A Systems Biology Approach Identifies SART1 as a Novel Determinant of Both 5-Fluorouracil and SN38 Drug Resistance in Colorectal Cancer

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Abstract
Chemotherapy response rates for advanced colorectal cancer remain disappointingly low, primarily because of drug resistance, so there is an urgent need to improve current treatment strategies. To identify novel determinants of resistance to the clinically relevant drugs 5-fluorouracil (5-FU) and SN38 (the active metabolite of irinotecan), transcriptional profiling experiments were carried out on pretreatment metastatic colorectal cancer biopsies and HCT116 parental and chemotherapy-resistant cell line models using a disease-specific DNA microarray. To enrich for potential chemoresistance-determining genes, an unsupervised bioinformatics approach was used, and 50 genes were selected and then functionally assessed using custom-designed short interfering RNA (siRNA) screens. In the primary siRNA screen, silencing of 21 genes sensitized HCT116 cells to either 5-FU or SN38 treatment. Three genes (RAPGEF2, PTRF, and SART1) were selected for further analysis in a panel of 5 colorectal cancer cell lines. Silencing SART1 sensitized all 5 cell lines to 5-FU treatment and 4/5 cell lines to SN38 sensitivity in the wider cell line panel. Further functional analysis of SART1 showed that its silencing induced apoptosis that was caspase-8 dependent. Furthermore, silencing of SART1 led to a downregulation of the caspase-8 inhibitor, c-FLIP, which we have previously shown is a key determinant of drug resistance in colorectal cancer. This study shows the power of systems biology approaches for identifying novel genes that regulate drug resistance and identifies SART1 as a previously unidentified regulator of c-FLIP and drug-induced activation of caspase-8. Mol Cancer Ther; 11(1); 119–31. ©2011 AACR.
transcripts confirmed as being expressed in colorectal cancer and normal colorectal tissue. This array contains transcripts that have not been available for previous expression analysis studies (12). The generation and utility of other disease-specific arrays using a similar technical approach have been previously reported (13–15). The power of this study is that we have used a systems biology approach of transcriptional profiling with functional testing of the identified genes to highlight potential novel drug targets and/or biomarkers, which could be used to potentially improve response rates for advanced colorectal cancer.

Materials and Methods

Patient samples

These have been previously described (16). Briefly, 20 patients with metastatic colorectal cancer were included in this study. All patients provided written fully informed consent as per Institutional Review Board guidelines in the University of Southern California and approval was granted from this body. These patients underwent biopsy of colorectal liver metastases before commencing irinotecan/5-FU chemotherapy on the IFL schedule: CPT-11 125 mg/m² i.v. over 90 minutes, leucovorin 20 mg/m² as intravenous bolus injection immediately before 5-FU and 5-FU 500 mg/m² as intravenous bolus injection administered weekly for 4 weeks and repeated every 6 weeks. CT imaging for response evaluation using WHO criteria was done every 6 weeks. Of these 20 patients, 1 had a complete response to treatment, 10 had a partial response to treatment, and 9 had progressive disease on treatment.

For the purpose of this study, we have further defined responders as those patients with either complete or partial response and nonresponders as those patients with progressive disease. We had specifically excluded patients with stable disease on chemotherapy from the study.

Materials

5-FU and SN38 were purchased from Sigma Chemical Co. and Abatra Technology Co., Ltd, respectively.

Cell culture

All colorectal cancer cells were grown as previously described (17). Following receipt, cells were grown up, and as soon as surplus cells became available, they were frozen as a seed stock. All cells were passaged for a maximum of 2 months, after which new seed stocks were thawed for experimental use. All cell lines were tested for Mycoplasma contamination at least every month. LS174T (2008), SW620 (2008), and RKO (2001) cells were obtained from the American Type Culture Collection [authentication by short tandem repeat profiling/karyotyping/isoenzyme analysis] and maintained in Dulbecco’s Modified Eagle’s Medium. The HCT116 human colon cancer cell line was kindly provided by Prof. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) in 2003 and was grown in McCoy’s 5A medium. LoVo cells were provided for AstraZeneca in 2003. HCT116, HT29, LoVo, and RKO cell lines were validated by short tandem repeat profiling by LGC Standards (18) in May 2011. The 5-FU- and SN38-resistant HCT116 sublines and were generated in our laboratory as previously described (19). The FLIP overexpressing HCT116 cell lines were previously described (20). All medium was supplemented with 10% dialysed fetal calf serum, 50 g/mL penicillin–streptomycin, 2 mmol/L l-glutamine, and 1 mmol/L sodium pyruvate (all medium and supplements from Invitrogen Life Technologies Corp.). All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Microarray analysis

