, RNA or protein) biomarkers have been proposed in the last decade for colon cancer follow up, TNM staging system, which focus on anatomical characteristics, is the only proven prognostic marker to identify aggressive colon cancer [18].

Metabolic alterations are key events in tumour transformation and progression leading to fulfil energetic and structural requirements of tumour cells. In our study, we found that the mucin glycosylation-related GCNT3 gene is under-expressed in stage II colon tumours in relation to human normal colon tissue (Fig. 1). This result confirmed the previously reported studies that compared the expression of GCNT3 in a small number of colon cancer patient samples of a commercial array [10]. In addition, it is consistent with the reduced expression of core 4 \( O \)-glycans in colon cancer cells [13], the high

![Fig. 1. GCNT3 expression in tumour and normal colon tissue. Down-regulation of GCNT3 gene expression (Q) in colon tumour samples in comparison to healthy colon tissue in training (a) and validation (b) series, expressed as mean ± S.E.M. Student’s \( t \) test was applied to determine statistically significant differences (\( p \leq 0.05 \)) between normal and tumour tissue samples.](image-url)
frequency of short O-glycans in cancer samples [10], the down-regulation of enzymes responsible for the synthesis of core 4 and I branch during adenocarcinoma development in vitro [22], the lower C2GnT-M activity found in colon cancer [9], and the inhibitory properties of GCNT3 on tumour growth and progression [10]. These results indicate that the modulation of GCNT3 gene expression may be involved in maintaining the tumour phenotype.

Moreover, we assayed the possible role of GCNT3 expression as a prognostic biomarker in stage II colon cancer patients from two different series, and we found that patients who presented lower expression of GCNT3 were significantly more likely to relapse than those with a higher expression (Fig. 2). This suggests that GCNT3 expression analysis of resected early-stage colon tumours might be a promising tool to provide additional information of the relapse risk of patients, thus contributing to the selection of the patients that will most benefit from adjuvant therapy. Consequently, this would help to avoid the adverse effects of chemotherapy in the patients that will not benefit from it.

Aforementioned results, as well as previous studies reporting the role of GCNT3 in inhibiting tumour proliferation and invasion [10], point to GCNT3 as a promising target in colon cancer therapy. Thus, agents that induce the expression of GCNT3 might be potential antitumour drugs for colon cancer treatment. In this work, we analysed the effect of three chemotherapeutic agents (5-FU, bortezomib and paclitaxel) at effective antitumour concentrations in GCNT3 expression in different human colon and breast cancer cell lines. The results showed a significant and dose-dependent over-expression of GCNT3 by the antitumour drugs in the sensitive cell lines, while 5-FU did not induce GCNT3 over-expression in colon cancer cells resistant to this drug (Fig. 3). These results suggest a role of this enzyme in carcinogenesis inhibition as well as in response to anticancer therapy.

Overall, our results indicate that low GCNT3 expression is a promising prognostic biomarker for colon cancer that could be used for the identification of early-stage colon cancer patients at high risk of relapse, and also might be a potential biomarker to monitor the response to chemotherapy.

**Author’s contributions**

ARM was responsible for conception and design of the study, supervision of the research and interpretation of the results. TV and MGV were responsible for experimental development, data collection and analysis, manuscript preparation under the supervision of ARM, and participated in the interpretation of the results. SM participated in the experimental development and data collection and analysis. JH designed and carried out the statistical analysis and participated in the discussion and writing of the report. JMR, EB, CA, AC and JF

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**Table 2**

Univariate and Multivariate Cox regression analyses for disease-free survival of the GCNT3 gene in the training and validation series of stage II colon cancer patients. HR (95% CI), hazard ratio and corresponding 95% confidence interval from adjusted or unadjusted Cox regression analyses. HR indicates the relative likelihood of disease-free survival in high GCNT3 expression versus low GCNT3 expression patients. P. Cox, p value from adjusted or unadjusted Cox regression analyses; N, number of patients; R, number of patients who relapsed.

<table>
<thead>
<tr>
<th>Series</th>
<th>Low expression</th>
<th>High expression</th>
<th>Unadjusted</th>
<th>Adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>R</td>
<td>N</td>
<td>R</td>
</tr>
<tr>
<td>Training series</td>
<td>18</td>
<td>10</td>
<td>59</td>
<td>12</td>
</tr>
<tr>
<td>Validation series</td>
<td>24</td>
<td>8</td>
<td>95</td>
<td>10</td>
</tr>
</tbody>
</table>

Fig. 2. Association between GCNT3 expression and disease-free survival in colon cancer. Kaplan–Meier plots for GCNT3 expression in colon cancer patients from training (a) and validation (b) series.
identified and recruited eligible patients, summarised the clinicopathological data of patients, and participated in the interpretation of the results and writing of the report. GR supervised the research and critically reviewed the manuscript. All authors had unrestricted access to the final study data on request, and approved the final version of the manuscript.

Role of the funding source

The sponsors of the study had no role in the study design, data collection, data analysis, data interpretation, or in the writing of this report.

Financial support

This work has been supported by the Spanish Ministry of Science and Innovation (Plan Nacional I+D+i AGL2010-21565, AGL2013-48943-C2-2-R, RyC 2008-03734, IPT-2011-1248-060000); Comunidad de Madrid (P2013/ABI-2728, ALIBIRD-CM); and European Union Structural Funds. We gratefully thank to Roberto Martín Hernández and Dr. Ricardo Ramos Ruiz for their help on gene expression analysis, and Dr. Ana de la Cueva for her help with the generation of 5-FU resistant cells.

Conflict of interest statement

None declared.

Acknowledgements

This work has been supported by the Spanish Ministry of Science and Innovation (Plan Nacional I+D+i AGL2010-21565, AGL2013-48943-C2-2-R, RyC 2008-03734, IPT-2011-1248-060000); Comunidad de Madrid (P2013/ABI-2728, ALIBIRD-CM); and European Union Structural Funds. We gratefully thank to Roberto Martín Hernández and Dr. Ricardo Ramos Ruiz for their help on gene expression analysis, and Dr. Ana de la Cueva for her help with the generation of 5-FU resistant cells.

References


