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Advances in Brief

Comparative Genomic Hybridization Analysis Detects Frequent, Often High-Level, Overrepresentation of DNA Sequences at 3q, 5p, 7p, and 8q in Human Non-Small Cell Lung Carcinomas[1]

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Abstract

Comparative genomic hybridization analysis was used to identify chromosomal imbalances in 20 non-small cell lung carcinoma (NSCLC) biopsies and cell lines. The chromosome arms most often overrepresented were 3q (85%), 5p (70%), 7p (65%), and 8q (65%), which were observed at high copy numbers in many cases. Other common overrepresented sites were 1q, 2p, and 20p. DNA sequence amplification was often observed, with the most frequent site being 3q26 (six cases). Other recurrent sites of amplification included 8q24, 3q13, 3q28-qter, 7q11.2, 8p11-12, 12p12, and 19q13.1-13.2. The most frequent underrepresented segment was 3p21 (50%); other recurrent sites of autosomal loss included 8p21-pter, 15q11.2-13, 5q11.2-15, 9p, 13q12-14, 17p, and 18q21-qter. These regions of copy number decreases are also common sites of allelic loss, further implicating these sites as locations of tumor suppressor genes. Although some of the overrepresented segments harbor known or suspected oncogenes/growth-regulatory genes, we have identified 3q and 5p as new sites that are very frequently overrepresented in NSCLC. These findings could represent entry points for the identification of novel amplified DNA sequences that may contribute to the development or progression of NSCLC.

Introduction

Lung carcinomas represent the leading cause of cancer mortality among both men and women in the United States, accounting for approximately 159,000 deaths in 1996 (1). Based on their biology, therapy, and prognosis, lung cancers are divided into two major classes: NSCLC[3] (75-80% of all lung cancers) and SCLC. NSCLCs consist of three major types: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma; however, many NSCLCs exhibit two or more histological patterns (2). Due to the inadequacies of current therapeutic protocols, less than 15% of NSCLC patients will be alive 5 years or more after diagnosis (1). Improvement in the efficacy of NSCLC therapy is a major public health goal. Thus, the identification of specific genetic alterations in a given tumor could provide molecular targets for individually tailored therapy.

NSCLC specimens often have a low mitotic index, and the rate of successful cytogenetic analysis is frequently less than 50% (reviewed in Ref. 3). Furthermore, the karyotypes are often very complex with widespread alterations, complicating efforts to identify consistent copy number changes having potential diagnostic or prognostic utility. Despite this fact, deletions of 3p, 9p, and 17p and gain of chromosome 7 have been found to be recurrent karyotypic alterations in NSCLC. LOH studies of NSCLCs have shown frequent allelic losses from various chromosome arms including 3p, 5q, 8p, 9p, 11p, 13q, 17p, and 18q (reviewed in Ref. 4). These sites harbor known or suspected tumor suppressor genes whose inactivation may play a critical role in lung tumorigenesis. Amplification and/or overexpression of certain proto-oncogenes (*e.g.*, *MYC* and *EGFR*) have also been implicated in NSCLCs (3, 4).

CGH is a valuable procedure for whole-genome scanning. It identifies chromosomal imbalances (gains, losses, or amplification of DNA sequences) in entire tumor genomes (5). Because such copy number changes are detected by CGH only if present in at least 50% of the cells, this method has the potential to identify consistent clonal aberrations associated with tumor development or progression. To date, there have been no reports of CGH analysis of NSCLCs. However, CGH has proven useful in identifying genomic alterations in SCLCs (6, 7). In addition to the expected frequent chromosome losses (*i.e.*, 3p-, 13q-, and 17p-) previously recognized in karyotypic and LOH studies of SCLC (3, 4), CGH analysis identified other recurrent abnormalities including frequent overrepresentation of 3q. Furthermore, several new recurrent amplification sites were described by this approach.

