

# Integrative Genomic Analysis of Small-Cell Lung Carcinoma Reveals Correlates of Sensitivity to Bcl-2 Antagonists and Uncovers Novel Chromosomal Gains

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## Abstract

Cancer is a highly heterogeneous disease in terms of the genetic profile and the response to therapeutics. An early identification of a genomic marker in drug discovery may help select patients that would respond to treatment in clinical trials. Here we suggest coupling compound screening with comparative genomic hybridization analysis of the model systems for early discovery of genomic biomarkers. A Bcl-2 antagonist, ABT-737, has recently been discovered and shown to induce regression of solid tumors, but its activity is limited to a fraction of small-cell lung carcinoma (SCLC) models tested. We used comparative genomic hybridization on high-density single-nucleotide polymorphism genotyping arrays to carry out a genome-wide analysis of 23 SCLC cell lines sensitive and resistant to ABT-737. The screen revealed a number of novel recurrent gene copy number abnormalities, which were also found in an independent data set of 19 SCLC tumors and confirmed by real-time quantitative PCR. A previously unknown amplification was identified on 18q and associated with the sensitivity of SCLC cell lines to ABT-737 and another Bcl-2 antagonist. The region of gain contains *Bcl-2* and *NOXA*, two apoptosis-related genes. Expression microarray profiling showed that the genes residing in the amplified region of 18q are also overexpressed in the sensitive lines relative to the resistant lines. Fluorescence *in situ* hybridization analysis of tumors revealed that Bcl-2 gain is a frequent event in SCLC. Our findings suggest that 18q21-23 copy number will be a clinically relevant predictor for sensitivity of SCLC to Bcl-2 family inhibitors. The 18q21-23 genomic marker

may have a broader application in cancer because Bcl-2 is associated with apoptosis evasion and chemoresistance. (Mol Cancer Res 2007;5(4):331–9)

## Introduction

Genetic heterogeneity of cancer is a factor complicating the development of efficacious cancer drugs. Cancers considered to be a single disease entity according to classic histopathologic classification often reveal multiple genomic subtypes when subjected to molecular profiling (1). In some cases, molecular classification proved to be more accurate than the classic pathology (2). The efficacy of targeted cancer drugs may correlate with the presence of a genomic feature, such as a gene amplification (3, 4) or a mutation (5, 6). For HER2 in breast cancer, detection of gene amplification provides superior prognostic and treatment selection information as compared with the immunohistochemical detection of the protein overexpression (7). It is therefore becoming clear that discovery of genomic stratification markers is an attractive approach that may improve the response rate of patients to targeted cancer therapeutics.

Lung malignancies are the leading cause of cancer mortality, which will result in ~160,000 deaths in the United States in 2006 (8). Small-cell lung carcinoma (SCLC) is a subtype of lung cancer, which represents ~20% of lung cancer cases (9). The remainder of lung cancer cases are non-small-cell lung carcinomas, a category that is composed of several common subtypes. In the past several years, there has been substantial progress in the development of targeted therapies for non-small-cell lung carcinoma, such as erlotinib and gefitinib (10). This progress has been facilitated by efforts in molecular profiling of non-small-cell lung carcinomas. Mutations and amplifications in the epidermal growth factor receptor (EGFR) kinase domain were shown to correlate with the response to erlotinib and gefitinib (6, 11-14). Unfortunately, no such progress has been achieved with SCLC, although genomic analysis of SCLC has been reported (15-18).

Comparative genomic hybridization (CGH; refs. 19-22) is a promising approach to genomic biomarker identification. Here we propose coupling genome-wide detection of gene copy number alterations by CGH with screening of therapeutic candidates to enable early discovery of genomic markers of drug sensitivity. We applied high-density genotyping arrays to screen a panel of SCLC lines used to screen a targeted SCLC

