Clinical and molecular insights into primary pediatric liver cancer
Weeda, V.B.

Citation for published version (APA):

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The Role of the SCCRO Family in Neddylation in Primary Liver Cancer

Weeda V, Sun H, Au J, LaQuaglia M, Fong Y, Singh B
ABSTRACT

Introduction. SCCRO a.k.a. DCUN1D1 functions as a component of the E3 in neddylation and exhibits an oncogene addiction phenotype. Results from an in vivo model and data from the literature suggest that SCCRO and neddylation may play a significant role in primary human liver cancer.

Methods. A pilot series of human hepatocellular carcinoma (HCC), hepatoblastoma (HB) and fibrolamellar hepatocellular carcinoma (FL-HCC) and matched normal liver samples were assessed for SCCRO family expression by real time qPCR and for SCCRO mediated neddylation by a novel tissue neddylation assay. Cell lines representative of these tumors were validated as an in vitro model and inhibition of SCCRO family mediated neddylation activity by the neddylation E1 inhibiting small molecule MLN4924 was tested in these cell lines.

Results. SCCRO2 overexpression correlates with increased neddylation activity in HCC. In HB and FL-HCC and liver cancer cell lines, a similar pattern was found for SCCRO2 and SCCRO5. LD50 and IC50 for MLN4924 were lowest in cell lines with high levels of SCCRO family expression and function and vice versa.

Conclusion. SCCRO family expression correlated with function in neddylation in human liver cancer tissue. High expression and function correlating with low LD50 and IC50 in liver cancer cell lines suggest the oncogene addiction phenotype of SCCRO family members can be exploited by selecting tumors to treat with MLN4924 by assaying their neddylation activity.
INTRODUCTION

Squamous Cell Carcinoma Related Oncogene (SCCRO, a.k.a. DCUN1D1) was identified in a systematic, positional cloning approach on position 3q26.3 and consecutively characterized as an oncogene (1, 2, 3). Amplification and overexpression of SCCRO in human squamous cell cancers correlates with poor clinical outcome (3). SCCRO is a member of a protein family with four other paralogues (SCCRO2-5, a.k.a. DCUN1D2-5) in the mammalian system. Based on their respective N-termini, the members are divided in subfamilies: SCCRO and SCCRO2 harbor a UBA domain, SCCRO3 has a myristoylation signal and SCCRO4 and SCCRO5 have a nuclear localization sequence. SCCRO, SCCRO2, SCCRO4 and SCCRO5 are putative oncogenes while SCCRO3 functions as a tumor suppressor (4, and Huang et al. Submitted). Knockdown of SCCRO using RNAi in cell lines with endogenous overexpression but not in cell lines without these features, leads to apoptosis, suggesting SCCRO has an ‘oncogene addiction’ phenotype (3, 5, and Ganly et al. Unpublished data). For SCCRO5 a similar phenomenon was observed (4).

Functionally, SCCRO is an essential component in cullin neddylation, facilitating the nuclear translocation of the substrate cullin and the transfer of the ubiquitin-like protein Nedd8 from the neddylation E2 (Ubc12) to cullin, thus fulfilling an indispensable role in the in vivo assembly of the Cullin RING E3 ligase (CRL), the rate limiting and substrate specificity determining component in ubiquitination (6-10). All human SCCRO paralogues function in neddylation. Evidence suggests that SCCRO3 functions as an antagonist to SCCRO by sequestering cullins away from the nucleus to the cell membrane employing its myristoylation signal (Huang et al. Submitted). CRL-based ubiquitination controls diverse cellular processes by altering protein activity and targeting a host of substrate proteins for degradation by the 26S proteasome (11-13).

CRL dysregulation is implicated in carcinogenesis (14-17). Upregulated ubiquitination activity has been associated with low survival in hepatocellular carcinoma (HCC), but mechanisms remain unknown (18).

