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Identification of SOCS2 and SOCS6 as biomarkers in human colorectal cancer

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Background: Over the past years, some members of the family of suppressor of cytokine signalling (SOCS) proteins have emerged as potential tumour suppressors. This study aimed at investigating the clinical significance of SOCS proteins in colorectal carcinoma (CRC).

Methods: We integrated publicly available microarray expression data on CRC in humans, analysed the expression pattern of SOCSs and assessed the predictive power of SOCS2 and SOCS6 for diagnostic purposes by generating receiver operating characteristic curves. Using laser microdissected patient material we assessed SOCS expression on RNA and protein levels as well as their methylation status in an independent CRC patient cohort. Finally, we investigated the prognostic value of SOCS2 and SOCS6.

Results: The meta-analysis as well as the independent patient cohort analysis reveal a stage-independent downregulation of SOCS2 and SOCS6 and identify both molecules as diagnostic biomarkers for CRC. We demonstrate a different methylation pattern within the SOCS2 promoter between tumour tissue and normal control tissue in 25% of CRC patients. Furthermore, early CRC stage patients with low expression of SOCS2 display significantly shorter disease-free survival.

Conclusions: Our data offers evidence that SOCS2 and SOCS6 levels are reduced in CRC and may serve as diagnostic biomarkers for CRC patients.

Despite progress made during the past decades, colorectal cancer (CRC) is still one of the most frequent and deadly cancers worldwide in both women and men. The prevention of this disease is, therefore, a significant public health issue. The primary cause of death is the development of distant metastases in organs such as liver and lungs; ~40–50% of patients who undergo curative surgery relapse and die of metastatic disease (Johnston, 2005). Strikingly, diagnosed in time, CRC can be cured in 9 out of 10 cases. Thus, it is highly important to identify more sensitive and specific CRC markers to strengthen the efficiency of early diagnosis as well as to improve therapeutic strategies.

Aberrant cytokine signalling has been associated with many diseases, including several cancers, disorders in haematopoiesis and autoimmune diseases. Cytokine responses have to be stringently controlled by a number of key regulatory proteins, such as the suppressors of cytokine signalling (SOCSs) family members. SOCS proteins are rapidly induced upon JAK/STAT signalling by activated signal transducer and activator of transcription factors (STAT) to negatively regulate cytokine signalling via a classical feedback loop (Inagaki-Ohara *et al*, 2013). Taking into consideration that ~20% of all malignancies are initiated or exacerbated by inflammation, it is not surprising that SOCS proteins are regarded as tumour suppressor-like proteins (Elliott *et al*, 2008; Culig, 2013). The SOCS family consists of eight proteins, namely SOCS1–SOCS7 and cytokine-inducible SH2-containing protein (CIS). Each of these proteins has a central SH2 domain, an amino-terminal

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domain of variable length and sequence, and a carboxy-terminal 40 amino-acid region called SOCS box (Inagaki-Ohara *et al*, 2013). The SOCSs can interact with a series of signalling intermediates through the binding of their SH2 domain to phosphorylated tyrosine residues, particularly those on cytokine receptors and JAKs, leading to the blockade of the signal (Inagaki-Ohara *et al*, 2013).

Among SOCS family members, SOCS1 and SOCS3 have been most widely studied. SOCS1 and SOCS3 have been shown to suppress cell growth and their expression is frequently downregulated in human cancers (Trengove and Ward, 2013). Low SOCS2 gene expression has been associated with hepatocellular, breast, pulmonary and ovarian cancers (Wikman *et al*, 2002; Sutherland *et al*, 2004; Farabegoli *et al*, 2005; Haffner *et al*, 2007; Qiu *et al*, 2013). SOCS4 shows a reduced expression in gastric cancer and is associated with a better clinical outcome in breast cancer patients (Sasi *et al*, 2010; Kobayashi *et al*, 2012) whereas loss of SOCS5 expression is correlated with poor prognosis in liver cancer (Yoon *et al*, 2012). SOCS6 has been reported to be downregulated in carcinomas of prostate, stomach, liver and colon (Lai *et al*, 2009, 2010; Qiu *et al*, 2013; Zhu *et al*, 2013). SOCS7 has a favourable prognostic value in breast cancer (Sasi *et al*, 2010). Altogether, these findings suggest the involvement of SOCSs in cancer.

In the present study, we examined the expression as well as the methylation status of SOCS proteins in primary colon tumours, its precursor lesions and distant normal tissue. We used laser microdissection (LMD) to select for a highly pure population of epithelial cells in primary CRC tumours as well as in normal colon tissue. Our results point towards the involvement of SOCS2 and SOCS6 in the carcinogenesis of CRC and demonstrate their potential use as biomarkers.

MATERIALS AND METHODS

Patients and samples. Institutional guidelines were followed. All human samples used in the scope of this work were donated freely and informed consent was obtained. Ethical approval was obtained from the Comité National d'Ethique de Recherche, Luxembourg (Reference 201009/09). Primary colon cancer tissue and matched distant non-neoplastic colon tissue (at the farthest longitudinal surgical margin) from 23 CRC patients were collected following the standard preanalytical code for biospecimens by the Integrated Biobank of Luxembourg (IBBL; Betsou *et al*, 2010) immediately after surgical excision and stored in liquid nitrogen before further processing. This collection contains high dysplasia ($n=3$), stage I ($n=3$), stage II ($n=10$), stage III ($n=6$), stage IV ($n=1$) tumour samples according to the TNM Classification of Malignant Tumours (TNM system, American Joint Committee on Cancer; Hari *et al*, 2013) as well as 23 normal tissue samples matching the corresponding tumour sample. To increase our CRC collection, we also received tumour specimens from stage 1 ($n=13$) and stage 4 ($n=30$) from the Ontario Tumour Bank (Ontario Institute for Cancer Research), so that in total our study includes 23 normal tissue samples and 66 CRC samples. The clinical and histopathological data were provided by a pathologist. In total, 66 CRC patients were included, 35 male and 31 female with a median age of 67 years (66.6 ± 12, range 30–89) (Supplementary Table 1). Tumours included in the study were of sporadic origin and restricted to the colon except for one sample located in the rectum. In the bioinformatic analysis the authors of the respective studies used the Dukes staging system, which ranges from A to D. Even if the TNM staging system is preferentially used nowadays we can still correlate both staging methods (Walters *et al*, 2013). Disease-free survival corresponds to the interval between the date of surgery and the date when recurrence is diagnosed or the date of last follow-up.

