Spinophilin loss correlates with poor patient prognosis in advanced stages of colon carcinoma

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Statement of translational relevance

Our data is the first report to show that Spn downregulation contributes to a more aggressive biological behavior induces chemoresistance and is associated with a poorer survival in patients with advanced stages of cancer, in this case colorectal carcinoma. Therefore the identification of the levels of Spn in advanced stages of colorectal tumor biopsies might contribute to select patients which could and could not benefit from current chemotherapy. Spn, also known as PPP1AR9B, is a regulator of PP1a, a phosphatase regulating many crucial aspects such as cell cycle G1 transition through the regulation of retinoblastoma phosphorylated status. Spn is located in the 17q21 region. 17q21 is frequently associated with microsatellite instability and loss of heterozygosity in cancer and therefore, our work might uncover the relevance of Spn, as relevant gene inside this locus, also in other tumors.
ABSTRACT

Background. The genomic region 17q21 is frequently associated with microsatellite instability and loss of heterozygosity in cancer, including gastric and colorectal carcinomas (CRC). This region contains several putative tumor suppressor genes, including Brca1, NM23, prohibitin and spinophilin (Spn, PPP1R9B, neurabin II). The scaffold protein Spn is one of the regulatory subunits of phosphatase-1 (PP1) that targets PP1 to distinct subcellular locations and couples PP1 to its target. Thus, Spn may alter cell cycle progression via the regulation of the phosphorylation status of the retinoblastoma protein, a direct target of PP1. Therefore, we analyzed whether Spn levels were reduced in CRCs and whether Spn levels correlated with prognosis or response to therapy.

Results. Spn was lost in a percentage of human gastric, small intestine and CRCs. In patients with CRC, tumoral Spn downregulation correlated with a more aggressive histological phenotype (poorer tumor differentiation and higher proliferative Ki67 index). Consistent with this observation, lower Spn protein expression levels were associated with faster relapse and poorer survival in patients with stage III CRC, particularly among those receiving adjuvant fluoropyrimidine therapy. We validated this result in an independent cohort of metastatic CRC patients treated with standard chemotherapy. While patients that achieved an objective tumor response exhibited Spn levels similar to non-tumoral tissue, non-responding patients showed a significant reduction in Spn mRNA levels.

Conclusion. Our data suggest that Spn downregulation contributes to a more aggressive biological behavior induces chemoresistance and is associated with a poorer survival in patients with advanced stages of colorectal carcinoma.
INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors worldwide (1). Despite advances in early detection, approximately one-third of patients present with metastatic disease at diagnosis, and ~40% of patients with early-stage tumors eventually relapse over the course of their disease (2). Systemic therapy is the mainstay of care for patients with metastatic CRC (mCRC). Several combination regimens, including fluoropyrimidines and oxaliplatin and/or irinotecan, with or without monoclonal antibodies targeting VEGF or EGFR, have been successfully developed and are associated with response rates of 40-60% and a median survival of 20-24 months (3-5). Despite the undeniable progress, a considerable proportion of patients do not respond to therapy, and reliable tools are needed to prospectively identify those patients who are more likely to benefit.

Colon carcinogenesis represents a stepwise progression from benign polyps to invasive adenocarcinoma that is believed to be the consequence of multiple sequential genetic and epigenetic alterations (6). Approximately 15 driver mutations have been identified to be relevant in CRC carcinogenesis. These mutations affect cell proliferation, migration, differentiation, adhesion, cell death and DNA stability and repair (7). The most commonly involved pathways include the Wnt/\(\beta\)-catenin, TGF-\(\beta\)/BMP, TP53, receptor tyrosine kinases, K-RAS and PI3K signaling pathways (8).