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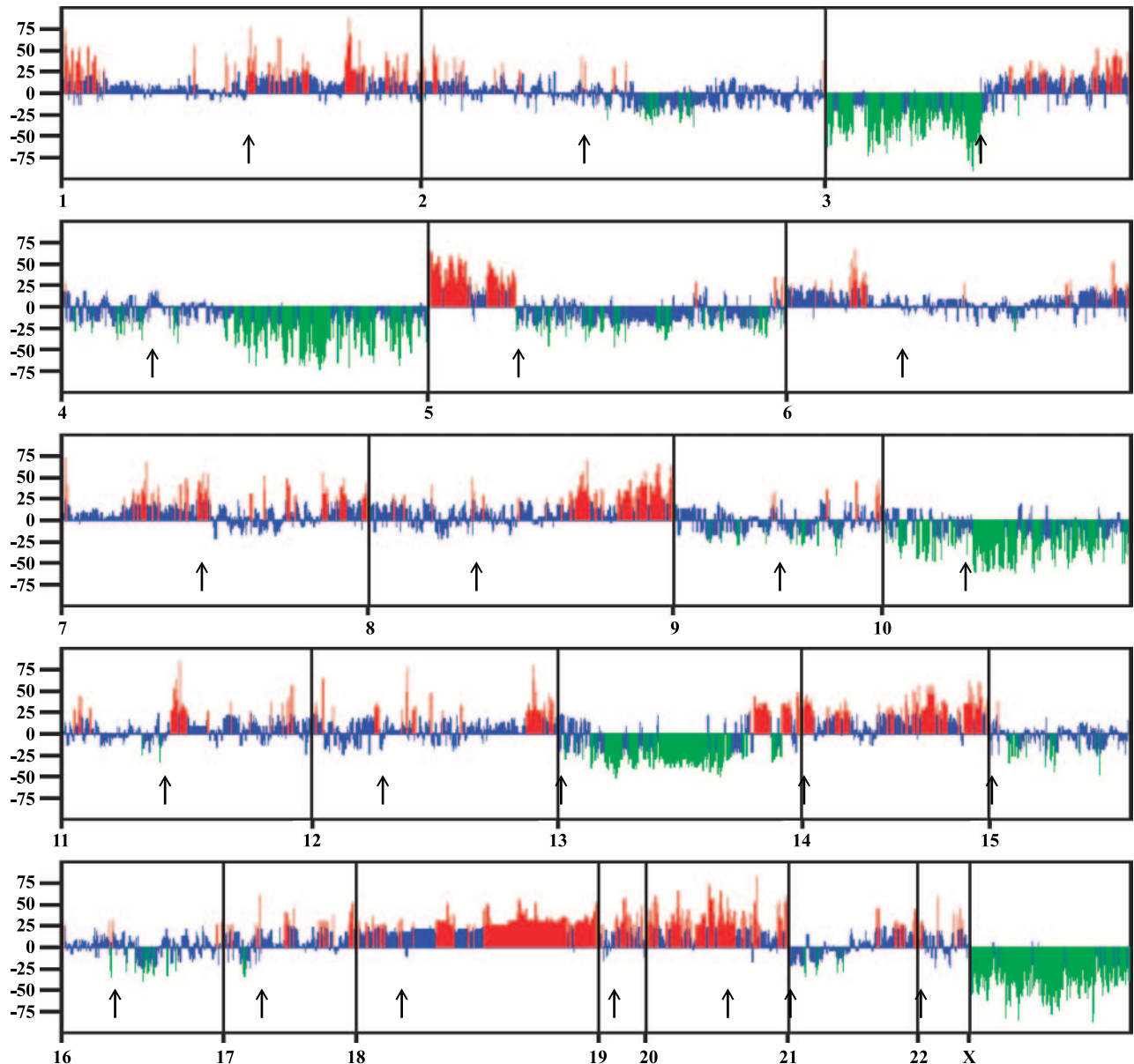
agent. A gain was identified on 18q that correlates with sensitivity of SCLC cells to the drug. The clinical relevance of this gain has been verified by fluorescence *in situ* hybridization (FISH) analysis of SCLC tumors. The genes residing in the marker region were shown to be overexpressed in the sensitive lines. Our findings validate the methodology and reveal a mechanism-based marker of drug sensitivity for SCLC.

## Results

### Identification of Recurrent Gene Copy Number Alterations in SCLC by CGH

A panel of 23 SCLC cell lines was profiled using high-density human single nucleotide polymorphism (SNP) geno-

typing arrays. The density of SNP coverage (~114,000 SNPs with a mean spacing of 23.6 kb) allowed for a very high resolution of the copy number scan. To validate the CGH method, we first sought to identify recurrent copy number abnormalities, as multiple aberrations have previously been defined for SCLC. Figure 1 presents a summary of DNA copy number alterations across the genome. The data are plotted as a frequency plot of copy number gains ( $\geq 3$  copies) or losses ( $\leq 1.5$  copies). Gains and losses with a frequency  $>25\%$  are shown in red and green, respectively. The threshold for losses of 1.5 was chosen because of the apparent signal compression on 100K SNP arrays in regions of DNA losses. In numerous cases, we observed long regions with a copy number of  $\sim 1.5$ .



**FIGURE 1.** Recurrent gene copy number abnormalities in a panel of SCLC cell lines. Y axis, frequency of copy number gains ( $\geq 3$  copies) or losses ( $\leq 1.5$  copies). Arrow, centromere. The p and q arms are shown to the left and to the right of the centromere, respectively. Gains and losses present in  $\geq 25\%$  of the lines are shown in red and green, respectively.