In this investigation, CGH analysis was carried out to identify genomic imbalances in tumor biopsies and early-passage cell lines from 20 NSCLC patients. One of the peculiarities of NSCLC is that contaminating normal stroma and infiltrating lymphocytes are a common feature. Therefore, we microdissected all tumor biopsies to enrich the samples for tumor cells, permitting an accurate assessment of CNAs. Previous CGH analyses carried out on microdissected cervical tumors have shown a highly consistent pattern of CNAs (8). In agreement with karyotypic and LOH studies of NSCLC, losses from 3p, 5q, 8p, 9p, 13q, 17p, and 18q were recurrent changes in the present study. In addition, CGH analysis discovered several sites of copy number increases whose high frequency had not been previously recognized. Prominent among these was overrepresentation of 3q (17 of 20 cases, 85%), suggesting a pivotal role of this aberration in most NSCLCs. Extra copies of part or all of 5p, 7p, and 8q were also very common, and in many instances, these overrepresented regions were present at high copy numbers. Eight recurrent sites of DNA sequence amplification were delineated, only some of which are at locations of known oncogenes. The remaining sites could represent entry points

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[3] The abbreviations used are: NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma; LOH, loss of heterozygosity; CGH, comparative genomic hybridization; CNA, copy number aberration; HLG, high level gain; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; EGFR, epidermal growth factor receptor; TGF-α, transforming growth factor α.

for the identification of novel amplified DNA sequences that may contribute to the development or progression of NSCLC.

Materials and Methods

Primary Tumor Specimens and Cell Lines. All 20 cases were newly diagnosed, untreated patients from whom primary tumor tissue was collected at the time of surgical resection. Ten frozen tumor specimens were microdissected by a single pathologist (E. G.). Frozen 12-μm sections were stained with H&E, visualized while immersed in drops of 80% ethanol, and microdissected with a 26-gauge needle to remove contaminating lymphocytes and stromal cells. Ten early-passage cell lines (median number, five passages) were prepared as described elsewhere (3). DNAs from cell lines and microdissected tumor samples were extracted according to our standard method (9). Clinical findings are summarized in Table 1.

CGH. Protocols for labeling tumor and reference DNAs, capture of gray-level images of fluorescence with a cooled charge-coupled device camera (Photometrics, Tucson, AZ), and digital image analysis were as described previously (10). For each tumor specimen, the mean values of individual ratio profiles were calculated from at least seven metaphase spreads. Averaged values were plotted as profiles alongside individual chromosome ideograms. The three vertical lines next to the individual chromosome ideograms indicate different threshold values between tumor DNA and normal reference DNA. The line on the left corresponds to a threshold value of 0.75, which would exist if 50% of the cells from a near diploid tumor had monosomy of a given chromosome. The center line indicates a balanced state. The line on the right represents a threshold value of 1.25, which would occur if 50% of the cell population exhibited trisomy for that chromosome. Such an overrepresentation was considered a gain, whereas overrepresentation exceeding a threshold value of 1.50 was designated a HLG. Overrepresentation defined by a sharp peak was considered indicative of amplification.

Results

All 20 cases exhibited numerous genomic imbalances (mean, 15.6/case), with every chromosome involved at least once in a CNA. The total number of imbalances/tumor specimen ranged from 6 to 18, compared to 6 to 26 in tumor cell lines (Table 1). A schematic summary of CNAs detected by CGH analysis in each case is shown in Fig. 1.

Overrepresentation of 3q was the most consistent CNA observed (85% of cases). Chromosome arms 5p (70%), 7p (65%), and 8q (65%) were also frequently overrepresented. In many instances, these four chromosome arms displayed HLGs and/or amplifications. Other commonly overrepresented sites included 1q (55%), 2p (50%), 20p (45%), 12q14–21 (45%), and 12p (40%). DNA amplification was identified at 19 different locations in 13 cases. The most frequent sites of amplification were 3q26 (six cases), 8q24 (four cases), and 3q13 (three cases). Amplification of 3q28–qter, 7q11.2, 8p11–12, 12p12, and 19q13.1–13.2 was observed in two cases each. Amplification of 2q32.2–32.3, 3p25–26, 5p13–14, 7p12, 7q31, 9p24, 10p14, 10q22, 12q14–15, 13q14, and 20p13 was detected in a single tumor each. The most common underrepresented site was 3p (10 cases; 50%), with the smallest region of overlap being 3p21. Autosomal loss at 8p21–pter, 15q11.2–13, 5q11.2–15, 9p, 13q12–14, 17p, and 18q21–qter was observed in four to six cases each (20–30%).

