SCG3 Transcript in Peripheral Blood Is a Prognostic Biomarker for REST-Deficient Small Cell Lung Cancer

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Abstract

Purpose: Specific markers of circulating tumor cells may be informative in managing lung cancer. Because the RE-1 silencing transcription factor (REST/NRSF) is a transcriptional repressor that is inactivated in neuroendocrine lung cancer, we identified REST-regulated transcripts (CHGA, CHGB, SCG3, VGF, and PCSK1) for evaluation as biomarkers in peripheral blood.

Experimental Design: Transcripts were screened across lung cancer and normal cell lines. Candidates were assessed by reverse transcription-PCR and hybridization of RNA extracted from the peripheral blood of 111 lung cancer patients obtained at clinical presentation and from 27 cancer-free individuals.

Results: Expression profiling revealed multiple chromogranin transcripts were readily induced on REST depletion, most notably SCG3 was induced >500-fold. The SCG3 transcript was also over-expressed by 12,000-fold in neuroendocrine compared with nonneuroendocrine lung cancer cells. In peripheral blood of lung cancer patients and cancer-free individuals, we found that SCG3 was more tumor-specific and more sensitive than other chromogranin transcripts as a biomarker of circulating tumor cells. Overall, 36% of small cell lung cancer (SCLC) and 16% of non-SCLC patients scored positively for normalized SCG3 transcript. This correlated with worse survival among SCLC patients with limited disease (n = 33; P = 0.022) but not extensive disease (n = 29; P = 0.459). Interestingly, the subcohort of 6 SCLC patients with resistance to platinum/etoposide chemotherapy all scored positively for peripheral blood SCG3 transcript (P = 0.022).

Conclusions: SCG3 mRNA, a component of the REST-dependent neurosecretory transcriptional profile, provides a sensitive prognostic biomarker for noninvasive monitoring of neuroendocrine lung cancer.

Lung cancer is the leading cancer killer in the United States and the United Kingdom, accounting for 22% of all UK cancer deaths and ranking highest for both incidence and mortality throughout most of the world. It is classified into small cell lung cancer (SCLC) or non-SCLC (NSCLC). Thirty-two percent of lung tumors show a spectrum of neuroendocrine differentiation, comprising mainly SCLC, together with carcinoids and neuroendocrine NSCLC. They express and secrete a variety of neuropeptides and hormones that can contribute to tumor progression through autocrine growth loops or paracrine signaling (1, 2). Unlike NSCLC, SCLC are frequently metastatic at presentation so not generally amenable to surgical resection and, while initially chemo- and radiosensitive, the majority rapidly recur. Although multimodality therapies have improved the control of this disease (3), the UK 5-year survival rate remains at 6%. Biomarkers for SCLC could be exploited to characterize primary or metastatic tumor samples but might more usefully be developed to assay blood or other body fluids to monitor tumors noninvasively. Potential applications include early detection of disease, initial diagnosis, clinical staging, prognosis, prediction of response to specific therapy, or monitoring remission and disease progression.

The neuroendocrine phenotype of SCLC distinguishes them from most other normal or neoplastic cells in the lung. Although routine pathologic diagnosis of SCLC from bronchoscopic biopsies relies on morphologic criteria, there are established immunohistochemical markers like neuron-specific enolase and neuronal cell adhesion molecule. Serum markers for SCLC include the secreted neuropeptides neuron-specific enolase (4, 5), arginine vasopressin (6), gastrin-releasing peptide precursors (5), and chromogranin A (CHGA; refs. 5, 7). However, their utility is limited by serum peptide stability, the variability in neuropeptides secreted by individual tumors, and the secretion of multiple processed peptides. Thus, there are currently no established serum markers used in prognosis or treatment of SCLC patients. Importantly though, shed lung
Translational Relevance