**Table 1. Summary of Novel Recurrent Chromosomal Aberrations in SCLC**

Abnormality	Coordinates	Length	Frequency in Cell Lines (%)	Frequency in Tumors (%)	Genes with Known Association with Cancer
Gain of 2q37.1-q37.2	235720690 to 236144659	420 kb	61	66	
Gain of 6p21.31	34998875 to 35354297	3.63 Mb	69	63	<i>CK2B, MSH5</i>
Gain of 7p22.1	5981609 to 6248486	270 kb	69	54	<i>RAC1</i>
Gain of 7p14.3	30702026 to 30743040	40 kb	75	41	
Gain of 7q11.21	64150926 to 64714134	560 kb	47	42	
Gain of 7q11.23	72200986 to 74708138	2.51 Mb	71	60	<i>RFC2, FZD9, BCL7B</i>
Gain of 7q22.1	98256212 to 98446355	190 kb	55	80	
Gain of 7q36	157764666 to 157892583	130 kb	72	54	<i>PTPRN2</i>
Gain of 9q34.11-34.12	128878693 to 130736916	1.86 Mb	58	63	<i>ABL1</i>
Gain of 9q34.2	133356411 to 133837324	480 kb	85	98	
Loss of 10q21.1	56640222 to 56976835	340 kb	53	42	
Loss of 10q21.1	57485108 to 59373358	189 Mb	57	44	
Loss of 11p11.12	48944115 to 49173275	230 kb	41	46	
Gain of 11q13.2-q13.3	68071140 to 68457277	390 kb	59	60	
Gain of 11q13.4	70119917 to 70508750	390 kb	88	81	
Gain of 11q23.3	118080390 to 118510298	430 kb	74	41	<i>DDX6, BCL9L, FOXR1, TMEM24</i>
Gain of 12p13.33	498351 to 546804	48 kb	52	96	
Gain of 12p13.31	6264939 to 6755337	490 kb	57	83	<i>TNFRSF1A, CHD4</i>
Gain of 12q13.12	48364391 to 48701968	340 kb	73	58	<i>BAX inhibitor-1, FAIM-2</i>
Gain of 12q14.2	63220192 to 63317157	98 kb	65	70	<i>RASSF3</i>
Gain of 12q24.11	108009640 to 108271491	260 kb	80	67	
Gain of 12q24.12	110170474 to 110352261	180 kb	86	46	
Gain of 12q24.13	112203187 to 112212500	10 kb	61	58	
Gain of 12q24.33	130872819 to 131618922	750 kb	55	85	<i>MMP17</i>
Gain of 13q34	112073788 to 113215972	1.14 Mb	59	53	
Gain of 14q11.2	21171249 to 21305730	130 kb	43	47	
Gain of 14q23.2	63829172 to 63899770	70 kb	48	40	<i>ER2</i>
Gain of 14q24.3	71984558 to 72392319	410 kb	46	45	
Gain of 14q24.3	74003876 to 75056332	1.05 Mb	54	47	
Gain of 14q24.3	76984427 to 77144559	160 kb	51	52	<i>CHES1</i>
Gain of 14q32.12	88752354 to 91109154	2.36 Mb	50	56	
Gain of 14q32.13-32.31	94993423 to 101036144	6 Mb	48	61	<i>TCL6</i>
Gain of 14q32.33	104475429 to 106312036	1.84 Mb	83	78	<i>TMEM121</i>
Gain of 17q21.33	46061156 to 46295421	230 kb	43	70	
Gain of 17q24.3-q25.1	68263039 to 70880595	2.62 Mb	53	77	
Gain of 17q25.3	73084146 to 74205146	1.12 Mb	59	61	
Gain of 18q12.2	33315195 to 33501543	190 kb	46	54	
Gain of 18q21.1	44239189 to 44613022	370 kb	48	51	
Gain of 18q22-q23	72742902 to 73144551	400 kb	46	88	
Gain of 20p13	546278 to 915957	370 kb	57	45	
Gain of 20p13	2692438 to 2884204	190 kb	59	49	
Gain of 20p11.23	18537737 to 18833682	300 kb	62	41	
Gain of 20p11.21	23097739 to 23887277	790 kb	52	40	
Gain of 20q11.21	30675396 to 30905617	230 kb	64	98	
Gain of 20q11.23	34166072 to 34443365	280 kb	35	56	
Gain of 20q12-q13.1	42528911 to 42717549	190 kb	43	98	
Gain of 20q13.12-q13.13	44838035 to 47292422	2.45 Mb	60	58	<i>PREX1, CSE1L</i>
Gain of 20q13.32	56373559 to 56412822	40 kb	42	84	<i>RAB22A</i>
Gain of 20q13.33	59186707 to 59832199	2.74 Mb	47	57	
Gain of 21q22.3	45373039 to 46844296	1.47 Mb	57	69	
Gain of 22q12	38036814 to 38102827	66 kb	65	61	

Real-time quantitative PCR established that all these regions had a copy number of 1 (data not shown). This effect can be attributed to the smoothing algorithm used by the GTYPE program.

The SCLC lines possess multiple recurrent genome alterations, with long regions of frequent gains on almost every chromosome (Fig. 1). Recurrent gains of genetic material (frequency  $\geq 25\%$ ) are observed on 1p, 1q, 3q, 5p, 6p, 7p, 7q, 8q, 10p, 11q, 12q, and 13q and throughout chromosomes 14, 18, 19, and 20. These hotspots of genomic instability in SCLC contain known oncogenes related to deregulation of cell growth, such as *EGFR* (26.1% of cell lines), *FGFR1* (52.2%), *c-myc* (56%), and *N-myc* (39%). The most frequent losses were found on 2q, 3p, 4p, 4q, 5q, 10, 13, 14, 16p, and 21p. These regions of

recurrent loss contain known tumor suppressor genes, such as *PTEN* (34%), *APC* (21%), and *FHIT* (30%).

A previously reported pioneering study of 70 lung tumors with 100K SNP arrays (18) included 19 primary SCLC tumors. However, recurrent aberrations in SCLC have not been specifically identified. Here, we obtained the data set by Zhao et al. (18) to validate our findings of recurrent aberrations in SCLC lines. To select only aberrations representative of tumorigenesis in SCLC, we analyzed our data set and the data set of Zhao et al. (18) concurrently using our CGH analysis program. Only abnormalities present in both data sets at a frequency of  $\geq 40\%$  were considered recurrent and selected for further analysis. Supplementary Table S1 summarizes the alterations recurrent in both data sets and lists the location,

length, and frequency of change for each region, as well as all the genes residing in the regions.

All of the SCLC aberrations reported to date were present in our data set, thus validating our CGH method. In addition, our data set also contained a number of novel recurrent copy number changes. Table 1 and Supplementary Table S1 (worksheet 2) summarize all copy number abnormalities present in  $\geq 40\%$  of our lines as well as in  $\geq 40\%$  of SCLC tumors from the data set of Zhao et al. (18) and not previously reported in the literature. The list of novel aberrations includes gains of 2q, 6p, 7p, 9q, 11p, 11q, 12p, 12q, 13q, 14q, 17q, 18q, 20p, 20q, 21q, and 22q and losses of 10q21.1.