Materials. All CRC cell lines were obtained either from the American Type Culture Collection (ATCC, Rockville, MD, USA) or the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and maintained in recommended culture conditions. LS174t and HT-29 were exposed to 5 μM 5-Aza-2′-deoxycytidine (5-aza-dC/DAC, Sigma-Aldrich, Diegem, Belgium) or vehicle (DMSO) for 4 days followed by RNA extraction.

Tissue processing and laser microdissection. We used LMD to maximise the purity of the epithelial tumour cell population. Briefly, frozen samples were mounted in Tissue-Tek OCT compound, serially sectioned at 10 μm in a cryostat at -20°C (Leica, Wetzlar, Germany), and mounted on prepared membrane slides (Leica mb slides PEN-membrane 2 μm). The parts containing areas of epithelial cells from tumours and normal tissue were identified using haematoxylin and eosin staining (Histogene LCM Frozen section staining kit, Applied Biosystems, Gent, Belgium) and analysed by a histopathologist. A Leica AS LMD microscope (Leica) was then used to select epithelial cells from tumour biopsies and their distant normal counterpart for each patient. Finally, sections were collected in a 0.5 ml microtube with RNA extraction buffer (All Prep Qiagen, Hilden, Germany) containing 1% β-mercaptoethanol.

RNA/DNA extraction and real-time PCR. RNA/DNA extraction was performed using commercially available kits and real-time PCR experiments were performed using TaqMan technology (Applied Biosystems) (Erickson *et al*, 2009). Details are provided as Supplementary Information to this article.

Methylation analysis. For the monitoring of the methylation pattern of the SOCS2 promoter, pyrosequencing and MassARRAY technology by Sequenom (San Diego, CA, USA) was performed at Varionostics GmbH, Ulm, Germany. The EpiTYPER assay (Sequenom) is a tool for the detection and quantitative analysis of DNA methylation using base-specific cleavage and matrix-assisted laser desorption–ionisation time-of-flight mass spectrometry (MALDI–TOF MS).

Western blot. Frozen tissues from CRC patients were homogenised in RIPA lysis buffer (ThermoFisher Scientific, Erembodegem-Aalst, Belgium) containing protease inhibitors. Beads were added to the homogenate, which was then lysed in a tissue lyser (Qiagen; 2×1 min). Proteins were run on a 12% SDS–PAGE gel and transferred to PVDF membranes (Pall Corporation, Port Washington, NY, USA). Membranes were blocked with 10% milk for 1 h at room temperature and were then incubated overnight at 4°C with antibodies directed against SOCS2 (1 : 1000, OriGene Technologies, Uden, Netherlands; TA307336), SOCS6 (1 : 1000, Santa Cruz Biotechnology, Heidelberg, Germany; sc-133058) and GAPDH (1 : 3000, Sigma-Aldrich, G9545). After washing, detection of the protein bands was performed using the Femto kit (SuperSignal West Femto kit, Thermo Scientific, Erembodegem-Aalst, Belgium). Detection was performed using Chemidoc XRS + imager (Biorad, Nazareth Eke, Belgium).

Immunohistochemical analysis. Tissue sections (4 μm) were cut from FFPE blocks from two patients with stage III to assess immunohistochemical staining for SOCS2 and SOCS6. Briefly, sections were deparaffinised followed by antigen retrieval and incubated with anti-human SOCS2 (Bioss, Woburn, MA, USA; bs-1896R, 1 : 300) and anti-human SOCS6 (Abcam, Cambridge, UK; ab53211, pre-diluted), respectively. The antibody dilution was determined after initial standardisation and specificity of the antibodies was verified by using positive controls. Sections on which the primary antibody was not applied served as negative controls.

Secondary detection was performed with a detection kit (ultraView Universal DAB detection kit, Ventana, Basel, Switzerland; ref. 760–500) and counterstain with Hematoxylin II (Ventana, ref. 790–2208) followed by application of bluing reagent (Ventana, ref. 760–2037). Each section was dehydrated by graded concentrations of alcohol before coverslips were applied.

Statistical analysis. GraphPad Prism 5 software (La Jolla, CA, USA) was used for statistical analysis. We used unpaired Student's *t*-test to compare expression levels between tumour and normal tissue. Kaplan–Meier curves were performed to assess if SOCS2 and SOCS6 levels correlate to prognosis of patients. For this, datasets GSE39582 and GSE14333 were downloaded from GEO (http://www.ncbi.nlm.nih.gov/geo/) and clinical information extracted (Jorissen *et al*, 2009; Marisa *et al*, 2013). For each dataset, gene expression values were grouped in two categories ('low' / 'high') using the *k*-mean algorithm. This clustering was performed in each dataset separately to avoid bias in gene expression values between the datasets. When only stages A and B are studied, the clustering was performed after removing patients that were not graded as stage A or B. After determination of the categories, both datasets were merged into a single one, which was used for further analysis. The survival analysis was performed using the survival package in R (http://cran.r-project.org/web/packages/survival/). Statistical significance of this correlation was assessed by log rank test. *P*-values <0.05 were considered statistically significant.