Furthermore, a number of chromosomal regions are altered in colorectal carcinomas (9). Among them, 17q21 is lost in a high percentage of colorectal and gastric carcinomas (10-13). This is a region frequently associated with microsatellite instability and loss of heterozygosity. This region contains several putative tumor suppressor genes, including Brca1, NM23, prohibitin and spinophilin. We have previously found that Spn mRNA is lost in a percentage of renal carcinomas and lung adenocarcinomas (14, 15). Interestingly, analysis of normal vs. tumoral Central Nervous System samples showed a clear downregulation of Spn in tumoral samples (14). Furthermore, lower levels of Spn mRNA correlated with a higher grade in ovarian carcinoma and chronic myelogenous leukemia (14).
Spinophilin (also known as neurabin II and PPP1R9B) was isolated independently by two laboratories and has been shown to interact with protein phosphatase 1 (PP1) and F-actin (16, 17). Genetic and biochemical analyses have revealed a number of distinct modular domains in Spn that govern protein–protein interactions (17-21). Spn may contribute to tumorigenesis through PP1/pRb regulation (22). Accordingly, the loss of Spn maintains higher levels of phosphorylated Rb. This effect contributes to an increase in p53 activity through an increase in the ARF protein. However, in the absence of p53, reduced levels of Spn increase the tumorigenic properties of cells. In vivo, Spn Knock-out mice have a reduced lifespan, an increased number of tumors and increased cellular proliferation (23). In addition, the combined loss of Spn and p53 activity leads to an increase in mammary carcinomas, confirming the functional relationship between p53 and Spn.

Taking into consideration the relationship of Spn with cell cycle regulation, the effect of Spn loss in KO models and its previously suggested role as a tumor suppressor in other tumors, the objective of the present study was to explore the role of Spn in colorectal cancer. With this purpose we assessed the functional consequences of Spn loss in CRC tumors and its impact in the clinical outcome of patients with stage II-IV CRC.

MATERIALS AND METHODS

Patients population and tumor samples

In the present work, we analyzed 2 different cohorts of patients, whose main characteristics are summarized in supplementary Tables 1 (cohort 1) and 2 (cohort 2). Cohort 1 included patients with stages II and III CRC, while cohort 2 comprised patients with metastatic stage IV CRC.

Cohort 1. Cohort 1 comprised 218 patients with surgically resected stage II and III CRC. High risk stage II and all stage III patients received adjuvant fluoropyrimidine chemotherapy following surgery, unless medically contraindicated. Tissue arrays were generated with formalin-fixed, paraffin-embedded surgical primary tumor specimens for immunohistochemical staining.

Cohort 2. Cohort 2 was used to study Spn mRNA levels. This cohort comprised 86 tumor samples and 33 non-tumor control samples from metastatic CRC patients.
(stage IV). Following surgical resection, tumor tissue samples were collected from all patients and immediately snap-frozen at −80°C until further use. All patients were treated with fluoropyrimidine-based chemotherapy plus oxaliplatin or irinotecan until disease progression. Patients were classified according to best response to chemotherapy in two groups: responders (N=46) or those that achieved an objective response (complete or partial response), and non-responders (N=40) or those whose best response to therapy was either stable or progressive disease. Tumor response was evaluated by conventional methods according to the standard RECIST 1.0 criteria: a complete response (CR) was defined as the disappearance of all measurable and evaluable evidence of disease; a partial response (PR) was defined as a > 30% decrease in the sum of the longest diameters of target lesions; stable disease (SD) was considered if the tumor burden decreased less than 30% or increased less than 20%; and progressive disease (PD) was indicated by a >20% increase in the sum of the longest diameters of target lesions or the appearance of any new lesion. The study protocol was approved by the Institutional Review Board and Ethical Committee of participating institutions. All patients provided informed consent.

**Statistical analyses.** All statistical analyses were performed using the SPSS statistical package (16.0 for Windows). The potential association between continuous or categorical variables was assessed by the Pearson correlation coefficient or by the Chi-squared or Fisher’s exact test, respectively. The distribution of quantitative variables among different study groups was assessed using parametric (Student t test) or non-parametric (Kruskal-Wallis or Mann-Whitney) tests, as appropriate. Disease-free interval (DFI) and overall survival (OS) of patients were calculated using the Kaplan-Meier method, and differences among subgroups were assessed by the log-rank test. The Cox proportional hazards method was employed for multivariate analysis to assess the influence of Spn expression on DFI and OS, while adjusting for other potential confounding factors such as age, TNM stage, tumor grade, or adjuvant chemotherapy. A p value of <0.05 was considered significant.