Real-time quantitative PCR was used to confirm these novel recurrent abnormalities. Each locus of interest was screened in three representative lines. The results (Supplementary Fig. S1) show a 94.8% agreement with lines that have amplifications above the 3.0 copy number threshold. Of all 59 gained loci examined, 53 were confirmed in all lines evaluated, in which the remaining loci were confirmed in two of the three lines. The copy number derived from quantitative PCR was slightly higher than that from CGH. This may be attributed to possible signal compression by 100K SNP arrays, as reported by others (18). The results for the four CGH-derived heterozygous deletions were inconclusive, reflecting the difficulty of detecting single copy loss by quantitative PCR. Overall, the quantitative PCR data validate the CGH results and confirm the existence of the novel copy number gains.

Most of the novel aberrations are relatively short (70 kb-3.6 Mb). It is therefore possible that they have not been previously detected because of the lower resolution of the previously used techniques (metaphase CGH, BAC array CGH, and FISH). The mean spacing between the SNPs on the 100K SNP array used in this study is 23.6 kb, thus permitting identification of very short regions of gains and losses. It is also possible that some of the newly detected recurrent copy number changes represent copy number polymorphisms (23). However, this is only a remote possibility because the copy number is determined relative to a panel of 110 normal individuals (24).

It is noteworthy that the novel regions on 7p22.1, 12q14.2, and 20q13.32 contain members of the Ras family [i.e., RAC1 (gains in 69% of lines and 54% of tumors), RASSF3 (65% of lines and 70% of tumors), and RAB22A (42% of lines and 84% of tumors)]. Other novel regions include antiapoptotic genes, such as *BI-1*, *FAIM-2* (12q13.12; gained in 73% of lines and 58% of tumors), and *RFC2* (7q11.23; gained in 71% of lines and 60% of tumors). The known oncogene *ABL1* on 9q34.1 was amplified in 72% of lines and 54% of tumors, indicating a potential role in tumorigenesis in SCLC.

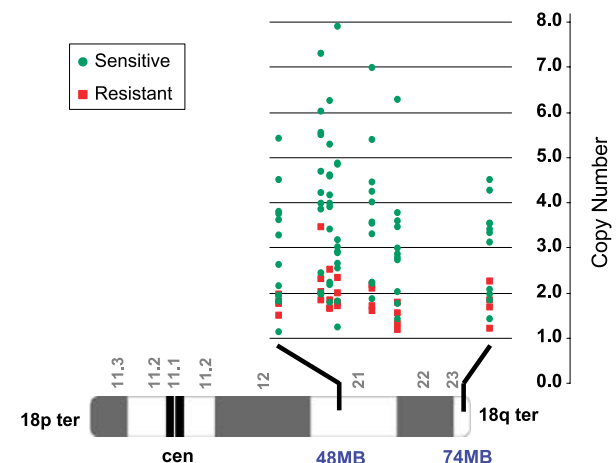
#### Identification of Gene Amplifications/Deletions Correlating with the Sensitivity to the Bcl-2 Inhibitor

Members of the Bcl-2 protein family are central regulators of programmed cell death (25). The family members that inhibit apoptosis are overexpressed in cancers and contribute to tumorigenesis (26). A small-molecule inhibitor of Bcl-2, Bcl-XL, and Bcl-w has recently been discovered at Abbott Laboratories and shown to induce regression of solid tumors (27). This compound (ABT-737) displayed selective potency against SCLC and lymphoma cells (27). Here, we tested the

compound against our panel of 23 SCLC lines as described in ref. 27 (Supplementary Fig. S2). Sensitive ( $10 \text{ nmol/L} < EC_{50} < 1 \text{ } \mu\text{mol/L}$ ) and resistant ( $EC_{50} > 10 \text{ } \mu\text{mol/L}$ ) lines were identified. The sensitive group consisted of NCI-H889, NCI-H1963, NCI-H1417, NCI-H146, NCI-H187, DMS53, NCI-H510, NCI-H209, NCI-H526, NCI-H211, NCI-H345, and NCI-H524, and the resistant group included NCI-H82, NCI-H196, SW1271, and H69AR. A structurally distinct Bcl-2 family antagonist yielded a very close inhibition profile for the same 23 cell lines (data not shown), indicating that the observed response is mechanism based.

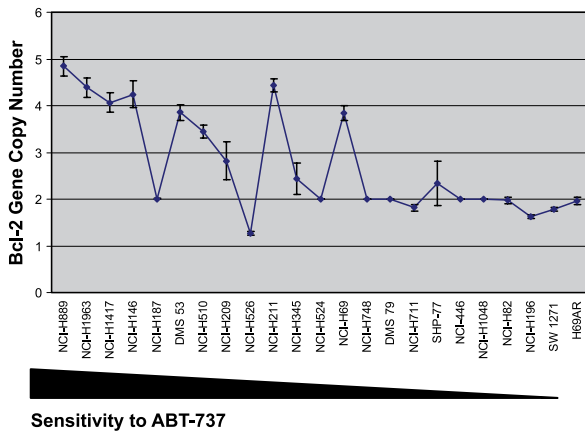
To identify potential genomic correlates of the sensitivity of SCLC cells to ABT-737, we developed a bioinformatics approach that identifies chromosomal aberrations that discriminate between the sensitive and resistant groups. Our program tested for statistical significance using Fisher's exact test to determine if a SNP shows preferential gain/loss in the sensitive or resistant group. The copy number thresholds for amplifications and deletions were set at 2.8 and 1.5, respectively. Contiguous regions of three or more probe sets (SNPs) with low table and two-sided  $P$  values were subjected to further analysis. Two regions (on chromosomes 18 and 19) were identified with  $P < 0.02$  (Supplementary Table S2). One region on chromosome 18q was of particular interest because of high copy numbers in the sensitive cell lines. The increased copy number region starts at position 45,704,096 and ends at position 74,199,087, and spans chromosomal bands from 18q21.1 through 18q23, with the section having the lowest  $P$  values ( $< 0.02$ ) located between 18q21.31 and 18q22.3.