Table 1 *Clinical and CGH findings in 20 NSCLCs*

Case no.	Sex	Histologic type[a]	Grade[b]	Total no. imbalances
Tumor specimens				
TU-1	M	SCC	PD	15
TU-2	M	SCC	MD	18
TU-3	M	SCC	PD	9
TU-4	M	AC	PD	8
TU-5	F	AC	MD	18
TU-6	M	AC	WD	11
TU-7	M	SCC	MD	17
TU-8	M	SCC	WD	6
TU-9	F	SCC	MD	9
TU-10	M	SCC	PD	15
Cell lines				
CL-1	F	SCC	PD	14
CL-2	F	SCC	MD	18
CL-3	F	SCC	WD	23
CL-4	M	SCC	MD	20
CL-5	M	SCC	PD	21
CL-6	M	AC	PD	26
CL-7	M	AC	PD	19
CL-8	M	AC	PD	6
CL-9	M	ASCC	MD	20
CL-10	F	ASCC	MD	20

[a] AC, adenocarcinoma; SCC, squamous cell carcinoma; ASCC, adenosquamous cell carcinoma.
[b] WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

Discussion

The CGH data presented here indicate a high incidence of overrepresentation of chromosome arms 3q, 5p, 7p, and 8q in NSCLCs. Each of these CNAs was observed in at least 65% of cases, and in many instances, these CNAs were HLGs suggestive of polysomy of these chromosome arms. In some cases, such gains could be due to isochromosome formation. In fact, we previously reported several recurrent isochromosomes in NSCLC karyotypes (3), including i(5p), i(8q), i(1q), i(3q), and i(7p), although the incidence of these abnormalities (8–14% of cases) had not been indicative of the high frequency of NSCLCs having extra copies of these chromosome arms.

Overrepresentation of chromosome arm 3q was the most prominent feature in this series. Nine cases exhibited a HLG involving 3q, which in some cases was associated with amplification of 3q26, 3q13, or 3q28–qter (Figs. 1 and 2). Amplification of 3q26 seems to be especially important, because it was observed in 6 of 20 cases. Amplification of 3q26 was identified in three of nine squamous cell carcinomas of the lung analyzed by reverse chromosome painting (11). Moreover, increased copy number at 3q26–27 has also been reported as a recurrent change in a variety of other tumors including SCLCs (6, 7) and advanced cervical carcinomas (8). One potentially relevant gene at 3q26.3 whose product may contribute to the control of cell proliferation and malignant transformation is *PIK3CA* (12). *PIK3CA* encodes the catalytic subunit of phosphatidylinositol-3 kinase, a critical component of several cell signaling pathways including those of epidermal growth factor, PDGF, and IGF. Another relevant gene at 3q26 encodes the transferrin receptor. Some NSCLCs express increased numbers of surface transferrin receptor and elevated levels of transferrin,[4] the locus for which is also in 3q at band q21. Amplification of 3q28–qter was identified in two of our NSCLCs, and a similar amplicon has been described in a case of SCLC (6).

Overrepresentation of 5p, the second most common CNA observed in this series, presented as a HLG in eight of our cases and as amplification of 5p13–14 in case CL-6. A potentially relevant gene, *SKP2* (5p13), encodes a protein associated with the CDK2/cyclin A complex. This protein has been found to be essential for S-phase entry (13). Overrepresentation of 7p was also a highly consistent CNA, and candidate loci include genes for EGFR (7p12), IGF binding proteins 1 and 3 (7p12–14), and PDGF-α (7p22). Southern blot analysis of case CL-9 revealed an approximately 20-fold amplification of *EGFR*.[5] EGFR and TGF-α (2p13) form an autocrine growth-regulatory loop in NSCLC (14). Interestingly, HLG or amplification encompassing 7p12 was observed in six of our NSCLCs, four of which also displayed HLGs at 2p13. *EGFR* has been found to be amplified in about 10% of NSCLCs (3, 4), and elevated levels of EGFR and TGF-α have each been reported in 50–60% of NSCLCs (14). PDGF-α is expressed in

[4] F. Cuttitta and J. M. Siegfried, unpublished observations.
[5] J. R. Testa, unpublished data.