HCC is a prevalent health issue, ranking third in causes of cancer-related death worldwide (19). Its prognosis is grim, especially in advanced stages where surgery is not feasible and other treatment modalities are applicable to small numbers of selected patients only (20). In primary pediatric liver cancers, prognosis in advanced stages is similarly poor, although underlying risk factors present in adult liver cancers are mostly absent. Knowledge of the signaling cascades and individual molecular components that are aberrant in both adult and pediatric liver cancers is essential to improve treatment strategies and develop new potential chemotherapeutics.
During development of a transgenic murine model for in vivo study of SCCRO’s function as an oncogene, overexpression as well as conditional overexpression led to early embryonic lethality. As an alternative, embryonic stem cells stably expressing SCCRO under control of the EF1-α promoter were injected into blastocysts and transferred to pseudopregnant mice to produce chimeric mice. Of two mice ultimately born, one spontaneously developed liver tumors. Pathology review was consistent with HCC and transgenic SCCRO expression in these tumors was confirmed by PCR (21). However, no additional mice survived using this approach, hindering reproduction of findings. Spontaneous development of HCC-like tumors in SCCRO chimeric mice, together with upregulation of ubiquitination activity in HCC as reported in the literature consistent with widespread findings of CRL dysregulation in cancer, led us to investigate the potential role of the SCCRO family of proteins in human primary liver cancer (14-17, 18, 21).

In this work we investigate SCCRO family expression and function in adult and pediatric liver tumors and use human liver cancer cell lines as an in vitro system to test the neddylation E1 inhibitor MLN4924.

METHODS

COLLECTION OF HUMAN TISSUE SAMPLES AND CLINICAL DATA
Twenty hepatocellular carcinoma (HCC) samples and matched normal liver tissue samples from adult patients, seven pediatric fibrolamellar hepatocellular carcinoma samples and four pediatric hepatoblastoma samples and matched normal samples from the same patients were obtained from the Memorial Sloan Kettering Cancer Center Tumor Bank. Informed consent was previously obtained from patients or, in the case of pediatric patients, their parents for research use of these samples. The institutional review board and human biospecimen use committee approved the research plan. Clinical data were collected and stored in a protected database.

CELL CULTURE
The HUH6 cell line was obtained from Riken Cell Bank (Tsukuba, Japan). The HUH7 and FOCUS cell lines were kind gifts of Dr. Wands at the Rhode Island Hospital Liver Research Center and Division of Gastroenterology, where the FOCUS hepatoma cell line was generated (22). HEPG2 was obtained from ATCC (American Type Culture Collection, Manassas, VA). HUH6 and HEPG2 were maintained in DME high glucose media with addition of 10% foetal calf serum (FCS) and antibiotics, whereas FOCUS and HUH7 were grown in MEM with addition of non-essential amino acids, 10% FCS and antibiotics. All cell lines were tested negative for mycoplasma.
REAGENTS
The following antibodies were used in this study: anti-Cul1 (Invitrogen, Grand Island, NY), anti-Cul3 (BD Biosciences, San Jose, CA), anti-ubiquitin (Santa Cruz, Santa Cruz, CA), anti-SCCRO (developed and validated as previously described (3)), anti-SCCRO3 (Abnova, Taipei City, Taiwan), anti-SCCRO5 (developed and validated as previously described (4)), anti-α-Tubulin (Sigma-Aldrich, St Louis, MO), and secondary antibodies conjugated to horseradish peroxidase (Santa Cruz, Santa Cruz, CA) with dilutions per manufacturer’s specifications.

SCCRO1-5 mRNA EXPRESSION ANALYSIS
Total RNA was extracted from freshly frozen tissue samples with TRIzol reagent (Life Technologies Inc., Grand Island, NY) following the manufacturer’s protocol and further purified with the RNeasy Mini kit (Qiagen, Germantown, MD). RNA samples were treated with DNase I to eliminate residual genomic DNA. Two microgram of total RNA per sample was reverse transcribed with MultiScribe Reverse Transcriptase (Applied Biosystems Inc., Foster City, CA). SCCRO and SCCRO paralogue specific gene assays and 18s gene assay were tested against SCCRO family plasmids and used as primer probe sets (Applied Biosystems). Reactions were prepared with TaqMan PCR Master Mix. Quantification of transcripts was performed by the 7900HT ABI prism (Applied Biosystems). Amplification was followed by melting curve analysis. The relative quantification of target genes was performed in comparison to reference 18s RNA (23). Reactions for each sample were done in triplicate. Data acquired by the 7900HT ABI prism using SDS2.3 software (Applied Biosystems) was analyzed with Microsoft Excel (Microsoft, Redmond, WA) according to the comparative cycle time (Ct) method (ΔΔCt = ΔCtSCCRO1-5 - ΔCt18S) (24) Detailed methodology has previously been published (2, 3).

