

Mapping of Multiple DNA Gains and Losses in Primary Small Cell Lung Carcinomas by Comparative Genomic Hybridization¹

Thomas Ried,² Iver Petersen, Heidi Holtgreve-Grez, Michael R. Speicher, Evelin Schröck, Stan du Manoir, and Thomas Cremer

Institute of Human Genetics, University of Heidelberg, INF 328, 69120 Heidelberg [T. R., H. H.-G., M. R. S., E. S., T. C.]; Institute of Pathology, University of Kiel [I. P.]; and Angewandte Tumoriologie, Deutsches Krebsforschungszentrum, Heidelberg [S. D. M.], Germany

ABSTRACT

Comparative genomic hybridization was applied for a comprehensive screening of under- and overrepresentation of genetic material in 13 autoptic small cell lung cancer specimens. The most abundant genetic changes include DNA losses of chromosome arms 3p, 5q, 10q, 13q, and 17p and DNA gains of 3q, 5p, 8q, and 17q. Amplification sites in these tumors were mapped to 22 chromosome bands. The most frequently involved band was 19q13.1 (4 cases). Bands 1p32, 2p23, 7q11.2, 8q24, and 13q33-34 were involved in two cases each.

INTRODUCTION

CGH³ is a new method to screen a tumor genome for genetic imbalances (1). DNA isolated from the tumor and reference DNA are labeled separately with different haptens. Equal amounts of both, tumor and reference genomic DNA, are hybridized to normal metaphase chromosome preparations together with an excess of unlabeled Cot1 fraction of human DNA. After the detection of the hybridized sequences with two fluorochromes, e.g., FITC for the tumor DNA and rhodamine for the reference DNA, the differences in fluorescence intensity values of the respective fluorochromes reflect the over- or underrepresentation of chromosomal sequences in the tumor genome. The technique has been used to detect genetic alterations of entire chromosomes or chromosomal subregions, as well as to map amplified genes on normal metaphase chromosomes in a variety of solid tumors (2-5). Also, formalin-fixed archived tumor material is suitable for this molecular cytogenetic technique because it requires solely tumor DNA (6).

Solid tumors account for about 90% of malignancies in man (7). Despite this high percentage, relatively few data on specific chromosomal aberrations are available as compared to hematological malignancies (8). This discrepancy is mainly due to the difficulties to apply cytogenetic banding techniques to solid tumor specimens. Therefore, alternative approaches to elucidate genetic changes in solid tumors have been introduced. These include interphase cytogenetics (9) of nuclei in tissue sections or isolated tumor nuclei using whole chromosome paint probes or region-specific probes (10, 11). Also, the detection of loss of heterozygosity by means of Southern blot analysis or PCR was applied to identify commonly deleted regions in tumor genomes (12). However, while these approaches have become very useful to evaluate copy number changes of certain chromosomal subregions or genes, they are not suitable for the routine assessment of genetic changes in entire tumor genomes.

Here we report on the identification of DNA alterations in 13 primary SCLC using CGH. Consensus deletion regions were identified for chromosomes 3p, 5q, 10q, 13q, and 17p, whereas a consistent overrepresentation of chromosomes 3q, 5p, 8q, and 17q was observed. The SCLC specimens revealed 22 different amplified regions. The chromosomal mapping positions of these amplified sequences were determined after CGH on normal metaphase and prometaphase chromosomes.

MATERIALS AND METHODS

Tumor Samples. Tumor specimens ($n = 13$) were collected at postmortem examination 3-24 h after the patients died. One aliquot of the sample was frozen in liquid nitrogen and kept at -80°C until DNA extraction. Genomic DNA was prepared by Proteinase K digestion and phenol-chloroform extraction (13). A second aliquot was submitted to formalin fixation and paraffin embedding. The diagnosis of a SCLC was established in every case according to the WHO guidelines on hematoxylin and eosin-stained formalin-fixed tissue sections (14). Multiple distant metastases were observed in 12 of 13 cases. One patient revealed only local lymph node metastases (case no. 11).