**Histopathology and immunohistochemistry.** Tissue or tumor samples were dissected and fixed in 10% buffered formalin. Formalin-fixed tissues were
embedded in paraffin, and a tissue microarray (TMA) was generated. For histopathologic analysis, TMAs were sectioned (2.5 μm) and stained with H&E. The slides were baked overnight at 56°C, deparaffinized in xylene, rehydrated, and washed with PBS. Epitope retrieval was performed using sodium citrate (pH 6.5). Endogenous peroxide activity was quenched with 1.5% hydrogen peroxide in methanol for 10 min. Incubation (40 min) was performed with anti-spinophilin, which was purchased from Chemicon (AB5669). The secondary antibodies used were as follows: horseradish peroxidase goat anti-rabbit IgG (Dako) diluted 1:50; horseradish peroxidase goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:50; and horseradish peroxidase rabbit anti-goat IgG (Dako) diluted 1:50. After incubation, immunodetection was performed with the peroxidase-based PAP system (DAKO) using diaminobenzidine as substrate. Incubations omitting the specific antibody were used as a negative control. Spn protein tissue expression intensity was assessed twice by an investigator blinded to the clinical information. A discrete value was assigned to each observation ranging from 0 (no Spn expression) to 3 (high expression, similar to normal colon tissue), and the average value calculated for each tumor. Score was obtained by multiplying the intensity by the percentage of positive cells.

RNA isolation from samples. Total RNA, containing small RNA, was extracted from tumor and normal tissue control samples by the mirVana miRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. The total RNA yield was determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Tech, DE, USA).

Spn Quantitative RT-PCR. Total RNA was extracted using TRI-Reagent (Sigma) according to the manufacturers’ instructions. After RNA isolation, DNAse I (Roche) treatment was performed using 40 units of enzyme in the presence of 40 units of RNAse inhibitor (Roche) per sample at 37°C for 1 h. Following phenol-chloroform purification, the RNA was quantified using a NanoDrop® 1000 spectrophotometer and quality-checked using a MOPS-1,2 % agarose gel. cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with 1 μg of total RNA as the starting
material for each sample. The PCR reactions were performed in 96-well optical plates (Applied Biosystems) with an ABI PRISM® 7900HT sequence detection system (Applied Biosystems). The reaction mix was prepared using 1x SYBRGreen Master Mix (Applied Biosystems), 50 nM of each primer [Spn: 5’ GCCCAGCTAATTCAGCAGAC 3’ (forward), 5’ GGAGCTCCTTGAACTTGTGC 3’ (reverse); GAPDH: 5’ GAGTCAACGGATTTGGTCGT 3’ (forward), 5’ TTGATTTTGGAGGGATCTCG 3’ (reverse)], and 100 ng of cDNA in a final volume of 50 µl. The cycling conditions were set to 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Finally, a dissociation curve was generated to check the amplification specificity. The results were analyzed, and the relative mRNA quantity was calculated using the qBasePlus software. Cyclophilin (ref. 4326316E), an endogenous control, was used to normalize variations in cDNA quantities from different samples. Each reaction was performed in triplicate with cDNA from tumor tissues from each patient.

Methylation-Specific PCR (MSP). Genomic DNA was isolated from fresh tissue using QIAamp DNA Mini Extraction Kit (Qiagen Inc, Valencia, CA, USA) and DNA concentration was measured by 260 nm absorbance. Total genomic DNA (1 µg) was treated with sodium bisulfite reagent using the EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA) following the manufacturer’s protocol. In each MSP reaction, 40 ng of final product was used in a 20 µL volume containing 10 µL 2x Zymo Taq DNA polymerase master mix (Zymo Research, Orange, CA), 0.25mM of dNTP and 0.3 µM of each primer in a G-Storm Thermal Cycler (Gene Technologies, Essex, UK). For human SPN, one pair of methylated (M) primers and one pair of unmethylated (U) primers covering the same region were used to analyze DNA methylation at the 5′ proximal region within the CpG island identified by UCSC Genome Bioinformatics (http://genome.ucsc.edu). The primers used in MSP are: MSP-M, forward, 5’- TTGGTTAGTCGTTTGGTGAAGG-3’, reverse, 5’- TAATAAAGCCTTAATACCTCGT-3’, and MSP-U, forward, 5’- AATTTGTTAGTTGTTGGTGAAGTG -3’, reverse, 5’- AATAAACCCCTTTAACACATCTCAT-3’. Each reaction was heated at 95 °C for 10 min, followed by 40 cycles of 30 sec at 95 °C, 30 sec at 62 °C (primers specific for methylated C) or 30 sec at 57°C (primers specific for unmethylated C), and 30 sec at 72°C, followed by a final 10-minute extension at 72°C. Ten microlitres of each PCR
reaction were loaded directly in 2% agarose gels, stained with ethidium bromide, and visualised under ultraviolet (UV) illumination.