Total RNA was extracted from 20 pretreatment metastatic tumor biopsies from patients with advanced colorectal cancer and also profiled on the colorectal cancer disease-specific array (Almac Diagnostics). In addition, in vitro analyses were also carried out using the colorectal cancer disease-specific array. Briefly, HCT116 parental cells were either untreated (0 h control) or treated with either 5 mol/L 5-FU or 5 nmol/L SN38 for 24 hours (acutely altered genes). Also, untreated 5-FU–resistant and SN38-resistant cells were analyzed to identify those genes that are basally deregulated between parental and resistant cells. Total RNA was isolated from 3 independent experiments using the RNA STAT-60 Total RNA isolation reagent (Tel-Test, Inc.) according to the manufacturer’s instructions. For both the clinical and in vitro studies, RNA was sent to Almac Diagnostics for cDNA synthesis, cRNA synthesis, fragmentation, and hybridization onto the colorectal cancer disease-specific array. Detailed experimental protocols and raw expression data are available within the ArrayExpress repository [ref. 21; Accession number E-MEXP-1692 (clinical analysis) and E-MEXP-1691 (in vitro analysis)].

Unsupervised classification analysis

Unsupervised classification analysis was carried out using Principal Components Analysis (PCA). All PCA was carried out using the Partek software (version 6.3, Partek Inc.). Briefly, each microarray experiment (5-FU basal, 5-FU inducible, SN38 basal, SN38 inducible, and 5-FU/irinotecan clinical) was initially normalized and then underwent minimal flag filtering before PCA analysis. Following PCA analysis of each experiment, the results were examined to determine which principal component (PC) lead to the greatest separation between the 2 treatment groups. The PC that lead to the maximal treatment group separation was then isolated and the top 10 positive and top 10 negative component loadings were listed for each experiment.

Quantitative reverse transcription PCR analysis

Total RNA was isolated as described above. Reverse transcription was carried out using 2 g of RNA using a Moloney murine leukemia virus–based reverse
transcriptase kit (Invitrogen) according to the manufacturer’s instructions. Quantitative reverse transcriptase PCR (RT-PCR) amplification was carried out in a final volume of 10 L containing 5 L of 2×SYBR green master mix (Qiagen), 4 L of primers (2 mol/L), and 1 L of cDNA using an Opticon DNA Engine Thermal Cycler (Bio-Rad Laboratories Inc.) using methods previously described (16). All amplifications were primed by pairs of chemically synthesized 18- to 22-mer oligonucleotides designed using the Primer3 primer design software (22).

Statistical analysis

All t tests and 2-way ANOVAs were calculated using the GraphPad software (Prism4). Specifically, t tests were unpaired, 2-tailed using 95% confidence intervals. Two-way ANOVA was calculated using 95% confidence intervals and a Bonferroni post hoc test.

Short interfering RNA plate analysis

All short interfering RNAs (siRNA) were supplied by Qiagen. siRNA screening was done using siRNAs targeting preselected genes identified from microarray analysis. AllStars Negative control and AllStars Death control were used as nontargeting (scrambled) and positive controls, respectively. Transfection conditions were optimized using siRNAs with known effect on cell survival (FLIP and XIAP; refs. 23–25). HCT116 cells were reverse transfected using HiPerFect transfection reagent (Qiagen) to a final concentration of 5 nmol/L siRNA. After 24 hours, drug/solvent control was added; 5-FU and SN38 (~IC_{50} doses). Forty-eight hours later, MTT (cell viability) and ToxiLight (cell death; Promega UK Ltd) assays were done. The measurement from each well was normalized to the scrambled control. ToxiLight values were divided by MTT values to give a measurement of the relative toxicity for each silenced gene. Significance and interaction effects were measured using t tests, 1-way ANOVA and 2-way ANOVA to assess statistical significance of changes compared with control siRNA.