There are presently no well-established prognostic or predictive biomarkers in SCLC and all patients receive standard chemotherapy for what is a heterogeneous disease. Although initially chemosensitive, recurrence is often rapid and survival rates are poor. Only a minority of patients with limited disease are currently eligible for concurrent chemoradiotherapy. The ability to detect SCLC biomarkers, such as SCG3 transcript, in the peripheral blood or other accessible body fluids could be used for minimally invasive longitudinal monitoring of disease. These have potential applications in early detection, diagnosis, staging, prognosis, predicting response to specific therapy, or monitoring response, remission, relapse, and disease progression for clinical management. Putative predictive factors may aid clinical trial development around emerging therapies; for example, biomarkers might be useful to monitor the subgroup of patients who would have de novo chemoresistance to standard therapy (20-30%). A predictor of poor response for limited disease patients might advocate alternative (nonplatinum) chemotherapy; for example, current trials suggest a role for Amrubin (anthracycline activity and topoisoasemerase inhibitor) in chemoradiotherapy SCLC at relapse. Furthermore, a prognostic factor would arm clinicians and patients with essential information on individualized treatment options and the required intensity of therapy. We therefore believe that the data reported here have identified SCG3 as a blood biomarker with the potential for future development in clinical practice.

Materials and Methods

Cell culture, small interfering RNA treatment, and immunoblotting. COR-L47, NCI-H727, NCI-H322, NCI-H647, NCI-H2170, MRCSVA (Cancer Research UK Cell Services), SV40 immortalized human keratinocytes (SVK), HeLa, NCI-H460, A549, and other SCLC (16) were cultured in RPMI 1640 or DMEM (Autogen Bicole) with 10% bovine calf serum (Pierce) at 37°C and 5% CO₂. Normal human bronchial epithelial cells (Cambrex BioScience) and BEAS-2B were maintained in complete small airway growth medium (Cambrex). NCI-H460 cells were transfected by electroporation (Biorad GenePulserII) with 600 pmol of the small interfering RNA (siRNA) sequences: sIREST1 (CAAGGAAUCCGUACGUAUUUA), siREST5 (CAUCCUAUAGAUAUUAUUAU), and siRNA (mokk) as described elsewhere. Cells were harvested at 3 days post-transfection for preparation of either protein or RNA. Protein was extracted by direct cell lysis in 2x Laemmlil sample buffer and quantified by BCA assay (Pierce). DTT was then added and samples were denatured. Protein [20 μg] was resolved on 8% SDS-PAGE gels and transferred to BiotranET membrane (VWR International). Replicate blots were probed with anti-REST (Upstate) or anti-actin (Sigma) antibodies. Proteins were visualized using SuperSignal West (Pierce).

RNA preparation and reverse transcription-PCR from cultured cells. Total RNA was prepared from untreated cell lines or NCI-H460 cells transfected with siRNA. Each PCR primer pair flanked an intron, and where appropriate, the reverse primer recognized the exon harboring an Affymetrix probe set. Sequences used to design primers were CGHA (NM_001275; ref. 8), CHGB (NM_001819.1), PCSK1 (NM_000439.3), SCG3 (NM_013243), VGF (NM_003378), and PCSK2 (NM_002594.2), and SCG2 (NM_003469). cDNA (100 μL) was first synthesized from 1 μg total RNA by reverse transcription (Promega). For semiquantitative reverse transcription-PCR (RT-PCR), 3 μL cDNA or reverse transcriptase-negative control were amplified with HotStar Taq (Qiagen) using standardized reaction conditions and optimized cycle numbers (35 cycles for VGF and 40 cycles for other genes). Electrophoresed PCR products were imaged at nonsaturating levels (Geneflash; Syngene) relative to GAPDH amplicons (25 cycles). Quantitative real-time RT-PCR (QPCR) was done in triplicate with 1 μL cDNA, 300 nmol primers, and IQ SYBR Green Supermix using an IQ5 real-time PCR detection system (Bio-Rad). Samples underwent 40 cycles of

with ANP, could contribute to hyponatremia in some SCLC patients (23). We recently undertook expression profiling in nonneuroendocrine lung cancer cells experimentally depleted of REST to better annotate the REST regulome in lung cancer.⁵ We reasoned that SCLC-associated transcripts, whose elevated expression is triggered by loss of REST, would be potential markers of SCLC tumors and should not be expressed in normal blood cells. We therefore screened for several genes induced in our model⁵ (CHGA, CHGB, SCG3, VGF and PCSK1) as candidate biomarkers. Despite transcriptional coregulation by REST, we found surprisingly diverse expression profiles for these mRNAs among lung cancer cell lines and in blood of cancer-free individuals (CFI). We identified SCG3 as the most sensitive and specific of these transcripts as a marker for CTC in SCLC. Furthermore, we found that it is prognostic of worse survival and was evident in patients with poor response to chemotherapy.

amplification at 94°C (30 s) and 60°C (60 s), fluorescence was read at 60°C, and melt curves analyzed. For each sample, the Ct values for test genes were normalized to the reference gene ACTB and relative expression represented as $2^{-\Delta\Delta Ct}$.