TISSUE NEDDYLATION ACTIVITY ASSAY AND IC50 NEDDYLATION ASSAY
Protein lysates from primary tumors and corresponding normal liver tissues were extracted with lysis buffer (Cell Signaling Technologies, Danvers, MA) and neddylation assays were performed to assess neddylation status in lysates of tumor and matched normal liver tissue through addition of neddylation components in molarities derived from those used in the previously developed and validated (in a clean system of SCCRO-wt and SCCRO-/- mouse embryonic fibroblast cell lysates) in vitro neddylation assay (6). APP-BP1/Uba3 (Neddylation Enzyme 1, 10 nM, Boston Biochem, Cambridge, MA), Ubc12 (200 nM, Boston Biochem), Nedd8 (2 µM, Boston Biochem) and ATP (100 mM, Sigma-Aldrich, St Louis, MO) were added to tumor lysate and neddylation buffer (50 mM Tris-HCl pH 7.6, 55 mM NaCl, 5 mM MgCl2, and 1 mM DTT) to a total volume of 50 µl, incubated for the indicated times and stopped by addition of 12 µl Laemmli reducing buffer. To determine IC50 following treatment of cells with MLN4924, a reaction mix without APP-BP1/Uba3 (the heterodimeric neddylation E1 complex which contains the enzyme inhibited by this compound) was used (Ubc12, 400 nM, Nedd8, 2 µM and ATP, 100 mM). Proteins were resolved on SDS-PAGE gel and subjected
to Western blot analysis probing with Cul1 or Cul3 antibody. Ponceau-S stain was used to confirm equal lane loading. Reactions were performed in triplicate for consistency and western blots were analyzed through densitometry with Discovery One software (BioRad, Hercules, CA) to determine the ratio of neddylated to unneddylated Cul1. For assessment of global ubiquitination, western blots from neddylation assays were stripped and reprobed with ubiquitin antibody.

LD50 ASSAY
To determine LD50, liver cancer cell lines HUH6, HEPG2, FOCUS and HUH7 were plated in 96-well plates at a density of 1000 cells/ well in 6-plicates for each condition and for each cell line. After allowing adherence time (24 h), cells were treated with MLN4924 (ActiveBiochem, Wan Chai, HongKong) at a log range of 10 nM – 5 µM, or DMSO only (Sigma Aldrich, St Louis, MO). After 96 h of treatment, cell viability was assayed through addition of MTS/PMS solution (Promega, Madison, WI), incubation for 3.5 h and plates were then read at 490 nM using a 96-well plate reader (Molecular Devices, Sunnyvale, CA). Cell viability was expressed relative to viability of cells treated with DMSO only. Averages of three independent runs of the experiment were used for LD50 calculations carried out with CompuSyn software (MSKCC HTS Core Facility).

STATISTICAL ANALYSIS
All statistical analyses were performed using the SPSS statistical software package version 19 (IBM SPSS Statistics, IBM, Armonk, NY). qRT-PCR results were expressed as mean ± SD and only tumor samples with values at least two-fold different from the matching normal liver sample as well as exceeding 2x SD of the mean of all normal samples of the same histology, were considered to overexpress or under express a particular gene. The nonparametric Wilcoxon test was used to compare the levels of mRNA expression between tumor and normal samples and to compute the relationship between SCCRO2 expression and neddylation activity. A p level of ≤ 0.05 was considered statistically significant, and all tests were two-sided.

RESULTS

EXPRESSION OF SCCRO AND ITS PARALOGUES IN HUMAN PRIMARY LIVER TUMORS
Spontaneous development of HCC in the SCCRO chimeric mouse model together with upregulation of ubiquitination activity in HCC and findings of CRL dysregulation in cancer led us to explore the potential role of the SCCRO family in HCC (14-17, 18, 21). We first assessed SCCRO family expression in these tumors. We investigated a pilot cohort of 20 human HCC tumor samples and matched normal liver tissue samples. Patients were between 34 and 81 years of age. Seven patients had cirrhosis, 3 patients were hepatitis B positive, 1 patient was hepatitis C positive, and 4 patients reported heavy alcohol use (Supplemental Table
1). Real time qPCR for SCCRO1-5 mRNA expression levels relative to 18s, which was determined the most adequate reference gene in liver tissue, revealed significant overexpression of SCCRO2 (Wilcoxon signed-rank test, \( p = 0.021 \)) but not SCCRO, SCCRO4 and SCCRO5, compared to levels in matched normal liver tissue samples (Figure 1A) (23). In contrast with previously published findings, significant underexpression of SCCRO3 could not be detected in this cohort of HCC samples (25). As SCCRO and SCCRO2 have highly homologous amino acid sequences, available antibodies cannot discern with certainty between the two proteins based on recognized epitopes, complicating efforts to use western blot analysis for confirmation of SCCRO2 overexpression at the protein level (data not shown). Furthermore, no significant relationships could be detected between SCCRO2 overexpression and clinical parameters such as outcome and risk factors (data not shown). As primary liver tumors in the pediatric setting have a dismal prognosis similar to HCC in adult patients but risk factors are starkly different, we wondered whether SCCRO family expression and activity could begin explaining poor outcome in these tumor types. We obtained a cohort of seven fibrolamellar hepatocellular carcinomas (FL-HCC) and four hepatoblastomas (HB) and matched normal samples from the same patients. Patient characteristics are summarized in Supplemental tables 2 and 3. Real-time qPCR for SCCRO1-5 relative to 18s as a reference gene showed overexpression of SCCRO in 1 HB sample, overexpression of SCCRO2 in 1 HB and 1 FL-HCC sample, no overexpression of SCCRO4 and overexpression of SCCRO5 in 2 HB and 2 FL-HCC samples relative to matched normal samples. Furthermore, SCCRO3 was under expressed in 2 FL-HCC samples. Average expression for the HB and FL-HCC cohorts is shown in Figure 1B and 1C.