Cell viability analysis

Cell viability was determined using MTT. For the siRNA plate analysis, the analysis was carried out at 72 hours posttransfection. For the combination index (CI) synergy calculation, the analysis was carried out at 24 or 48 hours posttransfection and drug treatment. CI values <1, 1, and >1 indicated synergism, additivity, and antagonism, respectively. For synergistic interactions, CI values between 0.8 and 0.9 indicated slight synergy, 0.6 and 0.8 indicated moderate synergy, 0.4 and 0.6 indicated synergy, and those <0.4 indicated strong synergy (26).

Western blot analysis

Western blot analysis was carried out as previously described.

SART1 (Abcam), FLIP (NF6; Alexis), caspase-8 (12F5; Alexis) and PARP-1 (eBioscience) mouse monoclonal antibodies were used in conjunction with a horseradish peroxidase–conjugated sheep anti-mouse secondary antibody (Amersham). Equal loading was assessed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AbD Serotec).

Flow cytometric analysis

Annexin V/propidium iodide (PI) analysis was carried out using the EPICS XL Flow Cytometer (Coulter). Cells were harvested and analyzed according to manufacturer’s instructions (BD Biosciences). Annexin V/PI staining was carried out at 72 hours posttransfection.

Results

Transcriptional profiling of metastatic colorectal cancer biopsies and drug-resistant cell lines

Using the colorectal disease-specific array, we carried out microarray expression profiling of pretreatment metastatic colorectal cancer patient biopsies (n = 20). After appropriate background corrections and normalizations using the Robust Multichip Average method (27), expression values from all the 61,528 probes were used for further analysis. In addition, we also carried out an in vitro transcriptional profiling experiment using the same platform. We used a HCT116 colorectal cancer cell line panel made up of parental drug sensitive cells and daughter cell lines with acquired resistance to 5-FU or SN38 (the active metabolite of irinotecan; ref. 19). The transcriptional profiles of the HCT116 parental cells following treatment with either 5 mol/L 5-FU or 5 nmol/L SN38 for 24 hours were examined. In addition, we also compared the basal transcriptional profiles of the HCT116 parental cells following treatment with either 5 mol/L 5-FU or 5 nmol/L SN38 for 24 hours were examined. In addition, we also compared the basal transcriptional profiles of the HCT116 parental cells following treatment with either 5 mol/L 5-FU or 5 nmol/L SN38 for 24 hours were examined. In addition, we also compared the basal transcriptional profiles of the HCT116 parental cells following treatment with either 5 mol/L 5-FU or 5 nmol/L SN38 for 24 hours were examined. In addition, we also compared the basal transcriptional profiles of the HCT116 parental cells following treatment with either 5 mol/L 5-FU or 5 nmol/L SN38 for 24 hours were examined. In addition, we also compared the basal transcriptional profiles of the HCT116 parental cells following treatment with either 5 mol/L 5-FU or 5 nmol/L SN38 for 24 hours were examined.

Unsupervised classification

For the unsupervised analysis, 5 experiments were created: clinical (responders vs. nonresponders), 5-FU basal (sensitive vs. resistant), 5-FU inducible (parental untreated vs. parental 5-FU treated), SN38 basal (sensitive vs. resistant), and SN38 inducible (parental untreated vs. parental treated). PCA was used as the unsupervised method, and all data from each of the 5 experiments was initially flag filtered. For the clinical analysis, it was the third PC (9.8% variability) that gave the best separation based on patient response, although within this, there were still a number of misclassifications (Fig. 1A). The top 10 probesets that correlated positively or negatively with PC3 were correlated positively or negatively with PC3 were correlated positively or negatively with PC3 were correlated positively or negatively with PC3 were correlated positively or negatively with PC3 were
selected (Supplementary Table S2). For the 5-FU *in vitro* experiments (basal and inducible), PCA of the flag-filtered data showed a clear separation of sample groups. In both cases, this distinction was evident from the first PC (Fig. 1B and C). The top 10 probesets from the extreme positive or negative values of the component loadings that correlated maximally with PC1 were selected (Supplementary Tables S3 and S4).