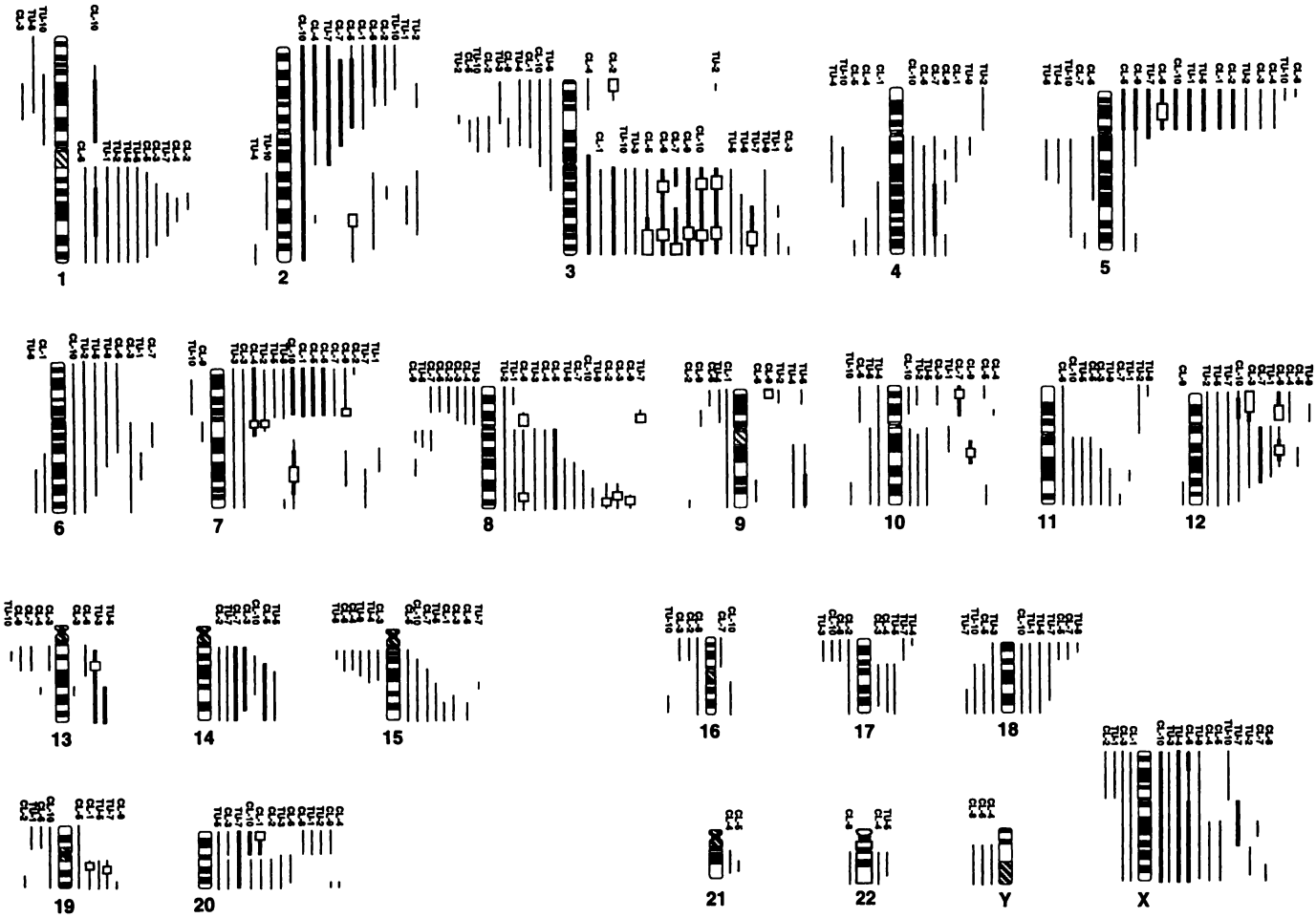

Fig. 1. Summary of CGH imbalances detected in 20 NSCLC tumor specimens and early-passage cell lines. *Vertical lines* on the *left* of each chromosome ideogram represent loss of genetic material in a given tumor, whereas those on the right correspond to gains. *Thick lines*, HLGs; *squares*, amplification. For each chromosome, smaller CNAs are placed furthest from the ideogram for ease of identification of minimal regions of overlap, *e.g.*, overrepresentation at 1q25–31, 2p16–21, 3q26–qter, 8q24, and 12p11.2–12.

more than 80% of NSCLC cell lines (15). Overrepresentation of 8q was observed in 13 of our cases, 4 of which showed amplification of 8q24, the location of the *MYC* oncogene. In case CL-9, Southern analysis revealed a 6-fold level of amplification of *MYC*.[5] *MYC* amplification has been reported in approximately 10% of NSCLCs (reviewed in Ref. 3). The smallest region of overlap of 1q gains observed in our investigation was 1q25–31 (Fig. 1). Breakage at or near the centromere of 1q and gains of 1q are often observed in karyotypic studies of NSCLCs (3). Candidate loci in 1q25–31 include the Abelson-related oncogene *ARG* and the protein tyrosine phosphatase receptor type c polypeptide gene (*PTPRC*).

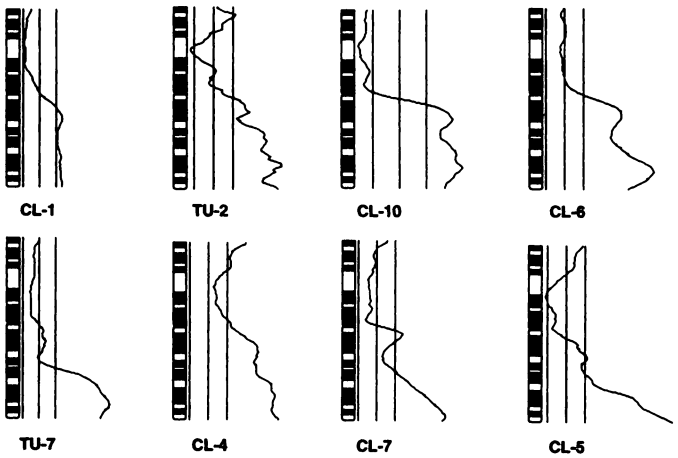

Fig. 2. Ratio profiles of chromosome 3 from eight selected NSCLCs, depicting 3q whole-arm gains as well as amplification at 3q26–qter, 3q13, and/or 3q28–qter.