DYSREGULATION OF THE SCCRO FAMILY IN HUMAN PRIMARY LIVER CANCERS CORRELATES WITH NEDDYLATION ACTIVITY AND IS ASSOCIATED WITH INCREASED UBIQUITINATION ACTIVITY

We continued by assessing whether dysregulation of SCCRO and its paralogues would have functional implications in human primary liver cancers. As SCCRO family proteins function in cullin neddylation, we sought to measure neddylation activity in these samples. This posed a significant challenge, as neddylation is a dynamic, reversible process and neddylated cullins are unstable. We thus developed and optimized a novel neddylation assay for use in human tissue lysates, based on the previously developed and validated in vitro activated neddylation assay (6). Through addition of purified neddylation E1 (APP-BP1/Uba3), neddylation E2 (Ubc12), ubiquitin-like protein Nedd8 and ATP to tissue lysates, neddylation was activated in tissue lysates. Reaction products were resolved on SDS-PAGE and subsequently immunoblotted with Cul1 antibody. Resulting western blots show an upper band (neddylated Cul1) and a lower band (unneddylated Cul1), an increase of density of the upper band over time indicating reaction efficiency and highest levels of neddylated Cul1 in the sample with high SCCRO2 expression level (example shown in Figure 2A, compare lane 3 and 6 to lane 9). The density of bands was measured and the ratio of upper to lower band was calculated and used as a measure of neddylation activity. All HCC tumor samples were assayed in this
manner and a pattern of increased neddylation activity with increased SCCRO2 level was found (Figure 2B). A subset of normal tissue samples were assayed for control purposes and showed scarce neddylated cullin (data not shown). Nonparametric Wilcoxon testing showed a significant correlation between SCCRO2 overexpression and increased neddylation activity in HCC samples ($p = 0.037$), implicating that SCCRO2 overexpression in HCC is functionally relevant.

HB and FL-HCC and matching normal liver tissue samples were assayed in the same manner. In hepatoblastoma increased neddylation of Cul1 was seen in samples with overexpression of SCCRO, SCCRO2 and SCCRO5 compared to samples without SCCRO family dysregulation (example shown in supplemental figure 1A, compare lane 6 to lane 3). Effects of aberration in SCCRO family expression in FL-HCC were most pronounced in neddylation of Cul3. Cul3 neddylation was increased in samples overexpressing SCCRO2 and SCCRO5 and underexpressing SCCRO3 in comparison with those samples without SCCRO family aberrations (Example shown in supplemental figure 1B, compare lane 9 in the left panel and lanes 3 and 6 in the right panel to lane 3 and 6 in the left panel and lane 9 in the right panel). Scarce neddylated cullin was detected in matched normal samples (data not shown). These results suggest that aberrant SCCRO family expression in primary pediatric liver tumors is associated with increased neddylation activity, like in adult HCC.

We then asked whether subsequent ubiquitination signaling would be affected by the level of SCCRO family deregulation. To address this question, immunoblots from activated neddylation assays were probed with ubiquitin antibody, showing a pattern of increased global ubiquitination with increasing SCCRO paralogue level, consistent with the pattern observed for neddylation, suggesting that increased neddylation activity leads to increased enzymatic activity in the subsequent ubiquitination pathway (Example shown in figure 2A lower panel).