Bioinformatic analysis. Microarray expression datasets from CRC, colon adenoma and inflammatory bowel disease (IBD) were retrieved from NCBI Gene Expression Omnibus (GEO) Database (http://www.ncbi.nlm.nih.gov/geo/). All the individual CEL files from datasets profiled on HG-U133 plus 2.0 (Affymetrix, Santa Clara, CA, USA) retrieved from GEO (GSE14333; GSE17538; GSE21510; GSE8671; GSE9254; GSE20916; GSE10714; GSE15960; GSE4183; GSE10961) and corresponding to different studies (Sabates-Bellver *et al*, 2007; Galamb *et al*, 2008, 2010; LaPointe *et al*, 2008; Jorissen *et al*, 2009; Skrzypczak *et al*, 2010; Smith *et al*, 2010; Tsukamoto *et al*, 2011) were integrated into one single global analysis, referred to as meta-analysis (Supplementary Table 2). A preselected set of arrays profiling normal colorectal tissue samples was chosen as control. This large number of arrays was subjected to multiple downstream processing steps. CEL files were preprocessed using the Robust Multiarray Analysis (RMA) algorithm with GC-correction on the commercial software Partek Genome Suite (Version 6.4, St Louis, MO, USA). In order to minimise multiple study expression artifacts, and therefore the technical variability between the different research laboratories, the batch effect was corrected by applying (a) $3':5'$ house-keeping gene ratio, which gives an indication on RNA quality (Gentleman *et al*, 2005); (b) principal component analysis (PCA) allows to determine potential sources of variability in a multidimensional data set; (c) relative log2 expression (RLE) summaries, which are sensitive to technical sources of variability that are large compared to biological variation (Bolstad *et al*, 2004). Twelve out of 843 arrays had to be withdrawn from the dataset because of insufficient quality. Non-informative gene features were removed by filtering out probe sets with log2 expression level never exceeding 6. An additional summarisation step was performed in order to remove not annotated transcripts and obtain a single expression value for each annotated gene. Statistical significance of the genes of interest was addressed by applying 95% confidence intervals for mean log FC. *P*-values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) method. Suitability of the potential biomarkers for discrimination of CRC and normal samples was addressed by calculation of the area under receiver operating characteristic (ROC) curves (AUC) using package caTools of R (http://CRAN.R-project.org/package=caTools).

RESULTS

A bioinformatic approach identifies SOCS2 and SOCS6 as potential diagnostic biomarkers. The integration of publicly available data of smaller studies into one meta-analysis increases the power to detect biologically relevant signals specific for the initiation or progression of a disease. Published microarray-based studies profiling human samples on IBD, colon adenoma, CRC, and normal colon tissue were chosen for the present study. Principal component analysis shows that the different disease conditions form well-separated clusters whereas no differential clustering was detected according to age, gender or location of the tumour (data not shown). All FDR values are indicated in Supplementary Table 3. To evaluate the biological significance of the microarray datasets, we analysed the expression profiles of known marker genes (*SOCS3*, *KIAA1199*, *osteopontin/SPP1*, *CDX1*) specific for IBD, colon adenoma or CRC (Agrawal *et al*, 2002; Kim *et al*, 2005; Rigby *et al*, 2007; Sabates-Bellver *et al*, 2007). Inflammatory bowel disease is characterised by a chronic recurrent colonic inflammation, which is associated with an increased risk of developing CRC. In our meta-analysis we found SOCS1 and SOCS3 expression to be upregulated in IBD samples compared to normal colorectal mucosa samples (2.94-fold and 3.10-fold, respectively, FDR <0.001, Supplementary Table 3 and Supplementary Figure 1A). Interestingly we find upregulation of all STAT factors except STAT6 in the IBD samples (Figure 1B and Supplementary Table 3). *KIAA1199* (colon cancer secreted protein 1) is 7.32-fold upregulated (FDR <0.001) in adenomas and stayed elevated in CRC samples from our meta-analysis (4.48-fold; FDR <0.001; Figure 1A). This is in line with a previous study by Sabates-Bellver *et al* (2007) who reported *KIAA1199* to be upregulated both in colon adenoma and stage independently in CRC patients. *Osteopontin/SPP1* a described progression marker for CRC (Agrawal *et al*, 2002), shows a higher expression in the CRC dataset compared to adenomas and normal mucosal tissue (Figure 1A). *CDX1* caudal type homeobox is defined as a differentiation marker in CRC (Kim *et al*, 2005). Accordingly, its expression is reduced by $\sim$35% (FDR <0.001) in CRC samples (Figure 1A).

As the selected control marker genes all showed the expression pattern previously described in the literature, we conclude that our meta-analysis, which contains a high number of microarray datasets, is biologically relevant and can be used in order to address the potential use of SOCS family members as new biomarkers or targets for CRC therapy. When comparing adenoma samples to normal tissue, we found SOCS2 expression to be $\sim$20% downregulated (linear fold change, FDR <0.001), whereas expression of the other members of the family are either not changed or slightly upregulated (Figure 1A). In CRC, CISH, SOCS2 and SOCS6 show a significant downregulation in CRC by 10%, 45% and 60%, respectively (Figure 1A). Interestingly, the expression of SOCS2 significantly decreases from adenoma to CRC ($P<0.001$, Figure 1A and B). A detailed analysis of their expression along CRC staging shows that their downregulation is stage independent (Figure 1B, Supplementary Figure 1B). For the remaining SOCS family members, the expression is also not influenced by the CRC stages (Supplementary Table 3). Importantly, expression of the control marker gene SPP1 increases stage dependently from CRC stage A to D by 1.72 (CRC.A); 2.98 (CRC.B), 3.14 (CRC.C) and 3.54 (CRC.D) fold, respectively; FDR <0.001) which is consistent with previous findings (Agrawal *et al*, 2002). Investigating the expression of the STAT family members, we observe a slight upregulation of STAT1 and a faint upregulation of STAT5A/B, an upstream regulator of SOCS2. Interestingly, we find STAT6 to be downregulated alongside SOCS2 and SOCS6 in CRC samples, albeit to a lesser degree (Figure 1B; Supplementary Table 3).

However, a direct link between STAT6 downregulation and the observed reduced expression SOCS2 and SOCS6 is unlikely as STAT6 activation has not yet been associated with the regulation of these two SOCS proteins. Further investigations including STAT protein expression analyses could help clarifying the significance of the observed STAT mRNA changes. We next analysed the predictive power of SOCS2 and SOCS6 expression levels for diagnostic purposes by generating ROC curves and *c* statistic (AUC; Figure 2). AUC values of 0.910 and 0.958 were observed for SOCS2 and SOCS6, respectively. Furthermore, the distributions of gene expression levels for SOCS2 and SOCS6 clearly separated into two distinct almost non-overlapping peaks. The high AUC values