**Cell culture, retroviral vectors and gene transfer.** The cells were grown and processed following previously described (24).

**Colony formation.** Briefly, CRC cells were transfected with the indicated construct. After 16 h, a glycerol shock was performed, and the cells were cultured at 37°C. Forty-eight hours after transfection, the cells were selected with 0.5 μg/ml of puromycin. After 12 days, the plates were fixed, and the colonies were stained with crystal violet.

**Growth in soft agar.** To measure anchorage-independent growth and foci formation, we followed a previously described protocol. Colonies were scored 3 weeks after seeding; all values were determined in triplicate.

**RESULTS**

**Loss of Spn in gastrointestinal tract tumors**

To first study whether Spn is downregulated in human CRC, we explored whether RNA or protein levels were decreased in tumor samples. To broadly analyze whether Spn RNA levels showed variations in CRC samples, we first determined Spn mRNA expression levels using quantitative RT-PCR in several paired normal/tumoral tissue samples from the same patient (cohort 2). Approximately 25% of CRC tumors showed a greater than 50% decrease in Spn mRNA levels compared to normal colonic tissue (Fig. 1A). Interestingly, a percentage of samples from gastric (35% aprox) and intestinal (15%) carcinomas also showed partial or complete loss of Spn mRNA expression (Fig. 1A).

To confirm this result by specifically exploring the presence of Spn protein, we generated a tissue array of 218 human CRC, 20 gastric and 20 small intestinal tumors at different stages and with different histopathological grades and compared them to normal tissue (cohort 1). We found that 70% of CRC
tumors displayed high Spn levels (mean values 2.5 - 3), similar to the values observed in normal colonic tissue, 20% showed intermediate Spn levels and approximately 10% of tumors showed no expression of Spn (Fig. 1B and 1C). Similar data were obtained with other gastrointestinal tumors. Small intestine tumor samples showed Spn protein levels similar to those of the CRC samples. However, a significantly greater proportion of gastric carcinomas showed a complete loss of Spn expression, as 30% of tumors had very low or null levels of Spn (Fig. 1B).

Due to the relevance of CRC and the availability of larger number of tumor samples, we focused on this malignancy to validate the role of Spn as a prognostic and/or predictive factor.

A similar proportion of cases showed reduced Spn levels independent of whether the analysis was performed on mRNA or protein, suggesting that Spn downregulation occurs in most cases through the regulation of mRNA levels. To further explore this result, we analyzed SPN promoter methylation in 32 metastatic CRC samples and found that this gene was not methylated in any of the tumor samples analyzed (Supplementary Fig. 1).

Causal effect of Spn loss in colon and stomach tumor cell lines

To evaluate the potential of Spn to act as tumor suppressor, we analyzed the effect of altering Spn levels by ectopically overexpressing Spn cDNA in colon tumor cell lines. Due to the functional relationship between Spn and p53 loss (22), we selected tumor cell lines with mutant or WT p53. To that end, we used the colon carcinoma cell lines COLO205 (with wild type p53) and HT29 and SW480 (with mutant p53). These cells express normal Spn mRNA (Fig. 1D). To evaluate the effect of Spn overexpression, we transfected the cells with Spn cDNA under constitutively expressing promoters. Overexpression of Spn significantly reduced the number of colonies that were able to grow in all cell lines independently of p53 status (Fig. 1E). Next, we selected a population of cells expressing the Spn gene and seeded them in soft agar to evaluate their ability to grow with contact inhibition. We observed that cells that overexpressed Spn had reduced growth in soft agar, forming fewer colonies (Fig. 1F) that were smaller in size (data not shown). On the other hand, the
reduction of Spn levels by means of overexpression of Spn shRNA increased the number of colonies growing at low density and in the presence of contact inhibition (Supplementary figure 2).

Therefore, our data suggest that Spn loss in CRC tumors might contribute to tumor cell malignancy and that the growth inhibitory effect induced by Spn overexpression is independent of the presence or absence of mutated p53 in colon tumor cell lines.