CGH Analysis. CGH analysis was done according to the protocol by Du Manoir *et al.* (2) with minor modifications. Genomic DNA from a healthy male donor was labeled in a standard nick translation reaction substituting dTTP by digoxigenin-11-dUTP (Boehringer Mannheim); tumor DNA was labeled accordingly with biotin-16-dUTP (Boehringer Mannheim). The DNase I concentration in the labeling reaction was adjusted in order to reveal an average fragment size of 500-1000 base pairs.

For CGH, 200 ng of digoxigenin-labeled reference DNA and 200 ng of biotin labeled tumor DNA were ethanol precipitated in the presence of 10 μg of salmon sperm DNA and 30 μg of the Cot1 fraction of human DNA (BRL). This excess of Cot1 DNA is required to prevent the ubiquitous hybridization of interspersed repetitive sequences contained in the tumor and reference DNA probes. The probe mixture was dried and resuspended in 10 μl hybridization solution [50% formamide, $2 \times \text{SSC}$ (0.15M NaCl, 0.015 M Na-citrate) and 10% dextran sulfate]. The DNA was denatured at 76°C for 5 min and allowed to preanneal at 37°C for 60 min. Reference metaphase spreads were prepared following standard procedures from peripheral blood lymphocytes of a healthy donor (46,XX). The chromosome preparations were denatured separately at 80°C in 70% deionized formamide and $2 \times \text{SSC}$ for 2 min and dehydrated through an ethanol series (70, 90, and 100%). The probe mixture was then applied to the denatured metaphase chromosomes under a coverslip (18 mm²), sealed with rubber cement, and hybridized for 4 days at 37°C .

Posthybridization steps were performed as described (15). The biotinylated sequences were detected with FITC conjugated to avidin (Vector laboratories). Probe sequences haptenized with digoxigenin were visualized with anti-digoxigenin Fab fragments conjugated to rhodamine. Chromosomes were counterstained with DAPI and embedded in an antifading agent (DABCO; Sigma Chemical Co.) to reduce photobleaching.

Digital Image Analysis. Fluorescence was detected using a Zeiss Axiophot epifluorescence microscope. The fluorescence banding pattern obtained after DAPI staining was used for chromosome identification. FITC and rhodamine fluorescence, specific for the tumor and the reference genome, respectively, were quantified as gray level images by a charge-coupled device camera (Photometrics, Tucson, AZ). After background fluorescence measurements, fluorescence ratio images (Fig. 1B) were calculated as described (2). The ratio profiles of individual reference chromosomes were determined by a program that was developed in our laboratory by S. du Manoir (5) and run on a Macintosh Quadra 950. Briefly, after the determination of the chromosomal

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² To whom requests for reprints should be addressed. Present address: National Center for Human Genome Research, NIH, Building 49, Bethesda, MD 20892.

³ The abbreviations used are: CGH, comparative genomic hybridization; FITC, fluorescein isothiocyanate; PCR, polymerase chain reaction; SCLC, small cell lung carcinoma; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; RFLP, restriction fragment length polymorphism; LOH, loss of heterozygosity; FISH, fluorescence *in situ* hybridization.