The CGH findings described here also suggest an association between overrepresented chromosomal regions and the sites of genes encoding other growth-regulatory proteins that have been implicated in the pathogenesis of NSCLC (16). Representative profiles depicting overrepresentation at such sites are shown in Fig. 3. Overrepresentation at 7p, mentioned earlier, is a prominent example. In addition to *EGFR*, this region harbors genes for the IGF binding proteins 1 and 3 (*IGFBP1* and *IGFBP3*) at 7p12–14, which was overrepresented in five cases. The region 2q33–34, which encompasses another IGF binding protein gene, *IGFBP2*, was also overrepresented in three cases. IGF binding proteins have been found to be secreted in large excess of the endogenously secreted IGFs in NSCLCs and may preferentially deliver synthesized IGF to its receptor (17). Interestingly, the region harboring the IGF receptor type 1 gene, located at 15q25–qter, was overrepresented in 7 of 12 cases exhibiting copy number increases at 7p12–14. Overrepresentation at 12p was observed in eight cases, three of which displayed a HLG or amplification. The parathyroid hormone-related peptide gene, *PTHLH*, located at 12p11.2–12.1, has been implicated in NSCLC (18, 19). *PTHLH* was found to be coamplified with the *KRAS2* oncogene in a human lung cancer cell line (18). Because *PTHLH* was present at a higher level of amplification than *KRAS2*, it was thought to be the target gene in this