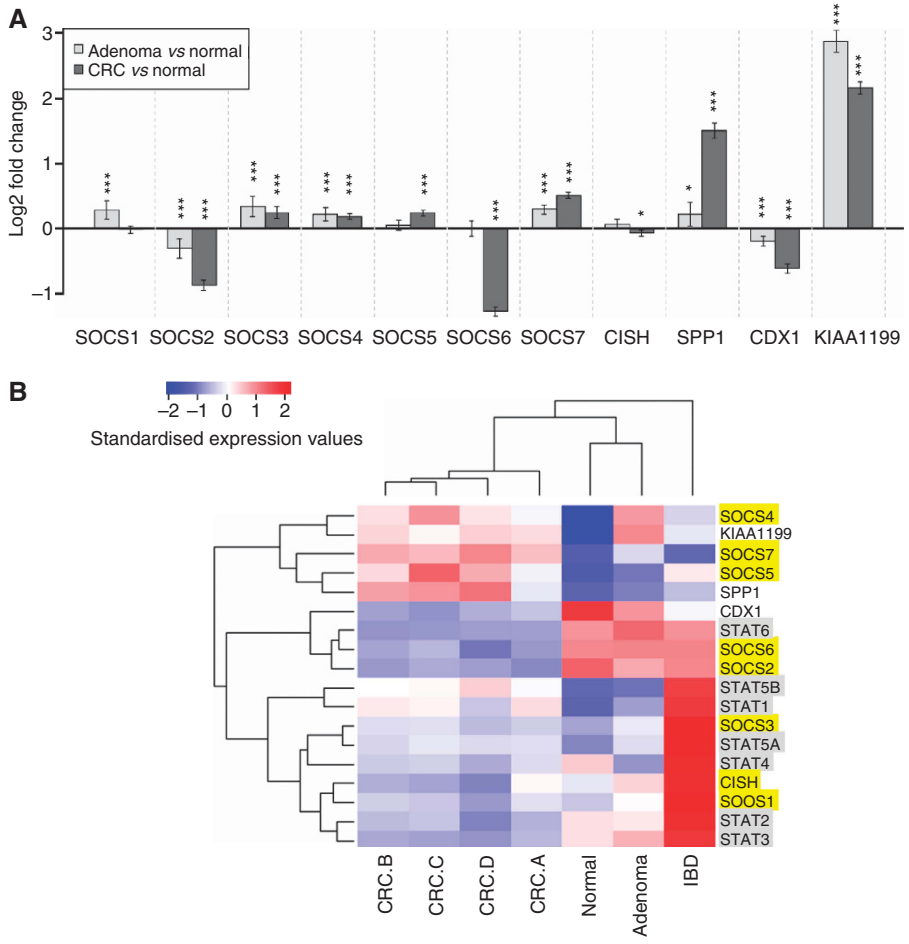

Figure 1. Bioinformatic analysis reveals SOCS2 and SOCS6 downregulation in CRC. (**A**) Bar plot showing the log2 FC values of the SOCS family genes and marker genes in adenoma and CRC samples compared to normal colorectal mucosa. Error bars correspond to 95% confidence intervals for mean log FC; *FDR<0.05, **FDR<0.01 and ***FDR<0.001. (**B**) Heatmap representation of SOCS (highlighted in yellow) and STAT (highlighted in grey) family genes as well as marker genes in normal tissues, IBD, adenomas, and CRC stages (A–D).

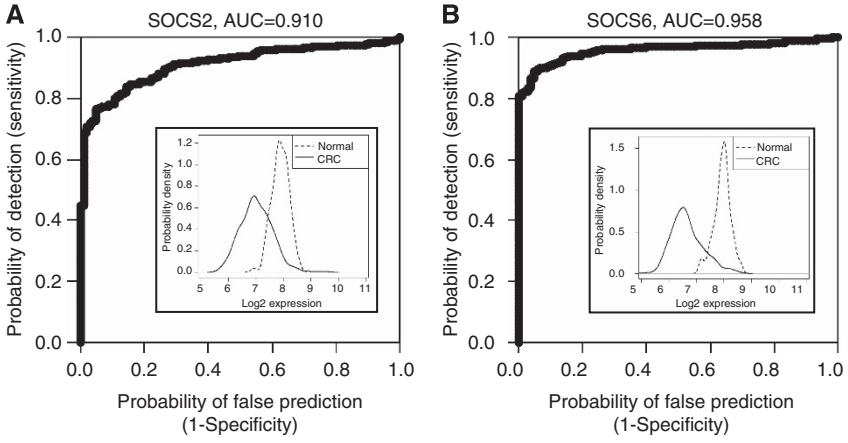

Figure 2. SOCS2 and SOCS6 are potential biomarkers in human CRC. ROC curves with corresponding AUC values for (**A**) SOCS2 and (**B**) SOCS6 when classifying CRC patients and healthy donors. All grades of CRC were pooled. Distributions of gene expression values for healthy and CRC patients are shown in the insets.

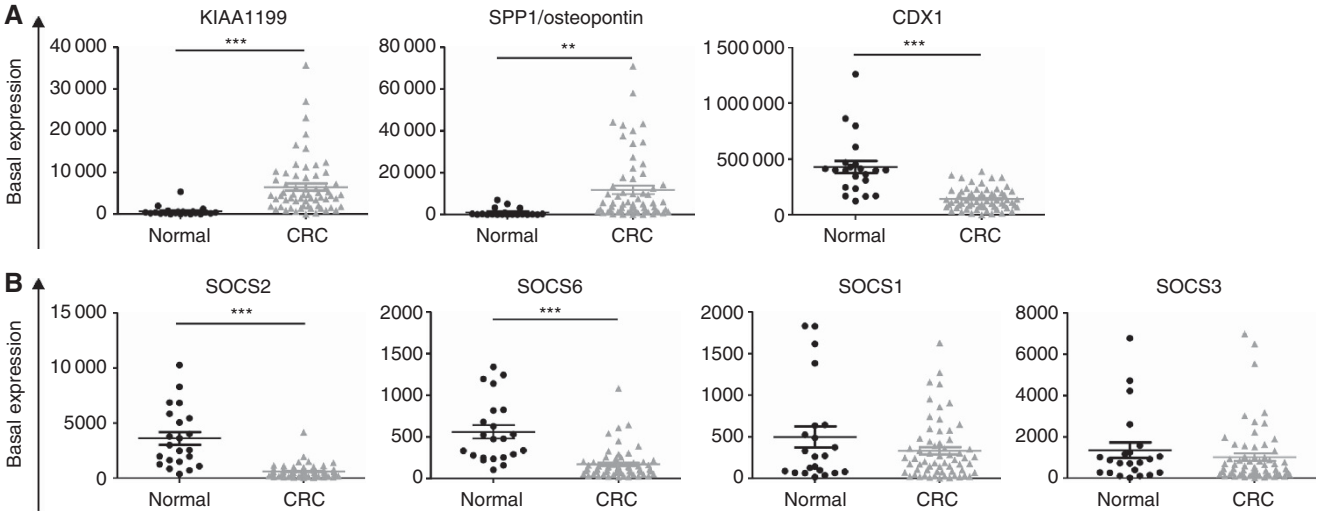

Figure 3. Expression of SOCS family members in primary human CRC samples. (**A**) Marker gene expression levels in CRC *vs* normal tissue of two patients. (**B**) SOCS1, SOCS2, SOCS3 and SOCS6 mRNA levels in CRC compared to normal mucosal tissue. Data are presented as mean ± s.e.m.; *$P<0.05$, **$P<0.001$ and ***$P<0.0001$.