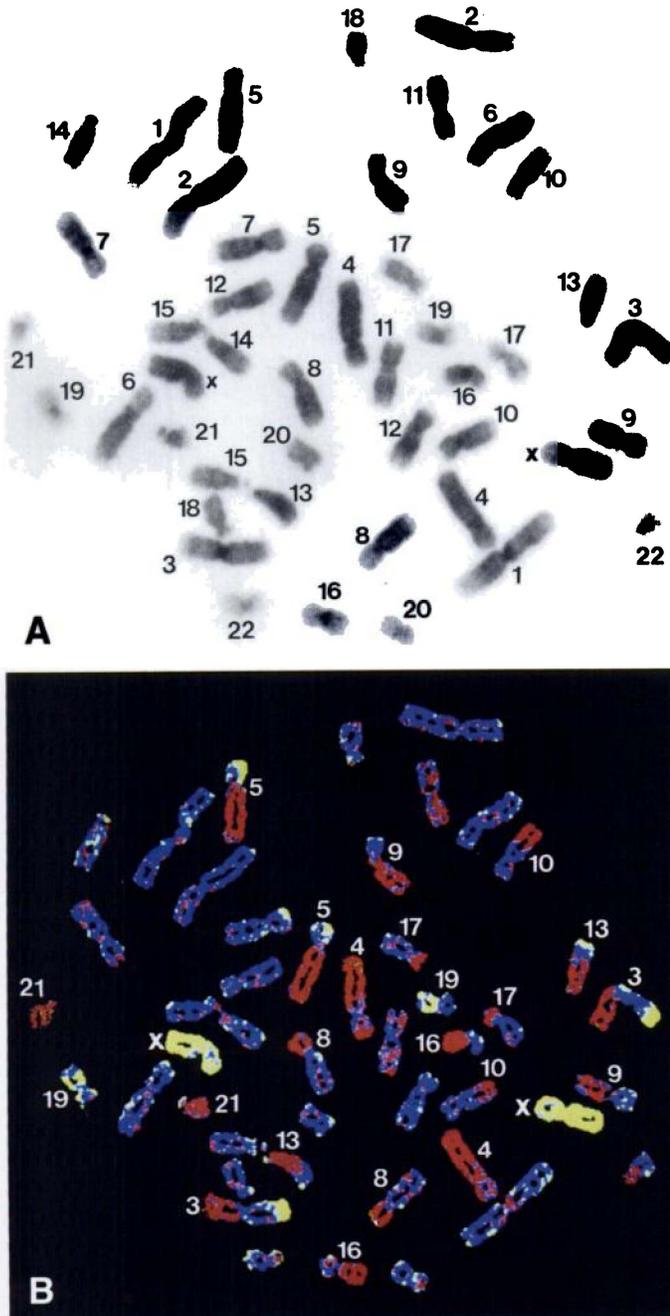


Fig. 1. Evaluation of the CGH analysis of SCLC case no. 12. A, DAPI-staining of normal metaphase chromosomes. The banding pattern was used for chromosome identification (numbers). B, display of fluorescence ratio image of the same metaphase spread as in A after CGH with SCLC DNA. A three-color look-up table was chosen for the visualization of pixel by pixel FITC:rhodamine ratios. Blue, balance between the FITC and rhodamine values. Green, overrepresentation of DNA in the tumor. Red, loss of tumor DNA at the corresponding sequences. The chromosomes involved in aberrations in this SCLC case are labeled with the respective numbers. Note the consistency of the CGH results by comparing the values on the homologous chromosomes.

axis for each chromosome in every metaphase, individual FITC and rhodamine profiles were calculated. These were used for the computation of the ratio (FITC:rhodamine) profiles. Theoretically, a monosomy of certain chromosomes in the tumor would result in a ratio value of 0.5 and a trisomy in a value of 1.5. Values above 2 were regarded as amplified regions. The mean of the individual ratio profiles from five metaphase spreads were calculated (Fig. 2). The three vertical lines on the right side of the chromosome ideogram represent different threshold values between the tumor and the reference DNA. The central line corresponds to a balanced state, as defined as the most frequently observed value. The right and left lines reflect the threshold values that would be present if 50% of the cell population carries either a monosomy or a trisomy for a

certain chromosome. Thus, these values are 0.75 and 1.25, respectively. The curve shows the ratio profile that was computed as the mean value of five metaphase spreads. The gray boxes in Fig. 2 represent heterochromatic chromosome regions that cannot be interpreted. In these regions, very low FITC and rhodamine fluorescence intensities were recorded due to the suppression with Cot1 DNA. Accordingly, fluorescence ratios indicating copy number changes could not be calculated. CGH experiments using, as the test genome, DNA extracted from healthy donors versus the reference DNA used in these experiments did not result in values beyond the defined thresholds.