For the SN38 *in vitro* experiments, the PC that accounted for the maximum variability in the dataset, PC1 (35.8%), was able to differentiate between the basal expression changes observed between parental and SN38-resistant daughter cells, although one of the parental cells was misaligned (Fig. 1D). However, in the case of SN38 treatment in the parental cells, separation based on drug treatment was only evident from the second PC (PC2: 25.6%) of the dataset, with one replicate from each class misaligned (Fig. 1E). In each case, the top 10 probesets from the extreme positive or negative values of the component loadings that correlated maximally with PC1 (SN38 basal) or PC2 (SN38 inducible) were selected (Supplementary Tables S5 and S6). For each of the above described experiments, 5 probesets from the top 10 positive and...
negative component loadings were selected for further functional analysis (Table 1); those that were omitted were either transcribed sequences or in antisense orientation.

Functional assessment of unsupervised genes

The clinical and in vitro genes identified from the unsupervised analyses (Table 1) were investigated further by RNAi for their functional relevance in mediating resistance to 5-FU and SN38. The results from the initial siRNA screen in the HCT116 cells are shown in Figure 2. The graphs show the relative toxicity, as assessed from MTT and toxiLight assays, for all positive genes from the clinical analysis (A), the 5-FU in vitro analysis (B), and the SN38 in vitro analysis (C). Displayed are those genes that show an interaction between gene silencing and either 5-FU or SN38 treatment as measured by 2-way ANOVA. Synergistic interactions are highlighted as †, P < 0.05; ‡, P < 0.01; ‡‡, P < 0.001.
Table 1. The top 50 genes identified from PCA of the clinical, 5-FU in vitro basal, 5-FU in vitro inducible, SN38 in vitro basal, and SN38 in vitro inducible transcriptional profiling experiments

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<tr>
<th>Probe ID</th>
<th>Gene symbol</th>
<th>Gene name</th>
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<td>SART1</td>
<td>Homo sapiens squamous cell carcinoma antigen recognized by T cells</td>
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sensitivity to either 5-FU or SN38. For the initial screening process, custom-designed siRNA plates included all genes for which siRNA sequences were available ($n = 50$). In each case, the effect of target gene silencing was examined alone and also in combination with either 5 mol/L 5-FU (IC$_{50}(48h)$) or 5 nmol/L SN38 (IC$_{50}(48h)$) in HCT116 colorectal cancer cells. For each experiment, 2 assays were carried out, cell viability (MTT assay) and cell death (ToxiLight assay). From this the relative toxicity was calculated as the ratio of cell death to cell viability. Cells were transfected with siRNA for 24 hours before 48-hour treatment with chemotherapy.

Of the genes identified from the clinical PCA analysis, $TNFS14$, $MAPK9$, $RAPGEF2$, and $RPS9$ silencing resulted in increased relative toxicity when combined with either 5-FU or SN38 compared with either treatment alone (Fig. 2A). Silencing of $TNFS14$ or $RPS9$ resulted in enhanced toxicity with 5-FU only, whereas silencing of $MAPK9$ enhanced the effect of SN38, but not 5-FU. Silencing of the genes identified from the $in vitro$ PCA, which were associated with 5-FU response identified a number of chemosensitizing genes: $GART$, $BTN3A2$, $ASSDHPIPT$, $RAD51AP1$, $PFR$, $PRKCBP$, $TSFM$, $MGA$, and $PSMA2$ (Fig. 2B). The silencing of either $RAD51AP1$ or $PTFR$ significantly enhanced the relative toxicity of both 5-FU and SN38 treatment, whereas silencing the remainder of these 5-FU response–associated genes only significantly increased sensitivity to 5-FU treatment. Analysis of the these 5-FU response–associated genes only significantly enhanced toxicity with 5-FU or SN38 compared with either treatment alone.