We applied real-time quantitative PCR to validate this region as a potential stratification marker derived by CGH. Two different primer sets run in triplicate were used to evaluate seven loci starting at 48 Mb (18q21.1) and ending at 74 Mb (18q23) within chromosome 18. The quantitative PCR results (Fig. 2) indicate segregation between the sensitive and resistant lines based on the copy number of the test locus ( $P < 0.0001$ , ANOVA), thus confirming the CGH data. The sensitive lines



**FIGURE 2.** Real-time quantitative PCR analysis of the copy number for the 18q21-23 marker region in the SCLC cell lines sensitive and resistant to ABT-737.





**FIGURE 3.** Correlation between the *Bcl-2* gene copy number and the sensitivity of SCLC cell lines to ABT-737 ( $R = 0.71$ ,  $P < 0.0002$ ). The cell lines are arranged in the order of decreasing sensitivity to ABT-737 (increasing  $EC_{50}$ ).

carry an amplification of the region under consideration (3-7 copies), whereas the resistant lines display a normal copy number.

Notably, the *Bcl-2* gene ( $P = 0.015$ , Fisher's exact test), the target of ABT-737, is located within this discriminant region on 18q21.3, suggesting that the sensitivity of a cell line to the drug may be determined by the amplification status of *Bcl-2*. Figure 3 illustrates the relationship between the *Bcl-2* gene copy number and the sensitivity of the SCLC cell lines. The cell lines are arranged in the order of decreasing sensitivity to the drug, as determined by  $EC_{50}$  values (Supplementary Fig. S2; ref. 27). The copy number for each cell line is presented as the mean  $\pm$  SD of the copy numbers for 17 SNPs within the *Bcl-2* gene. It is clear from the plot that the sensitivity of the SCLC cell lines correlates with the *Bcl-2* copy number ( $R = 0.71$ ,  $P < 0.0002$ ). The most sensitive lines (H889, H1963, H1417, and H146) have the highest *Bcl-2* copy number (4 or 5 copies).

Another apoptosis-related gene (*NOXA*), whose product promotes degradation of Mcl-1 (28), is located next to *Bcl-2* and has a similar copy number profile. There are two outliers in this data set, H187 and H526, which are sensitive but have a normal copy number of the *Bcl-2* gene. Their sensitivity to ABT-737 may be attributed to an extra copy of the *Bcl-w* gene on 14q11.2 (data not shown), which is also a target of the drug. Other genes located in the 18q marker region are listed in Supplementary Table S2. None of these genes seems to be linked to oncogenic processes, making *Bcl-2* and *NOXA* the main candidates for amplification targets in this amplicon. Thus, we established a correlation between the amplification of *Bcl-2* and *NOXA* on 18q21.3 and the sensitivity of SCLC cell lines to ABT-737. This observation is consistent with the mechanism of action of the drug and suggests that the single-agent sensitivity of a cell line to the drug may be determined by the amplification status of 18q21.3.

#### Analysis of the *Bcl-2* Gene Copy Number in SCLC Tumors

To determine the clinical relevance of the 18q21 gain, we analyzed the *Bcl-2* copy number in SCLC tumors by FISH. Our

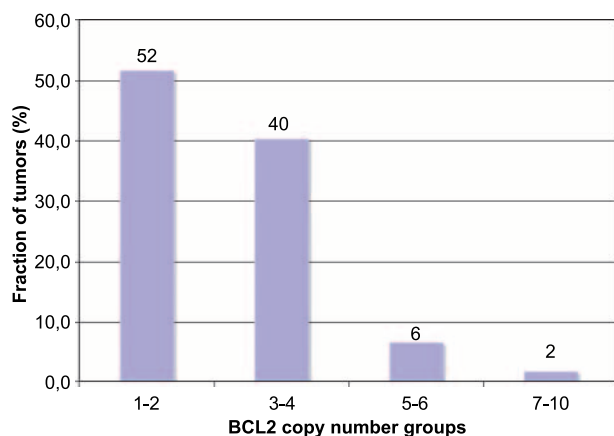
analysis included tumors from 62 patients arrayed on a tissue microarray. As can be seen in Fig. 4, low-level gains of the *Bcl-2* gene are present in 40% (25 of 62) of the patients and high-level gains are observed in 8% (5 of 62) of the tumors. This finding is consistent with our data from the SCLC cells, as most changes in the cell lines were also low-level gains. The percentage of lines carrying the aberration was also similar. Thus, we showed that an amplification of the *Bcl-2* gene is a frequent event in SCLC, implying that the correlation between the *Bcl-2* copy number and the sensitivity of SCLC cells to *Bcl-2* family inhibitors can be exploited in clinical trials.

#### Expression of the Genes Located in the Amplified Region on Chromosome 18

The 18q amplification observed in the sensitive SCLC cell lines is biologically relevant if it leads to higher expression of the genes located in the amplified region. To determine the relative expression of the 18q21-23 genes in the sensitive and resistant SCLC cell lines, we profiled the lines with expression microarrays. In Fig. 5, the 12 most sensitive cell lines are shown on the left and the 4 resistant lines are grouped on the right (in the order of decreasing sensitivity). Each row of the heatmap corresponds to a gene and each column corresponds to a cell line, whereas the color of each square reflects the relative expression of the gene in a particular cell line. The heatmap covers all genes located in the marker region on 18q21-23 and present on the U133A microarray. A continuum of expression levels is observed between highly sensitive, moderately sensitive, and resistant lines, with the expression levels decreasing with the sensitivity. Because the *Bcl-2* gene did not display a clear pattern of decreasing expression with decreasing sensitivity, we examined the expression of the *Bcl-2* protein in the sensitive and resistant cell lines. The *Bcl-2* protein expression pattern was consistent with the amplification pattern in the sensitive lines and the normal copy number in the resistant lines (29), suggesting that the lower expression in some of the sensitive lines shown by the expression microarray is likely to be an artifact of the microarray probe hybridization. Thus, the gene expression data provide further support for the selection of the 18q21-23 amplification as a stratification biomarker in SCLC. Additionally, this finding implies a significant degree of correlation between gene amplification and gene overexpression.