**Collection of clinical material and data.** Clinical material was collected prospectively between June 2003 and May 2006 under ethical (Wirral LREC 64/02, Liverpool LREC 04/01/272M/A, and St. Helen’s and Knowsley LREC 2003/2004-39) and hospital R&D approval with informed consent from SCLC and NSCLC patients presenting within the Merseyside and Cheshire Cancer Network at Clatterbridge Centre for Oncology, Liverpool Cardiothoracic Centre, and Whiston Hospital. Peripheral blood samples and clinical characteristics (age, sex, disease stage, performance status, treatment, response to therapy, and date of death) were collected from newly diagnosed lung cancer patients before chemotherapy. As unfit patients were not approached to participate in the study, the recruited cohort includes more limited disease patients with better performance status than the typical Merseyside and Cheshire Cancer Network lung cancer population. B-samples were collected from 20 SCLC patients in the cohort fit enough to be approached at follow-up at least 4 months after completion of chemotherapy. Patient blood was collected into PAXgene RNA tubes (Qiagen) for total RNA extraction. Control RNA samples were obtained from blood collected under ethical approval from healthy donors accompanying patients to clinic or purchased as total leukocyte RNA from CFI (AMS Biotechnology). Samples were processed in the same way for CHGA using the previously described primers and probe (8) and for SCG2 or PCSK2 with oligonucleotide probes (CATAAAATCTTTTCAACATGCTGGAAG and CTTGAGCGAACTCTTAAACTGAAG, respectively). Membranes were hybridized with probes (24) for 16 h, then washed to a stringency of 2 × SSC/0.1% SDS at 65°C, and exposed to autoradiography film at -80°C for 1 h up to 72 h. All patient samples were tested in duplicate for SCG3; samples were classed as positive if the transcript was detected at least once on 72 h exposure. SCG3 amplicon intensity was standardized to the positive control for each membrane using ImageJ and normalized to ACTB as determined by QPCR for each cDNA. Samples were scored for the intensity of the SCG3 amplicon above a statistically determined threshold. 

**Statistical analysis.** Receiver operating characteristic and statistical analysis of standard prognostic variables was carried out using SPSS. Sample size analysis was done using Statdirect. Survival data were calculated from date of diagnosis to date of death; 5 SCLC patients recruited to the study subsequent to initial diagnoses with lung cancer were excluded from these analyses. Treatment response was scored according to the treating clinician/radiologist assessment and was unavailable for four SCLC. Multivariate analysis was done using Cox regression. The Kaplan-Meier method was used to estimate survival and the log-rank test was used to correlate this with SCG3 based on scoring positively for the transcript at least once in the two replicate assays. Analysis of treatment response in SCLC where smaller patient subgroups were compared was based on detection frequency, but normalized scores followed the same trends.

**Results**

**SCG3 is highly transcribed in REST-deficient lung cancer.** We found by expression profiling that conditional depletion of REST in NSCLC cells using siRNA could alter transcription of several hundred genes, partly mimicking the transcriptional

| Table 1. Clinical characteristics for 111 recruited lung cancer patients |
|--------------------------|---------------------|---------------------|
| **Clinical characteristics** | **SCLC** | **NSCLC** |
| Patients recruited (n) | 67 | 44 |
| Median (range) age at diagnosis (y) | 63 (38-85) | 69 (40-84) |
| Sex (% female) | 46.8 | 34.1 |
| % Current or ex-smoker | 97 | 100 |
| Disease stage (n) | | |
| LD (%) | 62* | 39* |
| ED (%) | 53.2 | 46.8 |
| Stage I-II (%) | 5.1 | 49.4 |
| Stage III-IV (%) | 94.9 | |
| Performance status (n) | | |
| 0-2 (%) | 62* | 39* |
| 3-4 (%) | 95.5 | 97.5 |
| Treatment with at least one chemotherapy cycle (n) | 66* | 43* |
| Platinum/etoposide (%) | 97.0 | |
| Carboplatin/gemcitabine (%) | 93.2 | |
| Response to treatment (n) | | |
| Complete response (%) | 58* | 31* |
| Partial response (%) | 24.1 | 3.2 |
| Stable disease (%) | 65.5 | 67.7 |
| Progessive disease (%) | 3.5 | 12.9 |
| Survival (n) | | |
| Median (95% CI) survival (mo) | 12 (10-1.14.1) | 10 (7.0-13.0) |
| 1 y survival (%) | 48.4 | 41.0 |
| 2 y survival (%) | 21.9 | 15.4 |