Validation of positive hits in colorectal cancer cell line panel

One gene was selected from each of the analysis for testing across a colorectal cancer cell line panel. These 3 genes were selected based on displaying a synergistic interaction with both 5-FU and SN38 when silenced. Therefore, $RAPGEF2$ was selected from the clinical analysis, $PTFR$ from the 5-FU $in vitro$ analysis and $SART1$ from the SN38 $in vitro$ analysis. A range of colorectal cancer cell lines with varying mutational backgrounds were tested, LoVo, RKO, HT29, SW620, and LS174T. Cell death and cell viability assays were carried out as before and the relative toxicity measured. In this screen, 2 siRNA sequences were included. The results showed that silencing either RAPGEF2 or PTFR did not sensitize any cell line, other than HCT16, to either 5-FU or SN38 treatment; therefore, the effect of these genes seem to be cell line dependent (data not shown). However, in the extended cell line panel, silencing of SART1 with either siRNA produced similar results with either additive or synergistic increases in relative toxicity when combined with either chemotherapy treatment (Fig. 3A–E).

SART1 silencing induces apoptosis in colorectal cancer cells

SART1 protein expression was upregulated in response to chemotherapy treatment (Fig. 4A). Importantly, analysis of PARP cleavage (a hallmark of apoptosis) indicated that SART1 silencing induced enhanced chemotherapy-induced apoptosis after 48-hour treatment (Fig. 4A). To examine the levels of cell death induced following SART1 silencing alone and in combination with chemotherapy, Annexin V/PI staining was carried out in several colorectal cancer cell lines. In the HCT116 cell line, SART1 silencing alone resulted in approximately 43% cell death, and apoptosis was further increased when SART1 silenced cells were cotreated with 5-FU or SN38 (Fig. 4B). Similar results were shown with a second siRNA sequence for SART1 (data not shown). Moreover, similar results were obtained in other colorectal cancer cell line models, with SART1 silencing inducing apoptosis in LS174T and RKO cell lines (Fig. 4C and D).

Silencing of SART1 results in synergistic interactions with either 5-FU or SN38

To determine whether silencing of SART1 results in synergistic interactions with chemotherapy, cell viability assays were carried out, and CI values calculated. Synergistic interactions were observed between chemotherapy and SART1 silencing in the HCT116 (Fig. 5A and B), the LS174T (Fig. 5C and D), and the RKO (Fig. 5E and F) cell lines.

The cell death induced by SART1 silencing is caspase-8 and FLIP dependent

Previous experiments had shown that SART1 silencing resulted in apoptotic cell death; therefore, we investigated whether the mechanism was caspase dependent, using the pan-caspase inhibitor ZVAD. The cell death observed following SART1 silencing was apoptotic (Annexin V positive) and was completely abrogated following cotreatment with ZVAD (Fig. 6A). Analysis of caspase activity following SART1 silencing indicated that caspase-8 was highly activated (Fig. 6B). To assess whether SART1 siRNA-induced apoptosis was caspase-8 dependent, caspase-8–specific siRNA was cotransfected with SART1 siRNA. Apoptotic cell death induced following SART1 silencing was completely abrogated following caspase-8 cosilencing at both 24 and 48 hours (Fig. 6B). Caspase-8 activity assays showed that caspase-8 activity was inhibited following caspase-8 silencing (Fig. 6C). In addition, the increased caspase 3/7 activity that was observed following SART1 silencing at 24 and 48 hours was also completely abrogated following caspase-8 cosilencing (Fig. 6C). Furthermore, Western blotting showed that PARP cleavage following SART1 silencing was prevented by caspase-8 cosilencing (Fig. 6D). The decreased expression of procaspase-8 in
the SART1 silenced cells (Fig. 6D, lane 3) is indicative of procaspase-8 activation. Notably, the expression of the endogenous caspase-8 inhibitor c-FLIPL was observed to be downregulated following SART1 silencing. Importantly, c-FLIPL downregulation following SART1 silencing was not a downstream effect of apoptosis induction, as it was also observed in samples in which caspase-8 was cosilenced. Moreover, SART1 siRNA-induced apoptosis was attenuated in HCT116 cells stably overexpressing FLIP L (Fig. 6E). These results suggested that downregulation of c-FLIPL following SART1 silencing is an upstream effect that leads to caspase-8–dependent apoptosis and enhanced drug-induced apoptosis.