VALIDATION OF PEDIATRIC AND ADULT LIVER PRIMARY LIVER CANCER CELL LINES AS A MODEL FOR LIVER CANCERS WITH DIFFERENTIAL SCCRO FAMILY EXPRESSION AND ACTIVITY

As we found that aberrant SCCRO family expression has functional implications in these human liver cancers, we hypothesized that the oncogene addiction phenotype observed for certain SCCRO family members could be exploited by inhibiting SCCRO family mediated neddylation activity. Recent studies show that the new neddylation E1 inhibitor MLN4924, which currently is in phase I clinical trials for a wide range of human hematologic as well as solid cancers, is effective in human liver cancer cell lines (26, 27). To allow use of human liver cancer cell lines as a model to test inhibition of neddylation in relation to SCCRO family levels and enzymatic activity, we first assessed SCCRO family expression and function in these cell lines. For real time qPCR, the average of expression values of a cohort of normal liver tissue samples was chosen as a control based on gene expression profiling studies recently published showing expression of a wide range of genes in normal liver tissue is highly
concordant with expression in primary hepatocytes, which are considered gold standard for hepatic expression but are difficult to maintain for reliable results (28). Real time qPCR in HUH6, HEPG2, FOCUS and HUH7 relative to 18s showed pronounced overexpression of SCCRO5, overexpression of SCCRO2 and low expression of SCCRO3 in these cell lines relative to the average of the expression values of normal liver tissue samples (Figure 3A). In contrast to SCCRO2, SCCRO5 protein can be detected by a specific antibody. Protein analysis was performed showed an inverse relationship between levels of the oncoproteins (SCCRO/SCCRO2 and SCCRO5) and the tumor suppressor SCCRO3 (Figure 3B). Activated neddylation assay in these cell lines shows low neddylation activity as measured by the ratio of neddylated:unneddylated Cul1 in HUH6, intermediate activity in HEPG2 and FOCUS and high activity in HUH7 (Figure 3C). This neddylation assay was executed without addition of the neddylation E1, APP-BP1/Uba3, thus serving as baseline for further experiments with MLN4924, which inhibits the APP-BP1 subunit of this E1 enzyme. The above findings indicate aberrant SCCRO family expression and activity in these cell lines with similarities between findings in cell lines and human tissue samples, thus enabling us to use these cell lines as a model to evaluate these factors in relation with neddylation inhibition.

SCCRO FAMILY EXPRESSION AND ASSOCIATED NEDDYLATION ACTIVITY CORRELATE WITH RESPONSE TO THE NEDDYLATION INHIBITOR MLN4924 IN HUMAN LIVER CANCER CELL LINES

To quantitatively determine sensitivity of all four cell lines to MLN4924, we performed cell viability MTS assays with an increasing range of doses of the compound (Figure 4A). Conditions were based on concentration range and treatment duration in recent cell line studies with this compound and cells treated with DMSO only were used as control (29, 30). Calculation of the dose that was lethal for 50% of treated cells (LD50) showed highest values for HUH6 and HEPG2 (5.10 and 5.41 µM, respectively) and lower values for HUH7 and FOCUS (3.71 and 1.02 µM, respectively). Closely evaluating the pattern of reaction to the molecule, an immediate effect, even at lowest doses, is observed in HUH7 cells, no effect at the lowest doses but then a gradual decrease in viability is seen in FOCUS cells, steady viability and then a decrease only at highest doses in HUH6 and growth despite treatment before gradual decrease with higher doses in HEPG2, indicating that the latter cell lines are more resistant to the drug, and that HUH7 and FOCUS are more sensitive cell lines. We then determined IC50 through activated neddylation assay without E1 after treating cell lines with low (HUH6, HB) and high (HUH7, HCC) intrinsic neddylation activity with MLN4924 showing neddylated Cul1 was reduced to 50% with low doses of the small molecule in HUH7 whereas much higher doses were needed in HUH6 (Figure 4B). To lower the possibility of differences in histology as a cause for the difference in results, we also evaluated IC50 in HEPG2, a cell line that has HCC features (it may originate from a transitional HB/HCC tumor or from HCC) (31). This assay showed high doses were needed to lower Cul1 neddylation in HEPG2, excluding tumor type as an explanation for the observed differences (Supplemental Figure 2). Interestingly, inhibition of cullin neddylation was lowest in cells with
low intrinsic neddylation activity whereas MLN4924 was highly effective even at low doses in cells with high intrinsic neddylation levels, suggesting cells with higher intrinsic neddylation activity may be more affected in their function by this compound. Altogether, a pattern of high sensitivity to MLN4924 in cells with high SCCRO family levels and neddylation activity and low sensitivity in cells with low SCCRO expression levels and neddylation activity is observed, suggesting an inverse relationship between MLN4924 dose and SCCRO family levels and function at the extremes.
FIGURES