RESULTS

An example of the experimental data after CGH analysis of SCLC case no. 12 is presented in Figs. 1 and 2. The fluorescence banding pattern of a normal lymphocyte metaphase spread after DAPI staining is shown in Fig. 1A. This G-banding pattern was used for chromosome identification. Based on the values of the FITC and rhodamine fluorescence, a ratio image was generated and is displayed using a look-up table (Fig. 1B). Blue indicates equal copy numbers in the two genomes. Green represents an overrepresentation in the tumor genome, while red is DNA loss. The loss of chromosome 3p is obvious. The frequently observed loss of chromosome 5q was accompanied by an overrepresentation of 5p. Furthermore, amplification sites could be mapped to chromosome bands 3q27–28, 19q13.1, and Xq27. The average fluorescence profile that was calculated for each chromosome as the mean value of 5 metaphase spreads is exemplified for this case in Fig. 2. Shaded areas represent regions rich in repetitive DNA in which the profile could not be interpreted (see "Materials and Methods"). These average ratio profiles were used for the evaluation of chromosomal gains and losses in all cases.

An overview about all genetic alterations that were detected in 13 SCLCs is displayed in Fig. 3. Vertical lines on the left side of each chromosome ideogram represent loss of genetic material in the tumor, whereas those on the right side correspond to a gain. Changes in individual cases can be identified by the case number provided on the top of each line. Amplification sites are represented as solid squares or bars.

Chromosome 3 was most frequently affected and showed alterations in all except one case (no. 1). Total or at least partial loss of the short arm was a consistent finding. Additionally, the long arm showed an overrepresentation in 10 cases. Chromosome 5 revealed genetic imbalances in 10 of 13 cases. Again, the loss of genetic material on one arm (5q) was often accompanied by a gain on the other chromosome arm (5p). This combination occurred in 45% of the cases (6 of 13). The results of CGH analysis were compared with studies of loss of heterozygosity with three markers on chromosome 3p located at 3p13–14 (16) and 3p24 (17, 18) and two markers inside the *APC* gene that were assayed by PCR-RFLP (19). In seven cases that were informative for these markers, the results were confirmed by CGH-analysis, e.g., values specific for DNA losses were consistent with LOH. In one case, however, LOH on chromosome 3p was not confirmed by CGH (see "Discussion"). Chromosome 8 was involved in 7 of 13 cases (54%). In five of these, an overrepresentation of the long arm was observed. The short arm of chromosome 17 was deleted in 8 of 13 cases (61%).

Different amplification sites ($n = 22$) could be mapped to normal chromosomes. For high resolution mapping of the chromosomal location of the amplified regions, the CGH analysis was repeated on prometaphase chromosomes. Fig. 4 presents, as an example, a partial reference prometaphase spread with amplification sites visible at band 8q24.1 harboring the *c-myc* protooncogene (case no. 3). Most frequently, chromosomal band 19q13.1 was involved (4 cases). Sequences originating from chromosome bands 1p32, 2p23, 7q11.2, 8q24, and 13q33–34 were each amplified in two cases. Amplification