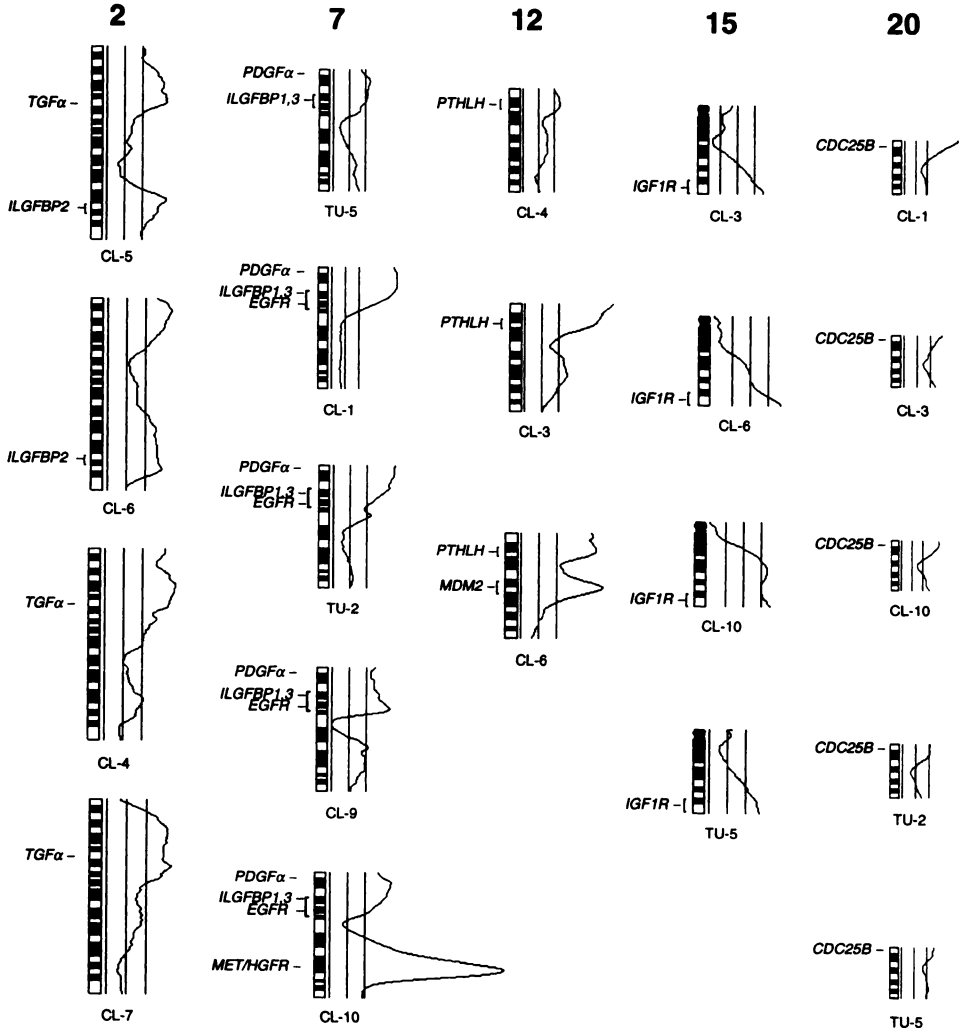

Fig. 3. Selected ratio profiles of chromosomes 2, 7, 12, 15, and 20 illustrating recurrent DNA gains, HLGs, or amplifications, along with candidate genes at these sites that have relevance to NSCLC. Included are genes encoding growth factors (PDGF-α, and TGF-α), growth factor receptors (EGFR, IGF1R, MET/HGFR), oncogene/cell cycle-regulatory proteins (MDM2 and CDC25B), and other peptides (ILGFBP and PTHLH).

amplicon. *PTHLH* encodes a potent osteoclast-activating factor thought to play an important role in bone metastasis (19). PTHLH has also been associated with hypercalcemia and leukocytosis in lung cancer patients (19, 20).