#### Discussion

Classification of cancers based on histopathology does not address the genetic heterogeneity of the disease. The response rates to novel cancer therapeutics can be improved by selecting patients based on the presence or absence of a genomic stratification marker. An early identification of such markers in the drug discovery process can provide a path for more successful clinical trials. Because early discovery is almost always done in cancer cell lines, genomic profiling of the model systems coupled with screening of candidate compounds represents a potentially very powerful strategy in genomic biomarker identification. In this study, we sought to identify genomic biomarkers of sensitivity to a *Bcl-2* family inhibitor (ABT-737) through CGH analysis of 23 SCLC cell



**FIGURE 4.** *Bcl-2* gene amplification in SCLC tumors as determined by FISH.

lines used to test Bcl-2 inhibitors *in vitro*. The compound is a selective and potent inhibitor of Bcl-2 proteins that exhibits single-agent mechanism-based killing of cells and induces regression of solid tumors (27). We used high-density SNP genotyping arrays for CGH analysis because of their high resolution and reproducibility. Their use in CGH analysis has recently been pioneered in a study of 70 lung cancers (18).

To validate our CGH method, we initially focused on recurrent gene copy number abnormalities in SCLC. A number of recurrent chromosomal aberrations in SCLC have previously been identified by conventional cytogenetics and array CGH. In particular, loss on chromosome 3p is considered the most frequent chromosomal aberration in SCLC (30-34). The fragile histidine triad gene (*FHIT*), the von Hippel Landau (*VHL*), and the protein-tyrosine phosphatase- $\gamma$  gene were suggested as candidate SCLC tumor suppressor genes on 3p (35, 36). Another common region of chromosomal loss is located on 5q (31, 33, 37). Losses on chromosomes 13 (31) and 17p are also common in SCLC, with the frequently lost regions overlapping at 17p13 (34). Studies by CGH have identified additional aberrations in SCLC including losses of 4q, 10q, and 16q and gains of 8q and 19q (36, 38). Frequent gains have been detected on 1p22-32 (*MYCL*), 2p23-25 (*MYCN*), 8q24 (*MYC*), 5p, 1q24, and Xq26, as well as losses on 17p13 (*TP53*), 13q14 (*RBI*), 3p, 22q12-13, 10q26, and 16p11.2 (39). Recurrent amplifications of regions containing known oncogenes *c-myc* and *N-myc* have been identified in SCLC (40-43). Using a submegabase-resolution tiling CGH array with overlapping BAC clones, Coe et al. (16) have detected a gain of a 350-kbp gene-specific region on 7p22.3 as the most frequent event in a panel of SCLC cell lines. Genome-wide analysis of 5 SCLC cell lines and 19 patient tumors used 100K SNP genotyping arrays similar to the arrays used in this study (18). High-level amplifications have been identified on 8q12-13 and homozygous deletions have been detected on 3q25 and 9p23-24.1.

In our study, we detected all the aforementioned aberrations, thus validating our CGH method. We also identified several novel recurrent copy number abnormalities (i.e., gains of 2q, 6p, 7p, 9q, 11p, 11q, 12p, 12q, 13q, 14q, 17q, 18q, 20p, 20q,

21q, and 22q and losses of 10q21.1). We only focused on frequent aberrations present in multiple cell lines (frequency > 40%) because they are more likely to reflect the oncogenic processes in SCLC. To identify aberrations most relevant to SCLC tumors, we selected only the abnormalities that were also present in a previously published data set on 19 SCLC tumors (18). The novel gains present in both cell lines and tumors were confirmed by quantitative PCR. The final list (Table 1; Supplementary Table S1, worksheet 2) thus contains verified novel recurrent copy number gains characteristic of SCLC. Additional studies will be required to determine the functional role of the genes residing in these regions in the pathogenesis of SCLC.