Discussion

In this study, we used a systems biology approach incorporating transcriptional profiling, bioinformatics, and functional analyses to identify key mediators of 5-FU and SN38 sensitivity in colorectal cancer that may represent novel drug targets and/or biomarkers. Following minimal filtering, unsupervised analysis was carried

Figure 3. Results from the cell line panel siRNA screen. The graphs show the relative toxicity, as assessed from MTT and toxiLight assays, for SART1, PTRF, and RAPGEF2 for the LoVo (A), RKO (B) LS174T (C), HT29 (D), and SW620 (E) cell lines. Displayed is the effect of gene silencing alone and in combination with either 5-FU or SN38 treatment. Statistical significance between gene silencing alone (siRNA) and gene silencing in combination with chemotherapy was assessed by an unpaired 2-way t test. Statistical significance are highlighted as *, *< 0.05; **, *< 0.01; ***, *< 0.001. Two siRNA sequences were included for each gene, indicated by (1) and (2). ns, not significant.
out on both the clinical and in vitro gene lists using PCA. The reason for using both clinical and in vitro data was to try and identify potentially clinically relevant targets that could also be validated within an in vitro system. In addition, PCA was chosen as the classification approach as it represents a completely unbiased approach. Furthermore, due to the fact that the clinical sample size used was small, a supervised method approach may have significantly overestimated the predictive ability of the identified genes. Using the unsupervised approach, PCA identified a total of 100 genes, 50 of which were taken forward for functional testing using a custom-designed siRNA screen. The siRNA screen incorporated multiple siRNA sequences across a panel of several colorectal cancer cell lines and identified squamous cell carcinoma antigen recognized by T cells or SART1 as a potential mediator of 5-FU and SN38 sensitivity in this disease setting.

We functionally tested 50 of the 100 transcripts by siRNA screening and found that a significant number of these did play a role in mediating chemotherapy response in the initial cell line model system. Many of the 50 untested transcripts represented hypothetical proteins, and some were antisense RNAs, which made testing their functional significance more difficult. However, although most of the remaining untested transcripts have not been previously implicated in either colorectal cancer progression or chemotherapy response, some potentially may have a role. Such genes include CSNK2A2 (5-FU in vitro related) and CCNDP1, TNFAIP8, and GDI2 (clinically related). CCNDP1 negatively regulates cell-cycle progression through the inhibition of the cyclinD1/CDK4 complex (28, 29). CCNDP1 is located on chromosome 15q15, which is associated with LOH in many tumor types, including colon (28, 29). It is downregulated in tumors compared with matched normal and, therefore, has been associated with tumor suppression (28). TNFAIP8 has been shown to regulate apoptosis in thymocytes (30). Interestingly, TNFAIP8 can block caspase-mediated apoptosis, contains a death effector domain, and has been hypothesized to be a novel member of the FLIP family (31). GDI2 has been identified in a number of proteomic studies as a potential biomarker for pancreatic and ovarian cancer (32, 33). Indeed, GDI2 has been identified as a marker for chemoresistance in ovarian cancer (32). CSNK2A2 is known to be overexpressed in cancers, including colorectal cancer. It has been associated with disease progression in colorectal cancer via a proteomic approach (34). Of interest, CSNK2A2 protects colon cancer cells from TRAIL-induced apoptosis (35). The prosurvival and inhibition of apoptosis that is associated with CSNK2A2 is mediated through its ability to induce survivin expression via the Wnt pathway (36). Although these targets were not assessed for
functional significance, they may still represent potential biomarkers or therapeutic targets for inhibiting drug resistance in this disease setting.

SART1 is a bicistronic gene that encodes 2 proteins, one of which is 800 amino acids long and contains a leucine zipper and the other that is 259 amino acids long that does not contain the leucine zipper. The SART1(800) protein is located in the nucleus of the majority of proliferating cells, whereas the SART1(259) protein is located in the cytosol of epithelial cancers. In this study, we focused on SART1(800), the main function of which is thought to be in the recruitment of the tri-snRNP to the prespliceosome (37). Therefore, SART1 may be critical for pre-mRNA splicing. SART1(800) protein is expressed in 100% of colorectal cancer cell lines, 55% of colorectal cancer tissue, and 0% of nontumor tissue (38). Hypoxia inducible factor (HIF) or HAF is the murine homolog of SART1, and several studies have shown that HAF binds to and regulates the activity of the EPO receptor, VEGF and HIF-1α (39, 40). HAF binds and regulates HIF-1α activity independent of oxygen levels (40). SART1 has also been identified as a novel SUMO1 and SUMO2 target protein (41–43).