Figure 1

A

HCC N=20

Relative expression SCCRO1-5

B

HB N=4

Relative expression SCCRO1-5

C

FL-HCC N=7

Relative expression SCCRO1-5

Figure 2

A

Time (min)

α-Cul1-Nedd8

α-Cul1

Ponceau-S

100kD

75kD

50kD

α-Ub

0 15 30 0 15 30 0 15 30

Sample 1 low  Sample 2 intermediate  Sample 3 high

SCCRO2 level

B

Ratio neddylation unneddylation Cul1

SCCRO2 overexpression

No SCCCRO2 overexpression
Figure 3

A

Relative expression SCCRO1-5

B

SCCRO/SCCRO2
SCCRO3
SCCRO5
α-Tubulin

Huh7, HePG2, FOCUS, Huh6

C

SCCRO/SCCRO2, SCCRO5 expression levels

low, intermediate, high

Cul1-Nedd8
Cul1
α-Tubulin
**Figure 4**

Supplemental Table 1 – HCC patient characteristics

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<tr>
<td>Hepatitis B</td>
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<tr>
<td>Hepatitis C</td>
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<tr>
<td>Cirrhosis</td>
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<tr>
<td>Alcohol use, heavy</td>
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<td>Tobacco use, present or past</td>
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<td>Moderately differentiated</td>
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<td>Poorly differentiated</td>
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# Supplemental Table 2 – HB patient characteristics and supplemental Table 3 - FL-HCC patient characteristics

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<tr>
<td>Comorbidity</td>
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<td>Beckwith Wiedemann</td>
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<tr>
<td>241088.6 ng/ml</td>
<td>(2217.4-599.000)</td>
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<td>Tumor size; mean (range)</td>
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<td>North American Post surgical staging system</td>
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<td>Female</td>
<td>5 (71%)</td>
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<td>Recurrence</td>
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<td>PreTEXT</td>
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<td>II (n=4) (E+, E+P+, V+, none)</td>
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<tr>
<td>III (n=2) (V+, E+P+V+)</td>
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<tr>
<td>North American Post surgical staging system</td>
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<td>II (n=1)</td>
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<td>III (n=1)</td>
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Supplemental Figure 1

A

Min
Cul1-Nedd8
Cul1
150kD
100kD
75kD
50kD
Ponceau-S

Sample 1 low
Sample 2 high
SCCRO, SCCRO2, SCCRO5 levels

B

Min
Cul3-Nedd8
Cul3
150kD
100kD
75kD
Ponceau-S

Sample 1 high
Sample 2 intermediate
Sample 3 low
SCCRO2/SCCRO5 levels (inverse SCCRO3 level)

Supplemental Figure 2

A

Min
α-Cul1-Nedd8
α-Cul1
150kD
100kD
75kD
50kD
Ponceau-S
MLN4824 (μM)
FIGURE LEGENDS

Figure 1. SCCRO1-5 expression in primary human liver cancers.  
A, Box plot showing fold increase in SCCRO1-5 mRNA expression, analyzed by qRT-PCR, in 20 hepatocellular carcinomas (HCC).  
B, Box plot showing fold increase in SCCRO1-5 mRNA expression, analyzed by qRT-PCR, in 4 hepatoblastomas (HB).  
C, Box plot showing fold increase in SCCRO1-5 mRNA expression, analyzed by qRT-PCR, in 7 fibrolamellar hepatocellular carcinomas (FL-HCC).

Figure 2. SCCRO2 overexpression in HCC correlates with neddylation activity.  
A, neddylation was activated in HCC samples with low, intermediate and high SCCRO2 mRNA expression levels through addition of APP-BP1/Uba3, Ubc12, Nedd8 and ATP to tissue lysates. Reaction products were resolved on SDS-PAGE and subsequently immunoblotted with Cul1 antibody. An example western blot is shown; the upper band is neddylated Cul1, the lower band is unneddylated Cul1. An increase of density of the upper band over time indicates reaction efficiency. Levels of neddylated Cul1 correlate with SCCRO2 expression level.  
B, All tumor samples were assayed as in A. The density of bands was measured and the ratio of upper to lower band was calculated and used as a measure of neddylation activity. Nonparametric Wilcoxon testing showed a significant correlation between SCCRO2 overexpression and increased neddylation activity in HCC samples ($p = 0.037$).  
C, Subsequent ubiquitination signaling was assessed by probing blots from activated neddylation assays with ubiquitin antibody, showing a pattern of increased global ubiquitination with increasing SCCRO2 level.