as well as the well-separated distribution profiles support the use for SOCS2 and SOCS6 as biomarkers. Taken together, bioinformatics and biostatistics represent a valuable tool for the analysis of complex human diseases and the identification of novel biomarkers like, in our case, SOCS2 and SOCS6.

SOCS2 and SOCS6 are downregulated at mRNA and protein level in primary human CRC samples. To validate our bioinformatic analysis in a different patient cohort we assessed the expression of SOCS family members in human CRC samples and their respective normal counterparts. 66 CRC tumour samples and 23 normal counterparts were used for the present study (Supplementary Table 1). Using LMD, we selected a highly pure malignant or normal epithelial cell population. This is particularly important for SOCSs as tumour-infiltrating inflammatory cells often express these proteins. Thus, taking bulk material may lead to a wrong estimation of the expression of SOCSs in tumour cells. First we checked the expression of marker genes within our primary human CRC collection. KIAA1199 and SPP1 are both upregulated in CRC samples ($P<0.001$ and $P<0.01$, respectively; Figure 3A). CDX1 shows a significantly reduced expression in CRC samples compared to the normal counterparts ($P<0.001$; Figure 3A). Thus, the marker genes show the same expression pattern as in the bioinformatic analysis. Further, among the SOCS family members, SOCS2 and SOCS6 expression is significantly downregulated ($P<0.001$) whereas SOCS1 and SOCS3 do not show a statistically relevant difference in expression compared to the normal mucosal tissue (Figure 3B). To analyse whether the SOCS family members show a different expression pattern along the progression of the disease, we separated the samples according to the TNM staging system. As expected SOCS1 and SOCS3 expression does not change between the different CRC stages (Supplementary Figure 2A). Furthermore, we do not observe a change in SOCS2 and SOCS6 expression along CRC staging (Supplementary Figure 2A). Thus, SOCS2 and SOCS6 are significantly downregulated in CRC samples in a stage-independent manner. Western blot analysis and immunohistochemical stainings confirmed that reduced expression of SOCS2 and SOCS6 also result in a decrease in protein levels (Figure 4A and B). These results validate our findings obtained with the bioinformatic approach in a second independent patient cohort and strongly support the use of SOCS2 and SOCS6 as potential diagnostic markers.

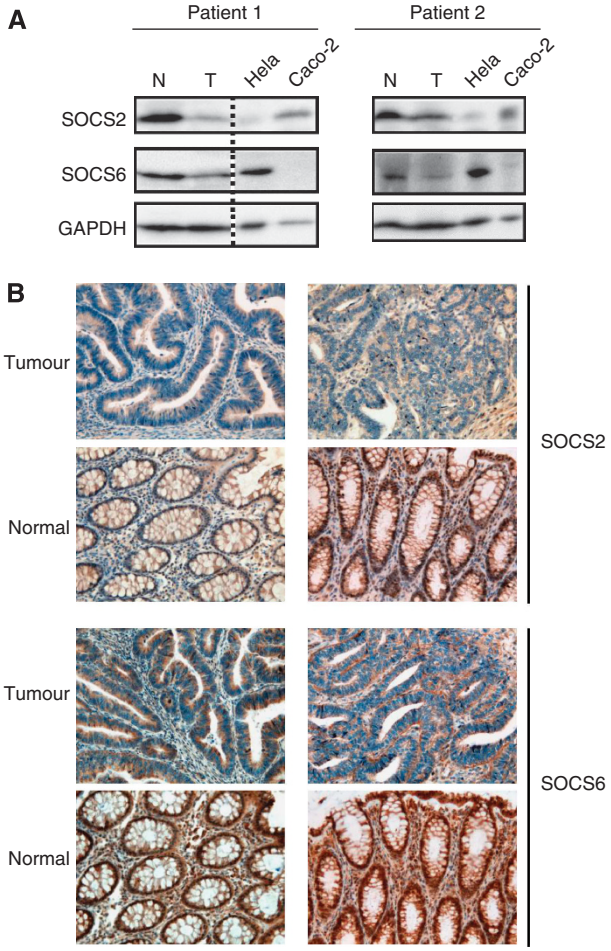

Figure 4. Protein levels of SOCS2 and SOCS6 in human CRC. (**A**) Western blot detection of SOCS2 and SOCS6 in CRC and distant normal counterpart tissues. Caco-2 and Hela cells served as positive control for SOCS2 and SOCS6, respectively. The samples were run on the same gel but the membrane was cut for representative purposes (dotted line). (**B**) Immunohistochemical staining for SOCS2 and SOCS6 in CRC and normal counterpart for two different patients, magnification × 200.