**Correlation of Spn loss with other clinical and pathological features in colon carcinoma samples.**

To evaluate whether Spn levels were associated with other clinical parameters, we correlated immunohistochemical staining results or mRNA levels with tumor stage, gender, age, tumor primary location or differentiation. We found no correlation between Spn levels (either protein or mRNA) and other clinical parameters used in this study except for tumor grade of differentiation. We observed that Spn loss was significantly correlated with a less differentiated phenotype (Fig. 2) in metastatic tumors (p<0.05), and a similar trend was observed in earlier stages of disease (data not shown).

Furthermore, as Spn loss might increase cell cycle progression by maintaining high levels of phosphorylated retinoblastoma protein and increasing the replicative capacity of cells, we assessed the correlation between Ki67 proliferative index in tumors and Spn expression levels. We found that Spn loss correlated with an increased percentage of cells showing nuclear Ki67 staining only in stage III tumors (stage IV tumors were not analyzed), and high levels of Spn in general correlated with lower Ki67 staining (Fig. 3). Although a large variability could be observed among samples, this correlation was significant (Pearson correlation, p=0.022) and indicated that cells with Spn downregulation seem to have a higher proliferative capacity.

Finally, we found no correlation between lower Spn levels and mutant p53 (data not shown).
Decreased Spn levels predicted a poor outcome in CRC patients with advanced stages of disease

To evaluate whether Spn levels were associated with clinical outcome, we correlated Spn immunohistochemical staining with patient disease-free interval (DFI) and overall survival (OS). Decreased Spn levels were associated with a poorer DFI (P=0.106) and OS (p=0.022) in patients with stage III CRC tumors, but not in those with stage II (Fig. 4). This negative effect of low Spn expression was particularly remarkable in stage III patients that had received adjuvant fluoropyrimidine chemotherapy following surgical resection (Fig. 5), suggesting Spn downregulation may also induce some degree of chemoresistance in tumor cells. However, multivariate analysis showed that a low Spn protein expression in tumors was associated with a shorter DFI (P=0.068) and OS (P=0.019), independent of other potentially confounding factors such as age or adjuvant chemotherapy (Table 1).

To further assess this hypothesis, we evaluated the association of Spn mRNA levels with tumor response to chemotherapy in an independent cohort of 86 patients with metastatic stage IV CRC tumors. The relative expression levels in responders versus non-responders to chemotherapy are represented in figure 6A. As shown, the transcription levels of Spn mRNA in colon tumor tissue samples of non-responders patients were significantly lower than those observed in tumors of patients that achieved an objective response to chemotherapy (P=0.017), and also lower than those detected in normal colon tissue samples (p=0.079).

These data suggest a potential role of Spn in modulating response to cytotoxic chemotherapy in advanced CRC. As a control, we measured BRCA1, a neighboring gene at the 17q21 location that is commonly related to the response to chemotherapy, and found that there was no variation in the BRCA1 mRNA levels between responders and non-responders (Figure 6B), highlighting the relevance of Spn as a predictive factor in metastatic CRC. Consequently, low levels of Spn mRNA were also associated with a shorter progression-free interval (PFI) and OS in this series of patients with mCRC (Fig 6C and 6D).
Discussion

We have investigated new genes whose alteration might contribute to pRb checkpoint deregulation and, therefore, to tumorigenesis. We explored the contribution of one of these genes, which is located in 17q21, to colon cancer tumorigenesis and therapy.

Spn is lost in a percentage of gastric, intestinal and colorectal carcinomas. Spn downregulation in colorectal carcinomas is correlated with de-differentiation and high Ki67 expression reflecting increased proliferation. The analysis of Spn downregulation and clinical outcome of patients with CRC showed that patients with stage III CRC with low levels of Spn exhibited lower survival and faster relapse, particularly in those receiving adjuvant therapy. Moreover, in this subset of patients, Spn expression significantly predicted a poorer survival independent of other relevant prognostic factors such as age or having received adjuvant chemotherapy. We validated this result in an independent cohort of metastatic CRC patients. While patients achieving an objective radiological response to chemotherapy had Spn levels similar to those of non-tumoral tissue, non-responder patients showed a significant reduction of Spn mRNA levels. In this independent cohort of patients with metastatic disease, our study also revealed that low Spn expression levels were associated with a shorter progression-free survival and a higher risk of death.