*Number of patients where data were available.
program of SCLC. Among the significantly induced transcripts were several members of the chromogranin and secretogranin family: CHGA, CHGB, SCG3, and VGF together with PCSK1, an enzyme that processes these proteins into peptides for secretion. Importantly, four of these genes were also highly transcribed in the SCLC cell line NCI-H69 relative to the NSCLC cell line NCI-H460 on DNA microarray analysis. Thus, we sought to further investigate their transcriptional profiles in lung cancer cells by RT-PCR, along with two related genes, SCG2 and PCSK2, which were not induced by REST depletion.

We first compared the transcriptional response to REST siRNA treatment of NCI-H460 cells (Fig. 1A). Basal transcript levels varied among the genes, but although mRNA was undetectable for SCG3 or PCSK2, CHGA was not substantially repressed in NCI-H460 cells. Both SCG2 and PCSK2 have been identified from genome-wide chromatin immunoprecipitation screening for REST binding sites (25, 26), but we confirmed that neither gene was regulated by REST in these lung cancer cells. Nonetheless, each gene identified from our microarray study was induced by depletion with both siRNA sequences targeting REST, confirming that CHGA, CHGB, SCG3, VGF, and PCSK1 were all REST-responsive. Notably, SCG3 transcription was exquisitely sensitive to loss of the REST repressor.

Whereas CHGA, CHGB and PCSK1 (5, 8, 27, 28) are established markers for SCLC, to the best of our knowledge, VGF and SCG3 have not been previously characterized in lung cancer. We initially screened a panel of SCLC, carcinoid (NCI-H727), nonneuroendocrine NSCLC and control cell lines by conventional RT-PCR to correlate transcription with REST status for these genes (Fig. 1B). The transcript most stringently restricted to neuroendocrine lung cancer cells was SCG3. Although CHGA transcript has been tested as a specific marker in SCLC (8), in this study, we detected CHGA transcripts in four of five nonneuroendocrine NSCLC cell lines. CHGB, VGF, PCSK1, and PCSK2 were also expressed in several NSCLC. In contrast, SCG3 was detected by conventional RT-PCR in neither NSCLC nor normal cells derived from lung epithelium or fibroblasts. By QPCR analysis, the mean differential expression of SCG3 between neuroendocrine and nonneuroendocrine lung cancer cell lines was >12,000-fold compared with 3,600-fold for CHGA (Fig. 1B). These data suggest the SCG3 transcript should be a specific and sensitive marker for tumors that lose REST.

**SCG3 mRNA can be used to detect CTC from neuroendocrine lung cancer.** The paucity of a candidate biomarker transcript in normal circulatory cells is a prerequisite for exploiting it to detect CTC. Therefore, we first screened peripheral blood RNA samples from CFI for the transcripts of interest. We excluded CHGB, VGF, and PCSK1 after the first screen, as mRNA was detected in at least one case by RT-PCR alone (data not shown). These data imply that only a subset of REST-regulated mRNAs will be candidate biomarkers for SCLC in blood. To improve the assay sensitivity and specificity for the other candidate transcripts, we conducted RT-PCR (using 6% of cDNA reaction) followed by hybridization with a cDNA-specific radiolabeled probe and autoradiography (Fig. 1C). In 20 blood samples from CFI, we found SCG2 mRNA in three individuals, but we did not detect CHGA, PCSK2, or SCG3 transcripts. SCG3 was selected for further evaluation, as this transcript was most highly expressed on REST depletion and among neuroendocrine lung cancer cell lines but was not detected in non-neuroendocrine NSCLC cell lines, normal lung cell lines, or peripheral blood of NSCLC cell lines, normal lung cell lines, or peripheral blood of NSCLC cell lines.