SART1 has been associated with the development and progression of head and neck squamous cell carcinoma. Again, in this disease setting, there was a much higher tumor expression compared with normal (44). Kittler and colleagues carried out an siRNA screen to identify genes that were essential for cell division in breast cancer. They identified SART1 as a gene essential for cell division in breast cancer, with SART1 depletion displaying similar defects to either CENPE or KIF11 depletion. These defects following SART1 depletion may indicate that SART1 plays a direct role in cell division or may have an indirect effect caused by defective pre-mRNA splicing (45). Following this study, a second study was carried out by Olson and colleagues, which examined whether the genes identified from the former study were associated with breast cancer risk.

Figure 5. Cell viability assays were conducted in HCT116 (A and B), LS174T (C and D), and RKO cell lines (E and F) in response to SART1 siRNA (0.5, 1 and 5 nmol/L) and either SN38 (1, 5, and 10 nmol/L) or 5-FU (1, 5 and 10 mol/L) for 72 hours. To evaluate the interaction between chemotherapy and SART1 silencing, we used the method of Chou and Talalay (26). CI values <1, = 1, and >1 indicate synergism, additivity, and antagonism, respectively. For synergistic interactions, CI values between 0.8 and 0.9 indicate slight synergy, 0.6 and 0.8 indicate moderate synergy, 0.4 and 0.6 indicate synergy and those less than 0.4 indicate strong synergy.
The results showed that 4 single nucleotide polymorphisms were present in SART1, and 2 were associated with increased risk of breast cancer, whereas the other 2 were associated with a decreased risk of breast cancer development (46). The authors concluded that genetic variation in SART1 may be associated with breast cancer development.

This study has shown that SART1 silencing sensitizes colorectal cancer cells to 5-FU or SN38 treatment via apoptosis induction. The mechanism of cell death is caspase dependent, specifically caspase-8 dependent. In addition, the results show that SART1 silencing leads to c-FLIP<sub>i</sub> downregulation. Our previous studies have shown that c-FLIP<sub>i</sub> is a key regulator of chemotherapy-induced cell death in colorectal cancer and other tumor types (20, 23–25, 47). The downregulation of c-FLIP<sub>i</sub> was not a consequence of cell death as we also carried out experiments in which caspase-8 and SART1 were

![Image of graphs and figures](image-url)
cosilenced and showed that the cells did not undergo apoptosis, however, c-FLIP, was still downregulated. Moreover, apoptosis induced by SART1 silencing was attenuated in c-FLIP overexpressing cells. These results suggest that SART1 expression is important for continued c-FLIP expression. c-FLIP regulation is highly complex and involves transcriptional and posttranscriptional regulation by a number of key signal transduction pathways, such as NFkB, JNK, c-myc, c-fos, and PKC (48). Preliminary data suggest that the effects of SART1 on c-FLIP expression are posttranscriptional (data not shown): the mechanistic basis of SART1’s regulation of c-FLIP is the subject of ongoing studies. This study has shown that silencing of SART1 enhanced chemotherapy-induced cell death via c-FLIP downregulation. Thus, c-FLIP and/or SART1 may represent predictive biomarkers of response to chemotherapy in colorectal cancer.

In conclusion, this study has used a systems biology approach to identify a number of novel regulators of chemoresistance in colorectal cancer, most notably SART1, which regulates expression of the critical apoptosis-regulating and drug resistance-associated protein c-FLIP. Future studies will assess the clinical relevance of SART1 expression as a prognostic and predictive biomarker in colorectal cancer.

Disclosure of Potential Conflicts of Interest

P.G. Johnston is employed by and has an ownership interest in Almac Diagnostics.

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References


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