The Spn gene is located in chromosome 17 at position 17q21.33. This genomic region contains a relatively high density of tumor suppressor genes including the known (BRCA1, NME1), the putative (JUP, prohibitin), and unidentified candidates distal to the BRCA1 locus. This area is frequently associated with microsatellite instability and loss of heterozygosity (LOH). The fraction of tumor samples in the cohort analyzed in our work with low (less than 0.5 in tumor /normal) or no Spn expression is approximately of 25% which is in good agreement with previous reports assessing the allelic imbalance of this region (10-13).

Most of the studies on the 17q21 locus have focused on BRCA1, which exhibits a variable frequency in LOH (25-27). The relevant data regarding spinophilin LOH came from a study on primary lung carcinoma (28). Extensive
LOH mapping in human lung cancers involved 15 highly polymorphic markers, 7 of which span the 17q11-24 region. The higher LOH value appeared with the D17S588 marker, which showed a 53% loss. The D17S588 marker locates exactly within the spinophilin locus at 45.57 Mb (28). This association appears to be specific because closer tumor suppressor genes, such as BRCA1, were not seriously affected (6-13% LOH). Furthermore, several genetic linkage studies have suggested the existence of a tumor suppressor gene distal to BRCA1 (29, 30), indicating the importance of this locus in cancer pathology. LOH in chromosome 17q21.3, not linked to BRCA1, has been observed in different tumors, including colorectal, gastric, and lung carcinomas (14). The analysis of BRCA1 in our samples indicates that this gene does not influence the response to treatment in metastatic CRC patients, highlighting the relevance of Spn gene in 17q21 LOH.

However, in addition to Spn, the NME1 gene, which has also been proposed as a tumor suppressor candidate, maps to this location. After its isolation, NME1 was described as a putative metastasis-suppressor gene on the basis of its reduced expression in murine melanoma cell lines with high metastatic potential. However, the implications of NME1 in cancer are still not clear and may have distinct, if not opposite roles, in different tumors.

As published in previous works (22) loss of Spn induces a molecular response very similar to that described in oncogen-induced senescence. This effect seems to be due to the inactivation of pRb which lead to E2F1 activation, p14ARF transcription and consequently p53 activation. In this context, inactivation of p53 allows cells with lower levels of Spn to bypass senescence. This has been shown in mice (23) and in human lung tumors (31). We have data that this functional relationship between Spn loss and p53 mutations also exists in other tumors such as stomach tumors (Data not shown). However, this functional correlation was not observed in stage II/III colorectal carcinomas. In colorectal carcinomas K-ras and B-Raf mutations, which occur in around 45% and 5-10% of tumors, are relative early events in the tumorigenic process (8, 32). Since oncogenic Ras or B-Raf also induce a senescence response, it is plausible that Kras- or B-Raf-mutated tumors need to bypass senescence to progress, and that this might occur by genetic alterations other than p53 mutations (ie: p14ARF methylation or INK4 locus loss). Since Spn loss
seems to be a late event, when this happens the senescence barrier is bypassed with no need for further p53 mutations to occur. This hypothesis therefore suggests that the correlation between Spn loss and mutations in p53 is dependent on the molecular context.

The literature relating loss of function of tumor suppressors and apparition of chemoresistance is large. Either by inactivating mutations such as the case of p53, or PTEN, allelic loss (i.e. PTEN or the INK4a locus), by posttranslational modifications leading to misslocalization (i.e. Foxo3A or p27kip1), or by promoter hypermethylation (i.e. p73, MLH1 or Apaf1), the loss of function of many tumor suppressors has been consistently related to decreased survival or tumor response in the presence of chemotherapeutic drugs in vitro and in vivo. The mechanisms through which they induce resistance have also been unraveled in many cases (33, 34). Recently, the downregulation of microRNAs have been also described playing a role in chemoresistance. For example, loss of miR-181a and miR-630 expressions might inhibit cisplatin-induced cancer cell death in NSCLC (35).