To improve detection of SCG3 transcript and to validate the assay sensitivity and reproducibility, we now increased the amount of template to 24% of each cDNA reaction. Peripheral blood samples were collected from a healthy volunteer into PAXgene tubes and spiked with a serial dilution of SCG3-expressing SCLC cells (Lu-165) before preparation of RNA. SCG3 transcript could be detected from ~10 SCLC cells/mL by RT-PCR and hybridization with the SCG3-specific radiolabeled probe (Supplementary Fig. S1), comparable with studies employing similar methodologies to detect arginine vasopressin, prepro-gastrin-releasing peptide (10), and CHGA (8) transcripts. These other studies did not address assay reproducibility. However, using replicate QPCR analysis for SCG3, we show that assay reproducibility decreased in line with the number of SCLC cells/mL of blood (Supplementary Fig. S1), suggesting the ability to detect these transcripts will reflect the patient CTC burden.

To determine the frequency with which SCG3 transcript could be detected in lung cancer patient blood, we now analyzed the whole cohort of 67 SCLC, 44 NSCLC, and 27 CFI. Each sample was tested in duplicate by PCR using 24% of the cDNA reaction and hybridization. Because assay reproducibility reflects the number of SCG3-expressing cells (Supplementary Fig. S1), patients were regarded as positive if SCG3 mRNA was detected at least once. The results for the SCLC cohort are shown in Fig. 2 and all data are summarized in Table 2. Overall, the SCG3 detection frequency among SCLC samples collected at diagnosis was 54%, with a third of samples positive in both replicates. Interestingly, SCG3 mRNA was also detectable in 75% of 20 SCLC follow-up samples (SCLC-B) and in 34% of NSCLC patients. Unexpectedly however, employing this more sensitive methodology now identified low levels of SCG3 transcript in 67% of CFI samples.

The reference gene ACTB was evaluated by QPCR for all peripheral blood RNA samples and the mean Ct value in CFI was 19.8 (SD = 1.10) compared with 22.0 (SD = 1.93) for SCLC and 21.5 (SD = 1.59) for NSCLC patients, showing that RNA recovered from CFI was of significantly higher quality than that from cancer patients (P < 0.0001). To account for this variable, we used ACTB transcript quantification by QPCR to derive a normalized SCG3 expression score (Fig. 3). Receiver operating characteristic analysis for the SCLC and CFI cohorts was then used to select the most suitable threshold value for scoring samples as positive and excluding false-negatives (Supplementary Fig. S2). The selected threshold of 0.043 excluded the entire CFI group, whereas the SCG3-positive rate in SCLC patients at diagnosis was 35.8% [24 of 67; 95% confidence interval (95% CI), 24.3-47.3%] compared with 15.9% (7 of 44) in NSCLC patients (95% CI, 5.1-26.7%; Fig. 3; Table 2). Based on the observed probability of scoring above the threshold in SCLC (0.36) and CFI (0.0), the sample sizes were predicted to be appropriately powered (55 cases and 22 controls: 95% power).
Fig. 1. Expression of chromogranin family transcripts in REST-depleted neuroendocrine lung cancer and peripheral blood of CFI. A, transcript induction on REST depletion for the chromogranin/secretogranin family and their processing enzymes. Semi-quantitative endpoint RT-PCR was carried out at optimized cycle numbers for each gene of interest and the reference gene GAPDH. Representative data for RNA collected 3 days post-transfection with control or REST siRNA sequences. An immunoblot is aligned to illustrate REST protein status (top). B, transcripts show differential expression across a panel of 17 SCLC, NSCLC, and normal control cell lines. Quantitative SCG3 (black bars) and CHGA (gray bars) expression profiles derived by QPCR are aligned (bottom), showing the mean expression relative to ACTB (log2 ΔCt; n = 3). Bars, SD. C, autoradiograms showing candidate transcript detection from the peripheral blood RNA of 20 CFI. CHGA, SCG2, PCSK2, and SCG3 were amplified from 6% of cDNA (+) or reverse transcriptase-negative (-) reactions by 40 cycles of PCR, blotted, and hybridized with gene-specific radiolabeled oligonucleotide probes. Dashed white lines, discontinuities in gels and blots.
Although not significantly different \( (P = 0.242) \), a positive score was more common among SCLC patients with extensive disease (ED; 41.4%; 95% CI, 23.5-59.3%) than those with limited disease (LD; 27.3%; 95% CI, 12.1-42.5%; Table 2), consistent with a higher level of CTC in individuals who already have overt metastases at presentation. In peripheral blood collected from 20 SCLC patients >4 months post-chemotherapy, the SCG3-positive rate rose to 60% (12 of 20; 95% CI, 38.5-81.5%) compared with 25% (5 of 20; 95% CI, 6.0-44.0%) of the same cohort at the time of diagnosis. These