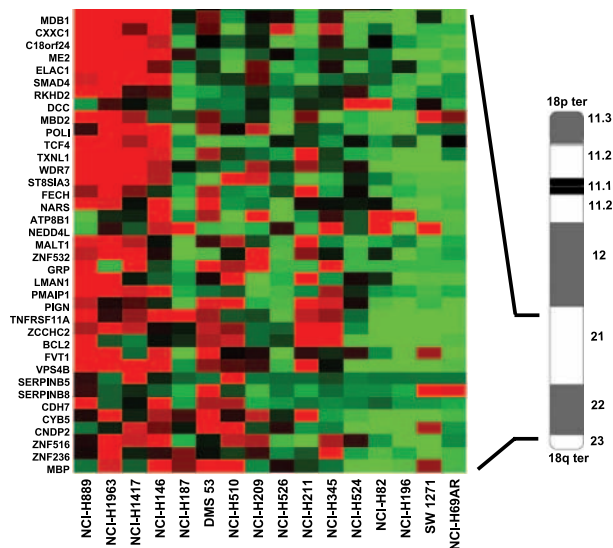
The key finding of this study is the discovery of a potential genomic stratification marker in SCLC, an amplification on 18q harboring the *Bcl-2* and *NOXA* genes. Overexpression of Bcl-2 in SCLC has previously been shown (44, 45). However, to our knowledge, amplification of 18q21 and, in particular, of the *Bcl-2* gene has not been reported in SCLC. We showed that an amplification of a region on 18q correlates with the sensitivity of SCLC cell lines to two novel anticancer drugs targeting Bcl-2 family members. The region contains one of the drug targets, Bcl-2, thus providing a likely explanation for the differential sensitivity of *Bcl-2* amplified lines. Additionally, the marker region contains *NOXA*, a gene regulating apoptosis by promoting degradation of Mcl-1 (28). Amplification of *NOXA* would enhance degradation of Mcl-1 and thus reduce the function of this antiapoptotic protein not targeted by ABT-737. We have previously shown that Mcl-1 functionally compensates for Bcl-2 and contributes to apoptosis resistance in SCLC cells, whereas NOXA overexpression sensitizes resistant cells to ABT-737 (29). Additionally, two recent studies have shown that overexpression of Mcl-1 induces resistance to ABT-737, whereas neutralization of Mcl-1 by NOXA sensitizes cells to ABT-737, establishing Mcl-1 as a major factor in cellular resistance to ABT-737 (46, 47). In our experiments, there was no correlation between the Mcl-1 copy number and the sensitivity to ABT-737 (a gain of one copy was observed in 4 of the 23 lines profiled), suggesting an alternative mechanism of regulation of Mcl-1 expression. Thus, we observed concurrent gains of Bcl-2 and NOXA in the sensitive but not in resistant lines, which is consistent with the findings that increased levels of NOXA sensitize cells to Bcl-2 antagonists by inactivating Mcl-1.

To investigate the 18q21-23 gain further as a genomic marker, we showed that the genes residing in the region are also overexpressed in the sensitive lines relative to the resistant lines. Next we sought to establish the clinical relevance of the 18q21-23 gain by studying the copy number of the *Bcl-2* gene in SCLC tumors. It was shown that *Bcl-2* is amplified in 48% of the patients. To our knowledge, the Bcl-2 gain has not been previously explored in SCLC. Taken together, our data suggest that the 18q21-23 copy number will be a clinically relevant predictor for sensitivity of SCLC to Bcl-2 antagonists as they enter clinical trials. The size of the subgroup of cell lines and patients carrying the 18q21-23 marker (~40%) is comparable to the fraction of lung cancer patients carrying the *EGFR* amplification (48, 49) or breast cancer patients displaying the Her-2 gain (50, 51), implying that a significant population of

SCLC patients would benefit from the Bcl-2 inhibitor, if the correlation seen *in vitro* reproduces in the clinic. The *Bcl-2* gene copy number in clinical samples can be determined by FISH using commercially available probes. In many cases, detection of gene amplification provides superior prognostic and treatment selection information as compared with immunohistochemistry (7). It is noteworthy that the protein product of another gene in the 18q marker region, pro-GRP, is elevated in the serum of some SCLC patients, thus raising the possibility that the levels of pro-GRP in plasma may be used to stratify SCLC patients (52).

It is also possible that the 18q marker may have a broader application in SCLC (and possibly other cancers) as *Bcl-2* amplification may correlate with sensitivity to other anticancer therapies. Indeed, Bcl-2 is a key protein regulating cell survival and its amplification may affect the sensitivity of cells to drugs. Indeed, overexpression of Bcl-2 in lung cancer cells induced resistance to cytotoxic agents (53). Furthermore, overexpression of Bcl-2 has been associated with resistance to therapy in ovarian (54, 55), cervical (56, 57), and gastric (58) cancers.

In summary, we used a genome-wide CGH screen to detect novel recurrent amplifications in SCLC and identify a genomic stratification marker for a targeted cancer drug. This work illustrates the potential benefits of CGH analysis of preclinical model systems as an approach to early codiscovery of targeted therapeutics and genomic diagnostics. As Bcl-2 antagonists are entering clinical trials, the discovered sensitivity marker will be used to predict patient response.



**FIGURE 5.** Expression of genes of the 18q21-23 marker region in the SCLC cell lines sensitive and resistant to ABT-737. The lines are arranged in the order of decreasing sensitivity to ABT-737. The gene names are shown on the right. The following genes also belong to the 18q21-23 marker region but are not present on the expression microarray: *CCDC11*, *MAPK4*, *MRO*, *STARD6*, *RAB27B*, *SE57-1*, *ONECUT2*, *ALPK2*, *SEC11L3*, *RAX*, *CPLX4*, *CCBE1*, *SDCCAG3*, *MC4R*, *CDH20*, *RFN152*, *KIAA1468*, *PHLPP*, *SERPINB12*, *SERPINB13*, *SERPINB4*, *SERPINB3*, *SERPINB11*, *SERPINB7*, *SERPINB2*, *SERPINB10*, *CDH19*, *TXNDC10*, *DOK6*, *CD226*, *RTTN*, *SOCS6*, *GTSCR1*, *CBLN2*, *NETO1*, *FBXO15*, *CNDP1*, *ZNF407*, *ZADH2*, *SDCCAG3*, and *GALR1*.

## Materials and Methods

### Cell Culture and CGH

The following SCLC cell lines were obtained from American Type Culture Collection (Manassas, VA): NCI-H889, NCI-H1963, NCI-H1417, NCI-H146, NCI-H187, DMS53, NCI-H510, NCI-H1209, NCI-H526, NCI-H211, NCI-H345, NCI-H524, NCI-H69, NCI-H748, DMS79, NCI-H711, SHP77, NCI-446, NCI-H1048, NCI-H82, NCI-H196, SW1271, and NCI-H69AR. All cells were cultured in the recommended media at 37°C. Genomic DNA was isolated using a DNAeasy kit (Qiagen) and run on 100K SNP genotyping array sets (Affymetrix, Santa Clara, CA). The arrays were run according to the manufacturer's protocol. The raw microarray data files have been loaded into Gene Expression Omnibus (accession no. GSE7068) and Array Express (accession no. E-MEXP-1008). The data were processed using the GTYPE software (Affymetrix) to create copy number (.cnt) files containing information on the inferred copy number for each probe set (SNP). The .cnt files contained combined information from both arrays in the set. These files were converted into .txt files and loaded into an internally developed software package for further analysis.