A large number of antineoplastic agents rely on DNA-damage as the main mechanism of action for cytotoxicity. Indeed, such is the case for the three commonly used agents in colorectal cancer 5-fluorouracil, irinotecan and oxaliplatin (36). Mutations in the DNA mismatch repair genes MLH1 and MSH2 are associated to resistance to DNA-damaging agents (37). Cisplatin resistance has been attributed to defects in the mismatch repair mechanism due to hypermetylation of the MLH1 promoter (38). Interestingly, cell lines resistant to cisplatin remain sensitive to oxaliplatin (39) which is attributed to the different DNA-adducts formed by the two drugs (40). P53 is a sensor of DNA-damage launching the appropriate cellular response, arrest, apoptosis or senescence.

The tumor suppressor p53 is mutated in approximately 50% of human cancers and its function is compromised by different mechanisms in most tumors with wild type p53 (41). Lack of functional p53 contributes to drug resistance likely due to the inability to undergo apoptosis. For example, loss of p53 function correlated to decreased sensitivity to 5-FU and oxaliplatin but not to irinotecan- nor paclitaxel-based therapies (42). Furthermore, data from clinical trials suggest that doxorubicin is more active in p53-wild type cancers. Spn could contribute to oxaliplatin and 5-FU resistance by regulating p53.
phosphorylation and activity which is regulated by PP1 (43). However more work is necessary to confirm this hypothesis. Disruption of apoptosis downstream p53 may also contribute to chemoresistance (41, 42). However, while p53 mutations contribute to resistance to chemo- and radiotherapy, BRCA1/2 dysfunction leads to increased sensitivity to DNA-damaging agents in breast and ovarian tumors (44, 45). Other p53 family members, p73 and p63, have been related to chemoresistance by different mechanisms (46).

Also, activation of AKT, which may occur by activation of tyrosine kinase receptors, PI3Ks or Ras, or inactivation of the tumor suppressor PTEN or proapoptotic proteins also has been related in many cases to chemoresistance in solid tumors. Some of these targets are regulated by PP1, such as PTEN, AKT, Bad, Bax or BRCA1 (43, 47) and therefore, Spn may contribute to resistance by indirectly targeting some of these targets to the phosphatase. More work is warranted to confirm the mechanism of resistance involved.

In summary, our data show for the first time that the protein spinophilin, Spn, has prognostic and predictive value for advanced stages of colorectal cancer. Together with other reports on Spn (17-20)(22), our data suggest that the scaffolding protein Spn is important for the regulation of PP1α and pRb and that its absence may contribute to tumorigenesis in colorectal carcinomas. Mechanistically, the loss of Spn may induce a proliferative response by increasing pRb phosphorylation, which may influence the malignant phenotype and contribute to the failure of therapy in advanced stages of colorectal carcinoma.

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Table 1: Multivariate analysis for stage III tumors

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Adj-CT: adjuvant chemotherapy; HR: Hazard ratio; CI: confidence interval. Statistics were assessed with COX regression model.
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**Figure Legends**

**Figure 1: Loss of Spn in human CRC tumors.** A) The percentage of human colon, gastric and intestinal tumors showing low or no levels of Spn mRNA is shown. The results were obtained using quantitative-RT-PCR for Spn. Then, the ratio of Spn levels in tumor/normal samples was calculated. Only the tumors with a ratio of <0.5 were considered positive. B) The percentage of human colon, gastric and intestinal tumors showing high, medium or low levels of Spn protein is shown. C) Representative pictures of normal crypt and different CRC tumors with different Spn levels are shown. The visual score is indicated in parentheses. D) The cells used in this study express Spn. The cells were grown to 80% confluence, and the mRNA was extracted. The figure indicates the RT-PCR results for Spn as indicated in the Materials and Methods section. E and F) The effects of Spn overexpression were determined. Colon cancer cell lines were infected with a retrovirus carrying Spn cDNA (grey bars) or empty vector (black bars). After selection, the cells were seeded at low density to measure clonogenicity (E) or the ability to growth in soft agar (F) as indicated in the Materials and Methods section. The graph shows the average of three independent experiments; the bars indicate the standard deviation.