Fig. 2. SCG3 mRNA is a peripheral blood biomarker in SCLC patients. Autoradiograms showing SCG3 transcript detection from the peripheral blood RNA of 67 SCLC patients at 48 h exposure. SCG3 was amplified in duplicate from 24% of cDNA (+) or reverse transcriptase-negative (−) reactions by 40 cycles of PCR, blotted, and hybridized with a SCG3-specific radiolabelled oligonucleotide. Duplicate analysis of each sample.
preliminary data show that SCG3 could be readily detected at patient follow-up, suggesting that it may be useful for longitudinal monitoring of disease.

**SCG3 transcript in peripheral blood of SCLC patients is prognostic for poor survival and associated with worse treatment response.** Based on the assumption that the normalized SCG3 transcript score reflects the CTC burden in patients, we used SPSS to test for differences in survival by the log-rank test. Cox regression found that disease staging ($P = 0.001$), treatment response ($P < 0.001$), and normalized SCG3 transcript ($P = 0.044$) were significant variables for survival of SCLC patients, but age ($P = 0.611$) and performance status ($P = 0.366$) were not. Thus, although overall survival was most highly correlated with treatment response, normalized SCG3 and treatment response were cumulative hazards. Kaplan-Meier estimates predicted a mean survival of 15.7 months (SE = 1.46) for the SCLC study cohort, which decreased among the group of SCLC patients positive for normalized SCG3 ($P = 0.095$) although not reaching significance ($P = 0.095$).

Overall survival among SCLC patients was highly correlated with the stage of disease at presentation ($P < 0.001$; Fig. 4A). To determine whether the SCG3 biomarker might be useful in further prognostic stratification among the LD or ED patients, we derived Kaplan-Meier estimates within each subgroup (Fig. 4B). SCG3-negative LD patients showed significantly better survival than those who scored positively for normalized SCG3 ($P = 0.022$; Table 2). Clinical characteristics of LD patients stratified by SCG3 status were similar (data not shown). In contrast, there was no correlation between SCG3 detection score and survival of ED patients ($P = 0.459$; Table 2). For NSCLC, there was no significant correlation between SCG3 mRNA score and mean survival ($P = 0.821$). Thus, the current assay for SCG3 transcript in the peripheral blood was of prognostic significance in SCLC patients with LD at presention, although the biomarker may also prove useful for monitoring other patient groups.

For both SCLC and NSCLC patients, survival correlated with treatment response ($P < 0.001$; Fig. 4C). Those that responded to standard platinum-based chemotherapy had a mean survival of 19.9 months (SE = 1.70) and those who did not respond had

### Table 2. SCG3 mRNA detection and scoring in peripheral blood with survival statistics for SCLC patients

<table>
<thead>
<tr>
<th>Cancer status</th>
<th>No. tested</th>
<th>SCG3 mRNA detected</th>
<th>Scored normalized SCG3</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>SCLC*</td>
<td>67</td>
<td>36</td>
<td>31</td>
</tr>
<tr>
<td>SCLC-B</td>
<td>20</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>NSCLC*</td>
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<td>15</td>
<td>29</td>
</tr>
<tr>
<td>CFI</td>
<td>27</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>SCLC b</td>
<td>62</td>
<td>33</td>
<td>29</td>
</tr>
</tbody>
</table>

**Survival (mo)**

<table>
<thead>
<tr>
<th></th>
<th>Median (95% CI)</th>
<th>Mean (SE)</th>
<th>$P$ (log-rank test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCLC LD</td>
<td>12 (10.0-14.1)</td>
<td>15.7 (1.46)</td>
<td>0.137</td>
</tr>
<tr>
<td>SCLC LD</td>
<td>17 (13.9-20.1)</td>
<td>19.8 (2.19)</td>
<td>0.194</td>
</tr>
<tr>
<td>SCLC ED</td>
<td>9 (7.3-10.7)</td>
<td>10.6 (1.21)</td>
<td>0.713</td>
</tr>
</tbody>
</table>

**Abbreviations:** LD, limited disease; ED, extensive disease.

*Number of SCLC and NSCLC samples collected at initial presentation.