Mechanisms contributing to reduced SOCS2 expression in colorectal carcinoma. Over the past years, hypermethylation of promoters has been reported for several SOCS family members (Elliott *et al*, 2008; Culig, 2013). Along this line, we decided to analyse whether promoter methylation could explain the observed downregulation of SOCS2 and SOCS6. First, we investigated the expression of SOCS2 and SOCS6 in CRC cell lines using quantitative real-time PCR. Caco-2 cells show a high expression of SOCS2 whereas HT-29 and LS174t have a low expression level of SOCS2 and SOCS6 (Supplementary Figure 3). To analyse whether methylation could explain the observed results, we treated HT-29 and LS174t with the demethylating agent 5-aza-dC/DAC. DAC treatment barely changed basal expression levels of SOCS6. In contrast, an increase in basal expression was observed for SOCS2 after DAC treatment in HT-29 and LS174t cells (Figure 5A), suggesting that methylation of the *SOCS2* promoter is responsible for the reduced expression. However, as DAC demethylates the entire DNA, an indirect effect cannot be excluded. Thus, we decided to analyse the methylation of 16-17 CpG sites within *SOCS2* and *SOCS6* promoter using pyrosequencing for an initial set of 12 patients. The sequence analysed for *SOCS6* did not reveal methylation, which correlates to the unchanged basal expression of *SOCS6* after DAC treatment (data not shown). However, *SOCS2* pyrosequencing analysis showed methylation in the analysed sequence (− 1271 to − 1166 from the start codon) for ∼17% (2 out of 12) of the patient samples (data not shown). Of note, methylation was not uniform among all the CpG sites analysed, indicating that the investigation

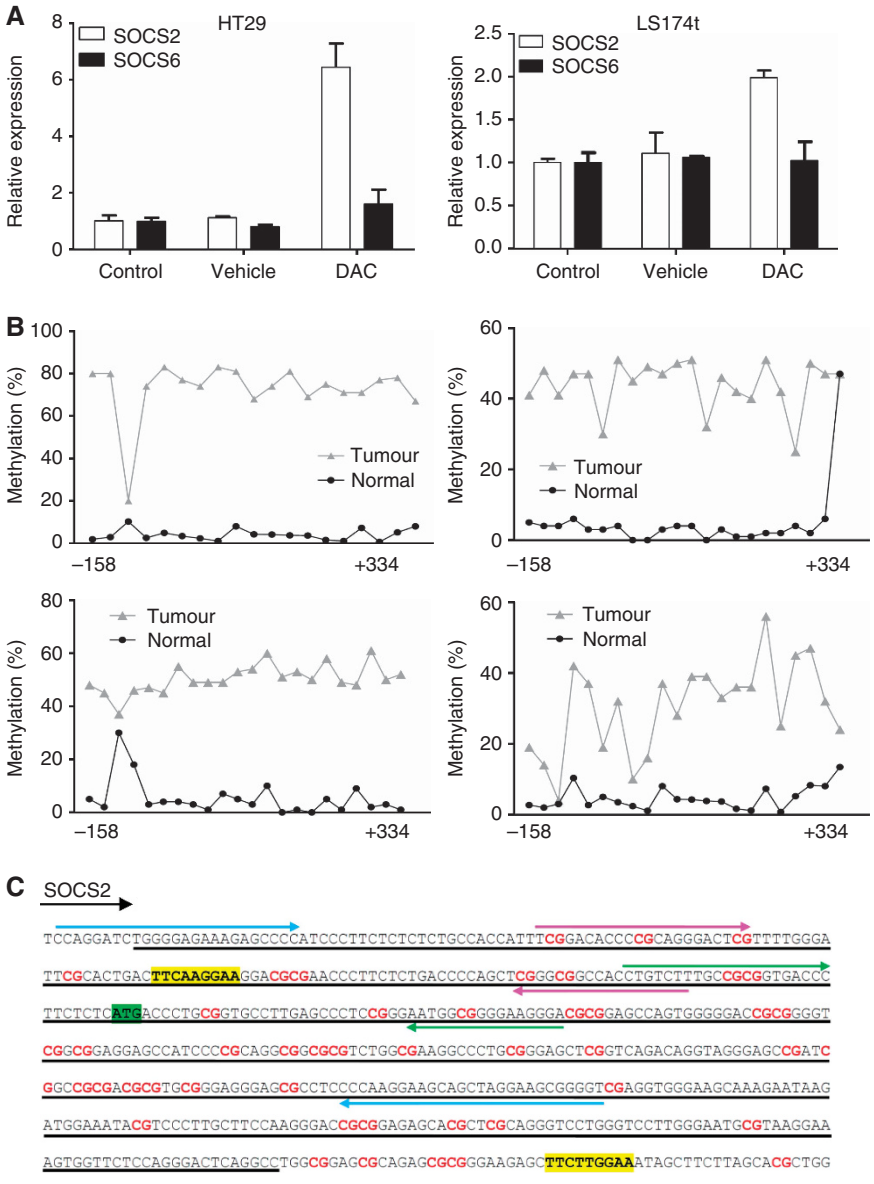

Figure 5. Methylation analysis of the SOCS2 promoter in primary human CRC samples. (**A**) Treatment with 5-aza-dC/DAC increases basal SOCS2 expression in LS174t and HT-29 colon cancer cell lines whereas SOCS6 levels are unchanged. Data are representative of four independent experiments and presented as mean ± s.d. (**B**) Representative histogram of the methylation pattern of four primary human CRC samples compared to their respective normal counterpart in a sequence − 158 to + 334 relative to the start codon. Each CpG site analysed is represented by a dot. (**C**) Representation of the *SOCS2* promoter region that shows differential methylation between tumour and normal control samples. The sequence analysed by mass array is underlined and the CpG sites are highlighted in red. Analysis of the methylated sequence shows the presence of 2 STAT GAS motifs (TTCnnnGAA) highlighted in yellow. The start codon is indicated in green. The primers used in other SOCS2 methylation studies are indicated (Liu *et al*: pink arrows; Fiegl *et al*: green arrows; Sutherland *et al*: blue arrows).

of a small number of CpG sites may in general be problematic. We thus decided to assess *SOCS2* promoter methylation in more detail. We used the mass array technology in order to determine the methylation pattern of the entire promoter sequence and analysed the methylation profile of 28 CRC patients as well as 20 normal non-tumour samples. Among these, we had 16 paired tumour/non-tumour samples. Tumour-specific methylation was detected in 3 out of 16 tumour samples from these paired samples in a sequence around the start codon (-158 to $+334$ from the start codon; Figure 5B and C). Importantly, all of the non-tumour samples showed an absence of methylation (defined by a methylation signal of $<5\%$). Overall, 7 out of 28 (25%) CRC samples were methylated in the *SOCS2* promoter region. Furthermore, the same sequence was also highly methylated in HT-29 and LS174t cells expressing low levels whereas no methylation was observed for the Caco-2 cells that express high levels of SOCS2 (data not shown). Strikingly, analysis of the methylated sequence revealed the presence of the consensus motif TTCnnnGAA, which all STAT members (except STAT6) recognise (Kang *et al*, 2013; Figure 5C). Taken together, methylation of the *SOCS2* promoter can partly explain the downregulation of SOCS2 expression in our patient collection.