**Figure 2: The relationship between Spn levels and the histological grade of metastatic CRC tumors was analyzed.** The levels of Spn mRNA were analyzed in metastatic CRC tumors (stage IV) using Q-RT-PCR. A correlation was established with the differentiation degree of each tumor. Statistical analyses showed borderline significance (p=0.062) using an ANOVA comparison among all 3 groups and strong significance (p=0.005) using Student’s T-test comparison between well and poorly differentiated groups. The upper pictures represent different tumors with different differentiation degrees and different Spn levels by immunohistochemistry. Right picture: well differentiated, centre picture: moderately differentiated, left picture: poorly differentiated.

**Figure 3: The relationship between loss of Spn and Ki67 nuclear staining in advanced CRC tumors.** Cohort I samples, containing stage II and III cases, were stained for Ki67, and the percentage of tumor cells showing nuclear staining were counted. This percentage of Ki67-positive cells correlated with Spn levels (Pearson correlation). A) The graphs indicate a lack of correlation between loss of Spn and Ki67-positive cells in stage II samples (r=0.049, p=0.577) and a correlation for stage III tumors (r=-0.251, p=0.022). B) Examples of pictures showing the Spn/Ki67 correlation in the same patient sample. Three tumors are shown (P1, P2 and P3) stained either for Spn or Ki67. Spn levels are as follows: Low: <100; Medium: 100≤x<200; High: ≥200.

**Figure 4: Kaplan-Meier curves of overall survival (A and B graphs) or disease-free interval (C and D graphs) according to Spn levels.** The analysis is grouped by tumor stage. A and B) Overall survival according to Spn levels is shown. The log rank test showed a non-significant correlation (Chi square =0.575; p=0.75) for stage II patients, while showing a significant correlation for stage III tumor patients (Chi square =7.677; p=0.022). C and D) Disease-free interval according
to Spn levels was analyzed. The log rank test showed a non-significant correlation (Chi square =0.263; p= 0.877) for stage II patients, while showing a borderline significant correlation for stage III patients (Chi square =4.487; p= 0.106). Spn levels are as follows: Low: <100; Medium: 100≤x<200; High: ≥200.

**Figure 5:** Kaplan-Meier curves of overall survival (A and B graphs) or disease-free interval (C and D graphs) with or without adjuvant chemotherapy according to Spn levels in patients with Stage III CRC. The analysis only shows stage III since all correlations in stage II were not significant. A) Overall survival according to Spn levels in patients without adjuvant chemotherapy. B) Overall survival according to Spn levels in patients treated with adjuvant chemotherapy. C) Disease-free interval according to Spn levels in patients without adjuvant chemotherapy. D) Disease-free interval according to Spn levels in patients treated with adjuvant chemotherapy. The log rank test showed a non-significant correlation for overall survival (p= 0.525) or disease free interval (p=0.472) for patients not treated with adjuvant chemotherapy, while showing a significant correlation for stage III tumor patients treated with adjuvant chemotherapy in overall survival (p= 0.001) or disease free interval (p=0.059). Spn levels are as follows: Low: <100; Medium: 100≤x<200; High: ≥200

**Figure 6.** The correlation of gene expression of Spn (A) or Brca1 (B) with response in metastatic CRC. Progression-free interval (C) and Overall survival (D) according to Spn mRNA levels. Spn expression levels were normalized to GAPDH in tumor tissue of non-responders and responders to chemotherapy and in normal colon tissue located near the tumor. The differences between the groups were tested using the T-test. R: Responders; Non-R: non responders.
Figure 3

(A) Stage II

K67 (% positive cells)

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<th>Spn levels (Score)</th>
<th>Low (&lt;100)</th>
<th>Medium (100 ≤ X &lt; 200)</th>
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<tr>
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<td>0</td>
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r = 0.049
p = 0.57

(B) Stage III

K67 (% positive cells)

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<th>Spn levels (Score)</th>
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<th>Medium (100 ≤ X &lt; 200)</th>
<th>High (≤200)</th>
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r = -0.251
p = 0.022

P1 Spn

P1 KI67

P2 Spn

P2 KI67

P3 Spn

P3 KI67
Figure 5: Cumulative survival plots comparing tumor-free interval (A-C) and overall survival (B-D) between AD ChT = NO and AD ChT = YES groups. Spn Levels: Low, Medium, High, Low-censored, Medium-censored, High-censored. Statistical comparisons are indicated by p-values: p=0.472, p=0.001, p=0.525, p=0.059.
Spinophilin loss correlates with poor patient prognosis in advanced stages of colon carcinoma


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