Other overrepresented regions highlighted by our CGH analysis seem to be sites of genes encoding phosphatases, some of which have been shown to be involved in human malignancy. Contrary to their putative role as tumor suppressors, in some instances, phosphatases have been found to be amplified and overexpressed and, thus, behave like oncogenes (21, 22). An example is the *CDC25B* tyrosine phosphatase gene located at 20p13, a frequent site of overrepresentation in our series. The human CDC25 tyrosine phosphatases trigger activation of CDC2 by removing inhibitory phosphates and have been suspected as potential oncogenes due to their role in promoting cell division (23). Besides 20p13, four other commonly overrepresented sites harbor genes for protein phosphatases. One of these, 10q22, represents the location of genes encoding the catalytic and regulatory subunits of protein phosphatase 3. The others, 7p15.1–15.2, 12p12–13, and 20q13.1–13.2, harbor genes for phosphoserine phosphatase, protein tyrosine phosphatase nonreceptor type 6, and protein tyrosine phosphatase nonreceptor type 1, respectively.

Other amplified regions observed in our series involved sites of known oncogenes. One such site, 19q13.1–13.2, was amplified in two cases. CGH evidence for amplification at 19q13 has also been reported in several SCLCs (6). A possible target is the *AKT2* oncogene, which has been shown to be amplified and overexpressed in 10–20% of ovarian and pancreatic carcinomas (9). The most striking illustration of amplification seen in our series was at 7q31 (Fig. 3). The *MET* oncogene, which encodes the hepatocyte growth factor receptor, is located at 7q31, and *MET* has been found to be expressed at high levels in a majority of primary NSCLCs (24). Another site in this chromosome, 7q11.2, was amplified in two cases, and a candidate gene at this location (*SKP1A*) encodes a protein associated with the CDK2/cyclin A complex. The region 12q14–15 was amplified in one adenocarcinoma case (CL-6) and overrepresented in another eight cases. The *MDM2* oncogene, located at 12q14–15, has been shown to be amplified and overexpressed in several lung adenocarcinomas (25).

Overall, loss of chromosomal regions was less frequent than gains in this CGH analysis. However, underrepresentation of 3p was a common occurrence, being observed in five tumor specimens and five cell lines, with the smallest region of overlap being 3p21. Previous LOH analyses have revealed that allelic loss from 3p21 is a frequent early change in NSCLC, and this chromosomal region is thought to harbor one or more tumor suppressor genes that may play an important role in the pathogenesis of this malignancy (4). Other recurrent underrepresented sites observed in this CGH study, *i.e.*, 8p21–pter, 15q11.2–13, 5q11.2–15, 9p, 13q12–14, 17p, and 18q21–qter, have also been reported to be common regions of allelic loss in NSCLC (4). Each of these segments is a known or suspected site of a tumor suppressor gene(s), *e.g.*, *TP53* at 17p13 and *CDKN2A* (*p16*) at 9p21, whose alteration may contribute to the development or progression of NSCLCs.

The consistency of genomic imbalances documented here in tumor specimens as well as in early-passage cell lines may be attributed to the use of microdissected specimens. Such manual microdissection seems to be an essential step in maximizing the sensitivity of CGH to detect CNAs in NSCLC. However, this procedure is very time-consuming, thus limiting the number of cases that can be studied. However, a recent technical innovation, laser capture microdissection (26), may greatly facilitate the rapid procurement of selected cell populations from heterogeneous tissue sections. This methodology holds considerable promise for enhancing the sensitivity and speed of CGH analysis in the future.

In summary, the recurrent copy number decreases identified in NSCLC by CGH analysis are supportive of previous allelic loss data, further implicating these sites as locations of tumor suppressor genes. In addition, many of the overrepresented chromosome segments observed in NSCLC are sites of known oncogenes/growth-regulatory genes implicated in lung tumorigenesis. Other overrepresented regions could be sites of novel amplified DNA sequences whose overexpression may provide a proliferative growth advantage in these aggressive neoplasms.

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