Most interestingly, recent reports have highlighted the crucial role of the glucocorticoid receptor (GR) for the induction of SOCS2 *in vivo* (Tronche *et al*, 2004; Martinez *et al*, 2013). We thus determined the expression levels of GR, which often acts as a cofactor of STAT5 for GH-induced genes, and found that they are also dramatically downregulated in our CRC samples (Supplementary Figure 2B).

SOCS2 has a prognostic value in early colorectal carcinoma. We next wanted to evaluate whether SOCS2 and SOCS6 have prognostic value for CRC. As patients affected by CRC are often already advanced in age at diagnosis and may die from another reason rather than from colon cancer, we decided to analyse the disease-free survival rates in CRC patients. For this we combined two datasets (GSE3958 and GSE14333), which provide follow-up data for the patients. Dividing the data into low and high SOCS2 or SOCS6 expression we observed a difference in disease-free survival for SOCS2, which reached statistical significance ($P=0.036$). (Figure 6A). We then extended our analysis towards the different CRC stages. Interestingly, while only taking early CRC stages into account, namely stages I and II (lymph node-negative cancer), we confirmed the significantly shorter disease-free survival time associated to patients with low expression compared to patients with high expression of SOCS2 (Figure 6B, $P=0.00066$). The strong increase in statistical significance for stage I/II patients argues for a prognostic value of SOCS2 expression in early CRC stages.

DISCUSSION

Selection of the most beneficial treatment regimens in CRC remains challenging due to lack of prognostic markers. In this study, we analysed the expression pattern of the SOCS family members in fresh-frozen primary samples of colon tumours. Laser microdissection was used to dissociate stromal from tumour cells and obtain highly enriched neoplastic cell populations, allowing us to specifically assess the levels of SOCS expression in tumour cells. Here we show for the first time that SOCS2 and SOCS6 display a reduced expression at the mRNA and protein level in CRC patients, identifying them as potential diagnostic markers. Furthermore, SOCS2 downregulation is partly due to methylation and is an independent predictor of shorter disease-free survival.

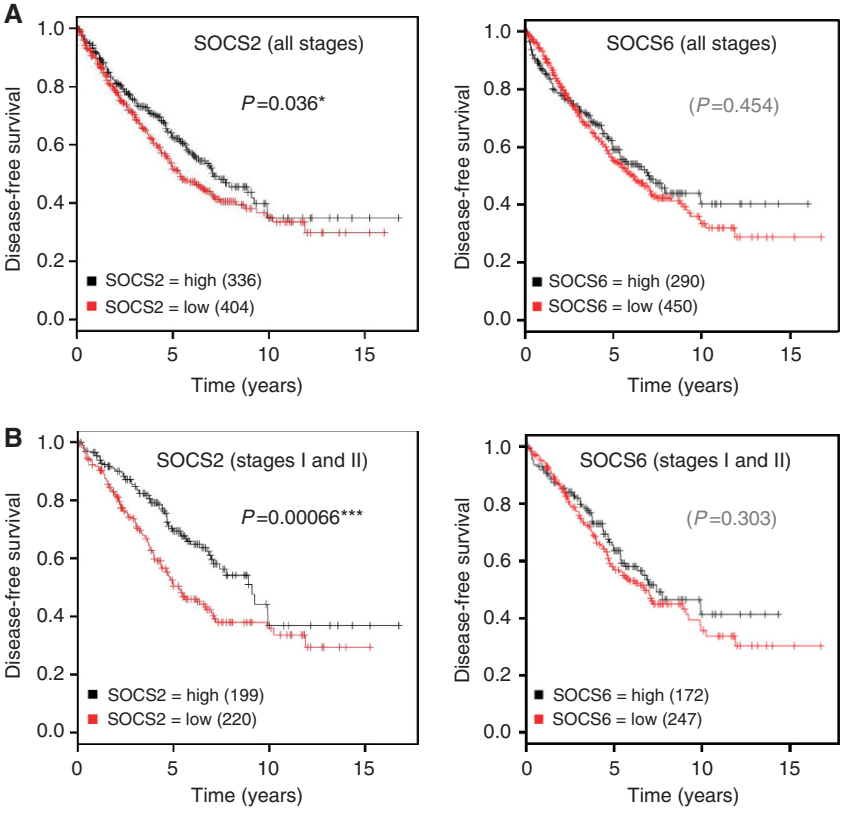

Figure 6. Prognostic value of SOCS2 and SOCS6 in CRC. Disease-free survival curves for patients with high and low expression of SOCS2 or SOCS6 in primary CRC including (**A**) all stages or (**B**) stages I and II only. The number of patients included in each group is mentioned within brackets. Significant *P*-values are indicated.

A bioinformatic study followed by a validation in a second group of fresh-frozen normal and colon cancer tissue specimens identified two potential biomarkers, SOCS2 and SOCS6, for CRC. Importantly, SOCS2 and SOCS6 expression levels allow us to discriminate between healthy and CRC cases, further emphasising their use as diagnostic biomarkers. These data validate and support bioinformatics and biostatistics as an appropriate starting point for the analysis of complex human diseases and discovery of novel biomarkers. SOCS2 has been associated with growth hormone (GH) signalling and is thereby involved in cell growth (Metcalf *et al*, 2000; Horvat and Medrano, 2001; Greenhalgh *et al*, 2005). Most importantly, *SOCS2* deletion in mice promotes the spontaneous development of intestinal tumours driven by mutations in the APC/β-catenin pathway (Newton *et al*, 2010). Along this line, it has been shown that forced overexpression of SOCS2 inhibits proliferation of the Caco-2 colon cancer cell line (Miller *et al*, 2004). Functional studies in mice have further shown that the disruption of one allele of SOCS2 in GH transgene mice leads to an increase in colon and jejunal crypt proliferation, thus favouring the formation of hyperplastic and lymphoid polyps in the colon (Michaylira *et al*, 2006). These findings provide evidence that SOCS2 normally limits tumour growth and strongly supports its tumour suppressive potential. Accordingly, low SOCS2 gene expression has been associated with diverse cancers (Trengove and Ward, 2013). However, until now there is no report on SOCS2 levels in human CRC. Here we show that SOCS2 levels are downregulated on mRNA as well as on protein levels in CRC patients. Interestingly, SOCS2 protein expression was associated with high differentiation and a low proliferation rate in breast carcinoma (Farabegoli *et al*, 2005). A low SOCS2 expression in prostate cancer is reported to be associated with an increased incidence of metastasis and SOCS2 mRNA levels decrease during prostate cancer progression (Hendriksen *et al*, 2006; Iglesias-Gato *et al*, 2014). However, SOCS2 expression in prostate cancer is controversial among the different studies (Hoefer *et al*, 2013; Zhu *et al*, 2013; Iglesias-Gato *et al*, 2014). In contrast to the studies conducted in breast cancer, there is no progressive decrease in SOCS2 expression along CRC staging. As SOCS2 is already downregulated in the adenoma samples, SOCS2 might be considered as an early diagnostic marker that enables diagnosis and treatment of patients susceptible to CRC at an early stage in cancer development. Furthermore, as we do not detect a progressive decrease in SOCS2 expression along the different cancer stages, our data suggest that reduced expression often occurs during the adenoma to CRC sequence. Altogether, *SOCS2* may not be a progression marker gene, but could represent an early diagnostic marker for CRC.