Figure 3. SCCRO1-5 expression and neddylation activity in human liver cancer cell lines.  
A, Box plot showing fold increase in SCCRO1-5 mRNA expression, analyzed by qRT-PCR, in 4 human liver cancer cell lines.  
B, Western blot analysis of SCCRO/SCCRO2, SCCRO3 and SCCRO5 protein levels in 4 human cancer cell lines showing an inverse relationship between SCCRO3 and SCCRO1/2 and -5. The epitope recognized by the SCCRO/SCCRO2 antibody occurs in both SCCRO and SCCRO2; there is no antibody for SCCRO4.  
C, Activated neddylation assay in these cell lines shows SCCRO family protein expression levels correlate with neddylation activity as measured by the ratio of neddylated: unneddylated cullin1. This neddylation assay was executed without addition of APP-BP1/Uba3, serving as baseline for further experiments with MLN4924, which inhibits the APP-BP1 subunit of this E1 enzyme.

Figure 4. LD50 and IC50 for MLN4924 are low in cell lines with high SCCRO family expression and function levels and vice versa.  
A, Cell viability MTS assay with an increasing range of doses of the compound. Cells treated with DMSO only were used as control. Calculation of the dose that was lethal for 50% of treated cells (LD50) showed highest values for HUH6 and HepG2 (5.10 and 5.41 µM, re-
respectively) and lower values for HUH7 and FOCUS (3.71 and 1.02 µM, respectively). B, IC50 through activated neddylation assay without E1 after treating cell lines with low (HUH6) and high (HUH7) intrinsic neddylation activity with MLN4924. A pattern of higher sensitivity to MLN4924 in cells with high SCCRO family levels and neddylation activity and lower sensitivity in cells with low SCCRO expression levels and neddylation activity is observed, suggesting an inverse relationship between MLN4924 dose and SCCRO family levels and function at the extremes.

Supplemental Figure 1.
A, Neddylation was activated in hepatoblastoma samples with low and high SCCRO, SCCRO2 and SCCRO5 mRNA expression levels samples through addition of APP-BP1/Uba3, Ubc12, Nedd8 and ATP to tissue lysates. Reaction products were resolved on SDS-PAGE and subsequently immunoblotted with Cul1 antibody. An example western blot is shown; the upper band is neddylated Cul1, the lower band is unneddylated Cul1. An increase of density of the upper band over time indicates reaction efficiency. Increased neddylation activity was seen with SCCRO, SCCRO2 and SCCRO5 overexpression. B, Fibrolamellar hepatocellular carcinoma tissue samples with low, intermediate or high SCCRO2 and/or SCCRO5 mRNA expression levels were assayed for Cul3 neddylation as in A. An example western blot is shown; Cul3 neddylation activity was increased in samples with SCCRO2 and/or SCCRO5 overexpression.

Supplemental Figure 2.
IC50 was measured through activated neddylation assay without E1 after treating HepG2, a cell line with HCC features and intermediate intrinsic neddylation activity, with MLN4924. In this cell line with intermediate neddylation activity, levels of neddylated Cul1 remained substantial with increasing doses of MLN4924.

Supplemental Table 1.
Hepatocellular carcinoma pilot cohort patient characteristics. Abbreviations: AFP = alpha-fetoprotein.

Supplemental Table 2.
Hepatoblastoma cohort patient characteristics. Abbreviations: AFP = alpha-fetoprotein; PreTEXT = PreTreatment EXTent of disease; V = involvement of IVC and/or hepatic veins.

Supplemental Table 3.
Fibrolamellar hepatocellular carcinoma cohort patient characteristics. Abbreviations: AFP = alpha-fetoprotein; PreTEXT = PreTreatment EXTent of disease; E = Extrahaepatic intraabdominal extension of disease; P = portal vein involvement; V = involvement of IVC and/or hepatic veins.
DISCUSSION

In this report we show for the first time that overexpression of SCCRO family members significantly correlates with increased neddylation activity in human primary liver cancer. Moreover, we show that SCCRO family mediated neddylation activity in an *in vitro* model for these cancers has an inverse relationship with LD50 and IC50 for the novel neddylation inhibitor MLN4924. SCCRO family mediated neddylation activity may thus potentially serve as a biological marker with predictive strength in the selection of patients for treatment with MLN4924.