1Number of SCLC-B samples collected from a subset of the SCLC cohort at follow-up >4 mo post-chemotherapy.

bNumber of SCLC samples collected at initial diagnosis where survival data were available.

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Fig. 3. Scoring of SCG3 mRNA in lung cancer patients and CFI. Quantification of hybridized SCG3 RT-PCR products in clinical samples normalized to ACTB. Values are a percentage of control expression and a threshold of 0.043 was applied to score peripheral blood mRNA samples positive. Sixty-seven SCLC, 44 NSCLC, 27 CFI, and 20 post-chemotherapy SCLC-B samples were analyzed in duplicate; all quantifiable products are shown.
a mean survival of only 9.7 months (SE = 1.73). The SCLC cohort received up to 6 cycles of carboplatin/etoposide; for 58 evaluable patients including both LD and ED, Fisher’s exact test showed a significant correlation between detecting SCG3 transcript in SCLC and overall response ($P = 0.022$). Although this study included only a small number of SCLC patients who failed to respond, it is interesting to note that all 6 patients were SCG3 positive (Fig. 4D). Thus, SCG3 transcript in peripheral blood may also be predictive for SCLC patients less likely to respond to standard chemotherapy.

**Discussion**

The application of tumor-associated transcripts as biomarkers of CTC in whole peripheral blood is dependent on their low basal expression in hematopoietic cells. Because REST was first described as a transcriptional repressor that silenced expression of neuronal genes in nonneuronal cells (12, 13), we hypothesized that transcripts restricted by REST, yet expressed in SCLC, should distinguish these CTC from hematopoietic cells. However, REST has transpired to be a complex transcriptional regulator, with dynamic, gene-specific, and cell-type specific factors determining the pool of repressed genes. Although collated data from recent studies of RE-1 prediction, REST overexpression, or genome-wide chromatin immunoprecipitation (25, 26, 29–36) suggested that each of the seven genes screened here are potential REST targets, we found that not all were induced by REST depletion in lung cancer. Furthermore, we found wide variation among the restriction of these genes to neuroendocrine

**Fig. 4.** Peripheral blood SCG3 mRNA is a prognostic biomarker in SCLC. A to C, cumulative survival curves derived from Kaplan-Meier survival estimates and log-rank tests for patients entering the study at their initial diagnosis with lung cancer. Crosses, censored individuals. A, survival of 62 SCLC patients according to stage of disease: LD and ED ($P < 0.001$). B, survival of 33 SCLC patients with LD according to scoring for normalized SCG3 transcript ($P = 0.022$). C, survival of 89 lung cancer patients with evaluable treatment response according to response to platinum-based chemotherapy ($P < 0.001$). D, detection of SCG3 mRNA in the peripheral blood of 58 SCLC patients entering the study at initial diagnosis who were evaluable for treatment response displayed by treatment responder type ($P = 0.022$, Fisher’s exact test): complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD).
lung cancer cells and among their expression in the peripheral blood of CFI.

Consistent with an earlier report that six SCLC-associated transcripts (neuronal cell adhesion molecule, PGP9.5, gastrin, gastrin receptor, synaptophysin, and gastrin-releasing peptide receptor) were evident in peripheral blood from CFI (10), we could detect several chromogranin transcripts in control blood samples. Although one CFI sample was positive for several transcripts screened, suggesting that we may not have realized an entirely cancer-free control group, these transcripts in CFI blood are most likely derived from leukocytes. In fact, the peripheral blood transcriptome represents ~80% of the human genome (37) and some tumor-associated transcripts are induced by lymphocyte activation (38).

REST is a putative tumor suppressor in cancers of nonneuronal origin (39). We identified the SCG3 transcript as an alternate candidate biomarker because it was the most highly induced transcript in genome-wide analysis of REST depletion in NSCLC and is transcribed at a high level in SCLC. This suggested that it could be exploited for detection of rare CTC in peripheral blood. In common with earlier studies of SCLC-derived transcripts in blood, we did not isolate CTC but instead monitored the nucleated cell fraction from whole blood. We saw a high differential in SCG3 transcript levels between SCLC and CFI, supporting our assumption that it is derived from tumor cells; furthermore, in our hands, SCG3 proved more sensitive than detection of CHGA transcript. Although application of the current assay in diagnosis is precluded by the low sensitivity, it should be noted that SCG3 was more frequently detected in SCLC patients with ED, who were statistically underrepresented in this study cohort compared with the larger patient population presenting in the Merseyside and Cheshire Cancer Network.