In CRC, loss of *SOCS6* gene copy number was found in 54.2% of the patients (Storojeva *et al*, 2005). SOCS6, by regulating insulin signalling, is thought to participate in CRC progression (Boyd, 2003). In addition, ectopic expression of SOCS6 was shown to suppress cell growth and colony formation (Lai *et al*, 2010). Accordingly, our study confirms downregulation of SOCS6 in CRC. In contrast to SOCS2 whose expression is downregulated in colon adenoma and more significantly in CRC, expression of SOCS6 is only downregulated in CRC. Thus, SOCS2 could have a higher diagnostic value as it may serve to diagnose CRC at a very early stage. Interestingly, a recent study by Qiu *et al* (2013) also showed reduced expression of the same two members of the SOCS family, SOCS2 and SOCS6, in hepatocellular carcinoma. This raises the hypothesis that both proteins could be regulated via a common mechanism and that both SOCS proteins may regulate hand in hand growth signalling in cancer.

In contrast to our study, several studies have shown a lower expression of SOCS1 and SOCS3 among other cancer types (Culig, 2013). Thus, their functions seem to be highly dependent on cell and tumour types. Interestingly, SOCS1 and SOCS3 may play an important role in inflammation-induced CRC as both were overexpressed in IBD according to our bioinformatic study. Along this line, SOCS1 and SOCS3 are often associated with inflammatory disorders in humans (Trengove and Ward, 2013).

One possible mechanism that explains downregulation of SOCS proteins in cancer is methylation in their gene promoter region. CpG islands of the *SOCS2* gene were shown to be hypermethylated in endometrial cancer (Fiegl *et al*, 2004), 6.5% of glioblastoma patients, 14% of primary ovarian cancers (Sutherland *et al*, 2004) and 43–63% of melanoma patients (Marini *et al*, 2006; Liu *et al*, 2008). However, methylation of *SOCS2* could not be found in human breast cancer patients (Sutherland *et al*, 2004). In our study, *SOCS2* methylation occurred in 25% of CRC patients. Importantly, the sequence throughout the *SOCS2* promoter, which showed methylation compared to the normal samples was already previously analysed and showed methylation in three studies performed on endometrial, ovarian and melanoma cancer patients (Fiegl *et al*, 2004; Sutherland *et al*, 2004; Liu *et al*, 2008). Strikingly, a detailed analysis of the methylated sequence revealed the presence of two STAT GAS motifs, which could explain the downregulation of SOCS2 in CRC as STAT factors are the classical inducers of SOCS family members (Kang *et al*, 2013). It is further suggested that allelic loss and promoter hypermethylation may account for the major mechanisms leading to SOCS6 inactivation (Lai *et al*, 2009, 2010). However, we could not identify *SOCS6* methylation in our CRC patient cohort. We identified a second mechanism that could contribute to the reduced expression of SOCS2 by demonstrating that the expression levels of the GR are reduced in CRC patients. As regulation of SOCS2 is also dependent on a synergistic action of STAT5 and GR (Tronche *et al*, 2004; Mueller *et al*, 2012; Martinez *et al*, 2013), SOCS2 downregulation in CRC patients may additionally be explained by the reduced expression of the coactivator *GR*. Further studies are needed to shed more light on the diverse mechanisms of SOCS2 downregulation. Growth hormone signalling components such as GH itself or its cell-surface receptor can also impact on SOCS2 expression levels. Initial results indicate that GH receptor is also downregulated in our CRC samples (data not shown) and may thus contribute to the reduced SOCS2 levels. Current investigations focus on the regulation of other GH signalling components in order to clarify the role of this pathway in CRC. In this context the extent of STAT5 activation will also be of interest as increases in STAT5 phosphorylation will most likely impact on SOCS2 expression (Greenhalgh *et al*, 2002).

A significantly higher disease-free survival time was observed in patients with high compared to low SOCS2 expression in early CRC stages (stages I and II). It can be speculated that patients with especially low expression of SOCS2 at the early CRC stage may display an exaggerated response to growth-promoting signals such as GH. At later stages this signal may lose some of its significance as the growth-promoting mechanism may increasingly drive tumour development. Along this line, patients with high SOCS2 expression had an improved survival rate and high SOCS2 expression proved to be an independent predictor for good prognosis in breast cancer (Haffner *et al*, 2007). Furthermore, reduced expression of SOCS2 and SOCS6 correlate with poor prognosis in hepatocellular carcinoma patients (Qiu *et al*, 2013). However, in our study SOCS6 was not associated with disease outcome. This is in line with two studies performed in prostate cancer and CRC, for which no correlation between SOCS6 mRNA expression and patient survival was found (Storojeva *et al*, 2005; Zhu *et al*, 2013). Importantly, up to 30% of stage II patients relapse after surgery and many of them will die due to metastatic disease. Therefore, it is highly important to identify clinical and molecular

determinants of outcome in order to target treatments to those patients who are destined to relapse. Our data thus suggest that SOCS2 is an early diagnostic marker for CRC and might be useful as a prognostic marker to delineate a high-risk population at an early CRC stage.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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