Overexpression of SCCRO correlating with poor clinical outcome in human squamous cell cancers has previously been shown by our group and overexpression of SCCRO in non-small cell lung cancer correlating with occurrence of metastasis to the brain was recently found by others (3, 32). Furthermore, an association between alterations in Cul1 neddylation signaling and aggressiveness of lung tumors has previously been shown and suggested as a therapeutic target (33, 34). The data presented here constitute the link between SCCRO family overexpression and increased neddylation activity in human tumors. The observations in several tumor types indicate that increased SCCRO family mediated neddylation in cancer is a broadly occurring phenomenon. At the same time, these observations reinforce the notion that knowledge of molecular background of a tumor is at least as important as anatomical location in precision medicine in human tumors (35).

Although the significant correlation between SCCRO2 expression and increased neddylation activity in a pilot cohort of HCC samples is encouraging, confirmation in a larger patient cohort is warranted to firmly establish these findings.

The observed patterns in pediatric liver tumors parallel findings in adult HCC. However, both pediatric liver cancer cohorts were too small to perform any valid statistical analyses or to draw conclusions that may answer the question posed: whether aberrations in SCCRO family mediated neddylation-ubiquitination signaling may explain dismal outcomes. Thus, evaluation of the rare pediatric liver tumors investigated in this study should be regarded as exploratory and should guide the direction of further mechanistic molecular biology research rather than be regarded as definitive data. Nonetheless, confirmation of a significant role of increased SCCRO family mediated neddylation activity in HB could provide a rationale to select patients with resistance to the commonly used cisplatin-based regimens, for treatment with cisplatin/MLN4924 (36). Recent studies indicate efficacy of the combination of cisplatin with MLN4924 in cisplatin-resistant ovarian cancer (37).

As yet, it is unknown whether there is a difference in impact of the different SCCRO family members, i.e. whether simultaneous overexpression of several oncogenic SCCRO family members has an increased oncogenic effect as compared to overexpression of just one fam-
ily member and whether the four oncogenic SCCRO family members have differential levels of oncogenicity. Data from our group indicate that SCCRO3 functions as a tumor suppressor that antagonizes the function of SCCRO (Huang et al. Submitted). Potentially, SCCRO3 also antagonizes other oncogenic SCCRO family members. These questions remain to be answered in future work.

The panel of cell lines used represents both pediatric and adult liver cancers and contains HB and HCC. HUH6 is derived from HB. Regarding HEPG2 a debate is ongoing whether it may be derived from a transitional HB/HCC tumor or from HCC (31). FOCUS and HUH7 are HCC cell lines. Moreover, FOCUS contains inactive hepatitis B virus. Unfortunately, there is no FL-HCC cell line available, prohibiting further exploration of the more pronounced neddylation of Cul3 in tissue samples of this tumor type. Possibly, some of the observed relationships between mRNA/protein expression, functional aberrations and sensitivity for MLN4924 in these cell lines as well as the differences in SCCRO and SCCRO paralogue overexpression between tumor cohorts and cell lines, may have their origins in the described diversity in the panel of cell lines. This diversity may induce unknown factors into the model system in addition to conventional issues with cell lines such as representation of molecular abnormalities in a sole tumor, and dependence on environmental stability for reproducibility of findings. However, at present, cell lines remain the best available option for initial determination of drug LD50 and IC50. Also, results of an additional IC50 assay to exclude tumor type induced differences (Supplemental figure 2) did not suggest such differences.

Clinical testing will have to show if the observations described here are meaningful in clinical practice and if doses are tolerable and correlate with in vivo needs. Generally, drug doses up to 10 µM are feasible clinically, as in HCC treatment with the small molecule tyrokinase inhibitor sorafenib, and definitive data for MLN4924 are expected in the near future (38).

Similar to experience with previously developed targeted therapies such as imatinib (BCR-ABL inhibitor) and gefitinib and erlotinib (EGFR inhibitors), evidence for mechanisms of potential resistance to MLN4924 when used as a single agent has been found in vitro (39, 40). MLN4924 is currently in phase I clinical trials (26, 39). Future studies will show whether expected resistance occurs in patients in a similar manner.

Targeted treatment of human cancers involves screening for molecular driver aberrations leading to a personalized treatment regimen and screening for occurring resistance during treatment (41). Tools to identify driver aberrations are particularly useful (42). Given the oncogene addiction phenotype observed for SCCRO family members, SCCRO family aberrations may represent driver aberrations. Investigation of SCCRO family expression levels and associated enzymatic neddylation activity may constitute a set of biological markers to predict efficacy of MLN4924 in human liver cancers in clinical practice.
REFERENCE LIST