The frequency of SCG3 scoring in NSCLC patients (16%) would be consistent with the percentage of tumors expected to show neuroendocrine differentiation, although diagnostic biopsies were not available for confirmatory immunohistochemical staining of neuroendocrine markers in this study. In fact, studies using sensitive immunohistochemical staining suggest that up to 45% of NSCLC may express one or more neuroendocrine marker (40). Thus, SCG3 transcript may also be able to identify those patients with neuroendocrine NSCLC and CTC. Although the clinical significance of neuroendocrine differentiation in NSCLC remains contentious, some recent studies report a worse prognosis (41, 42).

Most interestingly, the SCG3 transcript correlated with survival among LD SCLC patients, suggesting that it may help to identify those with more aggressive disease. In contrast, there was no significant correlation among SCLC patients with ED, perhaps because the number of circulating tumor cells may have less effect on survival in patients who already have overt metastases. In previous studies, other SCLC-associated transcripts in peripheral blood have not proven useful for prognostication. Although neuromedin B receptor-positive patients showed a trend toward worse survival, by log-rank test, this was not significant in their cohort of 44 SCLC patients or among the 21 patients with LD (11). Furthermore, although CHGA transcript was reported to be associated with an increased lethal risk, patients positive for CHGA exhibited a better mean survival than CHGA mRNA-negative patients (8). Although the extent of disease at diagnosis is important in determining survival, this is only used to group SCLC patients as LD or ED and there is potential additional benefit in using a biomarker like SCG3 to stratify the LD patients into better or worse survival groups.

Furthermore, response to standard chemotherapy is important in determining survival but at present cannot be predicted at the time of diagnosis. The survival correlation with SCG3 we report here may, at least in part, be attributable to the association with response to chemotherapy. Although the numbers are small, all the SCLC patients who did not respond to conventional treatment in this cohort were positive for SCG3 transcript in peripheral blood. A validated test for subgroups of SCLC patients with LD who are less likely to respond to standard chemotherapy might advocate alternative combined chemotherapy regimes or entry into clinical trials for novel agents. Thus, whether SCG3 positivity predicts for worse survival because of worse response to treatment or for other reasons (more aggressive tumors), it may be developed as a useful biomarker to stratify the patient population.

SCG3 has cellular functions in scaffolding neuroendocrine secretion and as a secreted neuropeptide (43–45). Although, like other neuropeptides, it may be involved in autocrine regulation in SCLC, this remains to be explored and there is no obvious mechanistic association between SCG3 expression in tumors and response to platinum-based therapy or topoisomerase II inhibitors. However, we are using SCG3 as surrogate readout for tumor cells that have lost REST and we have shown elsewhere that transcription of several hundred genes is markedly dysregulated in REST-depleted NSCLC cells. Therefore, one or more genes within this transcriptional profile may increase the aggressive potential of the tumor or modulate the drug sensitivity of REST-depleted tumors. For example, the cell adhesion molecule CD24 is associated with more aggressive disease in a range of tumor types including SCLC (46) and was significantly induced by REST depletion. Furthermore, the transcriptional response to carboplatin treatment of drug-resistant ovarian cancer cell lines includes modulation of the REST corepressor RCO1 (47) and recent expression profiling of cisplatin-resistant breast cancer cell lines identified eight significantly induced transcripts (48), two of which, DUSP4 and SLC7A1, were also significantly increased by conditional REST depletion in NSCLC.

SCG3 is just one component of the neurosecretory and oncogenic transcriptional profile that characterizes lung tumors where transcriptional repression by REST is lost. However, because of the extremely high level of SCG3 transcript expression in REST-deficient lung cancer cells, coupled with relatively tight transcriptional restriction in other cell types, it provides a useful marker of SCLC and perhaps other neuroendocrine tumors. We show here that not only can SCG3 mRNA be used as sensitive biomarker in the peripheral blood for noninvasive monitoring of neuroendocrine lung cancer but also that its detection might be of prognostic significance in patients with LD.

Disclosure of Potential Conflicts of Interest

J. Coulson: inventor for related patent application.

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SCG3 Transcript in Peripheral Blood Is a Prognostic Biomarker for REST-Deficient Small Cell Lung Cancer

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