This internal program was used for the graphical display and analysis of multiple copy number files. The data were displayed as a histogram of copy number versus SNPs ordered sequentially along the chromosome. For each SNP, the predicted cytogenetic band, as well as any genes between this and the next adjacent SNP, was reported. The gene coordinates and cytogenetic band positions were inferred from the build 35 of the human genome. Data summaries and plots can also be exported to other desktop software. When a region is selected on the histogram, a summary file can be exported that contains the coordinates of all probe sets (SNPs) with the corresponding copy numbers, cytogenetic bands, gene IDs, names, and the coordinates of all the genes residing in the region.

To facilitate identification of recurrent aberrations, the frequency of copy number change is calculated and plotted for each probe set (SNP) after the user defines thresholds for gains (e.g.,  $\geq 3$  copies) or losses (e.g.,  $\leq 1.5$  copies). The program also enables identification of copy number abnormalities that correlate with a predefined class label within a data set. The cell lines were classified into sensitive and resistant and a threshold was set for gains ( $\geq 2.8$  copies) and losses ( $\leq 1.5$  copies). Fisher's exact test was used to identify aberrations associated with the sensitivity of cell lines to a Bcl-2 inhibitor. For each SNP, a  $2 \times 2$  contingency table was constructed for testing the significance of an increase or a decrease in copy number in the two groups. A minimum of three contiguous SNPs meeting the *P* value threshold were considered to be one region.

### Real-time Quantitative PCR

Primers were designed using Vector NTI (Invitrogen, Carlsbad, CA), purchased from IDT (Coralville, IA), and tested to ensure amplification of single discrete bands (Supplementary Table S3). Real-time PCR was conducted on an iCycler (Bio-Rad, Richmond, CA) using SYBR Green quantitative PCR supermix UDG (Invitrogen). Each reaction was run in triplicate and contained 10 ng of purified genomic DNA and 300 nmol/L of each primer in a final volume of 50  $\mu$ L. The cycling conditions were as follows: 95°C for 3 min; 35 cycles of 95°C for 10 s;



57°C for 45 s. Melting curves were done to ensure that only a single amplicon was produced and samples were run on a 4% agarose gel (Invitrogen) to confirm specificity. Data analysis was done with the linear regression software DART-PCR v1.0 (59) using raw thermocycler values. Normalization of sample input was conducted with geometric averaging software GeNorm v3.3 (60) to GAPDH,  $\beta_2$ -microglobulin, YWHAZ, RPL13a, and PLP-1. The copy number for each locus was determined by establishing the normalized quantitative PCR output for the sample, dividing this value by the normalized quantitative PCR output for a control genomic DNA (Clontech, Palo Alto, CA), and multiplying the resulting value by 2. Each quantitative PCR copy number estimate is the average value for two independent primer sets (mean coefficient of variation, 11.5%).

### FISH

A tissue microarray containing primary SCLC tumors from 62 patients was analyzed by FISH using a dual-color FISH probe targeting 18q21 (LSI Bcl-2 Break-apart probe, Abbott Molecular, Des Plaines, IL). The slides were deparaffinized for 10 min in xylol, rinsed in 95% ethanol, air-dried; incubated in a pretreatment solution for 15 min at 80°C, rinsed in water; incubated in a protease buffer for 2.5 to 5 h, rinsed; dehydrated in 70%, 80%, and 95% ethanol, and air-dried. The probe (10  $\mu$ L) was applied onto the slide, and the slide was covered, sealed, heated to 72°C for 5 min, and hybridized overnight at 37°C in a wet chamber. The slides were then washed with 2 $\times$  SSC containing 0.3% NP40 (pH 7-7.5) for 2 min at 75°C, rinsed with water, air-dried, mounted with a 4',6-diamidino-2-phenylindole solution and a 24  $\times$  50 mm coverslip, and examined under an epifluorescent microscope. For each tissue sample, the number of red and green FISH signals (both corresponding to the Bcl-2 locus) was estimated in at least 20 cells. An average copy number per spot was then calculated based on the minimal and maximal numbers of Bcl-2 FISH signals per cell nucleus in each tissue spot. Copy number groups were built based on the absolute gene copy numbers according to the following criteria: 1 to 2 signals, average copy number <2.5; 3 to 4 signals, average copy number  $\geq$ 2.5 and <4.5; 5 to 6 signals, average copy number  $\geq$ 4.5 and <6.5; and 7 to 10 signals, average copy number  $\geq$ 6.5.

### Expression Microarray Analysis

Total RNA was purified on RNeasy columns (Qiagen, Valencia, CA). Labeled cRNA was prepared according to the microarray manufacturer's protocol and hybridized to human U133A 2.0 arrays (Affymetrix). Data files were loaded into the Rosetta Resolver software for analysis and the intensity values for all probe sets were normalized using the Resolver Experimental Definition. The intensity values for the probe sets corresponding to genes within the amplified regions were normalized across each gene and compared in heatmaps using Spotfire software.

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## Integrative Genomic Analysis of Small-Cell Lung Carcinoma Reveals Correlates of Sensitivity to Bcl-2 Antagonists and Uncovers Novel Chromosomal Gains

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