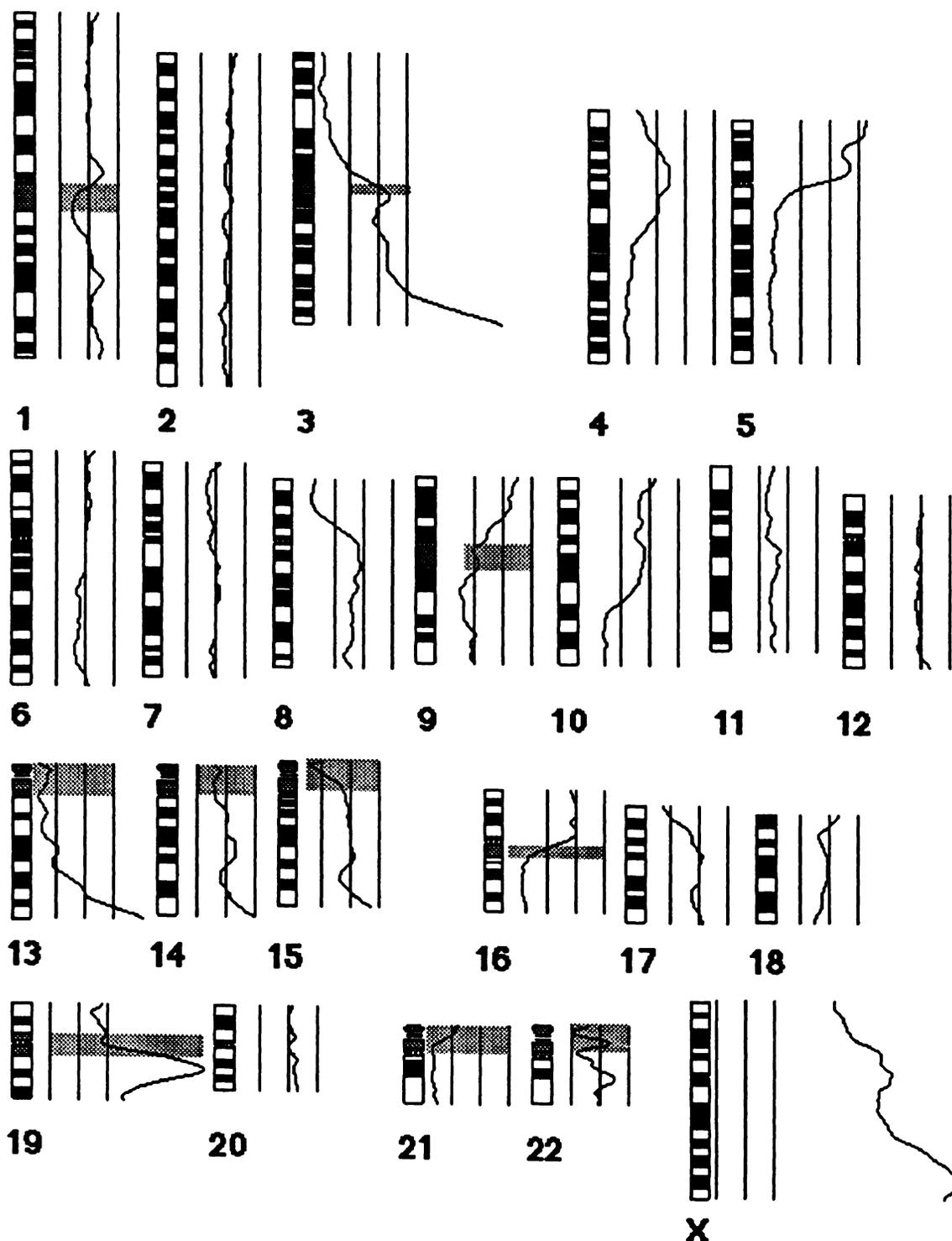


Fig. 2. Mean of the ratio profile calculation of five metaphase spreads of case no. 12. The *three vertical lines* on the *right side* of the chromosome ideograms represent different values of the fluorescence intensities between the tumor and the reference DNA. The values are 0.75, 1, and 1.25 from *left to right*. The ratio profile (curve) was calculated as a mean value of five metaphase spreads. *Gray shaded boxes*, chromosomal regions rich in heterochromatin that could not be interpreted due to the abundance of highly repetitive DNA. All genetic changes that were visible in Fig. 1B were confirmed after mean ratio profile measurements.

sites that occurred only once were observed at the following locations: 1q24, 1q32, 3q27-28, 5p15, 5p14, 6q21, 6q25, 8q21, 9q22, 11q13, 12p, 14q12-21, 15q24-25, 21q21, 22q11.2, Xp11.2, and Xq.

DISCUSSION

CGH has recently evolved as a powerful tool for the identification of chromosomal gains and losses in genomic DNA from tumor

samples (1-6). Using CGH, we identified numerous chromosomal gains and losses as well as DNA amplification sites in 13 cases of primary SCLC.

Loss of genetic material was observed in decreasing frequency on chromosomes 3p, 5q, 17p, 13q, 10q, and 4q (Fig. 3). Most consistently, a loss of the short arm of chromosome 3 was observed in 90%. This is also the most consistent finding that has been reported previously by means of cytogenetical (20-24) and molecular biology

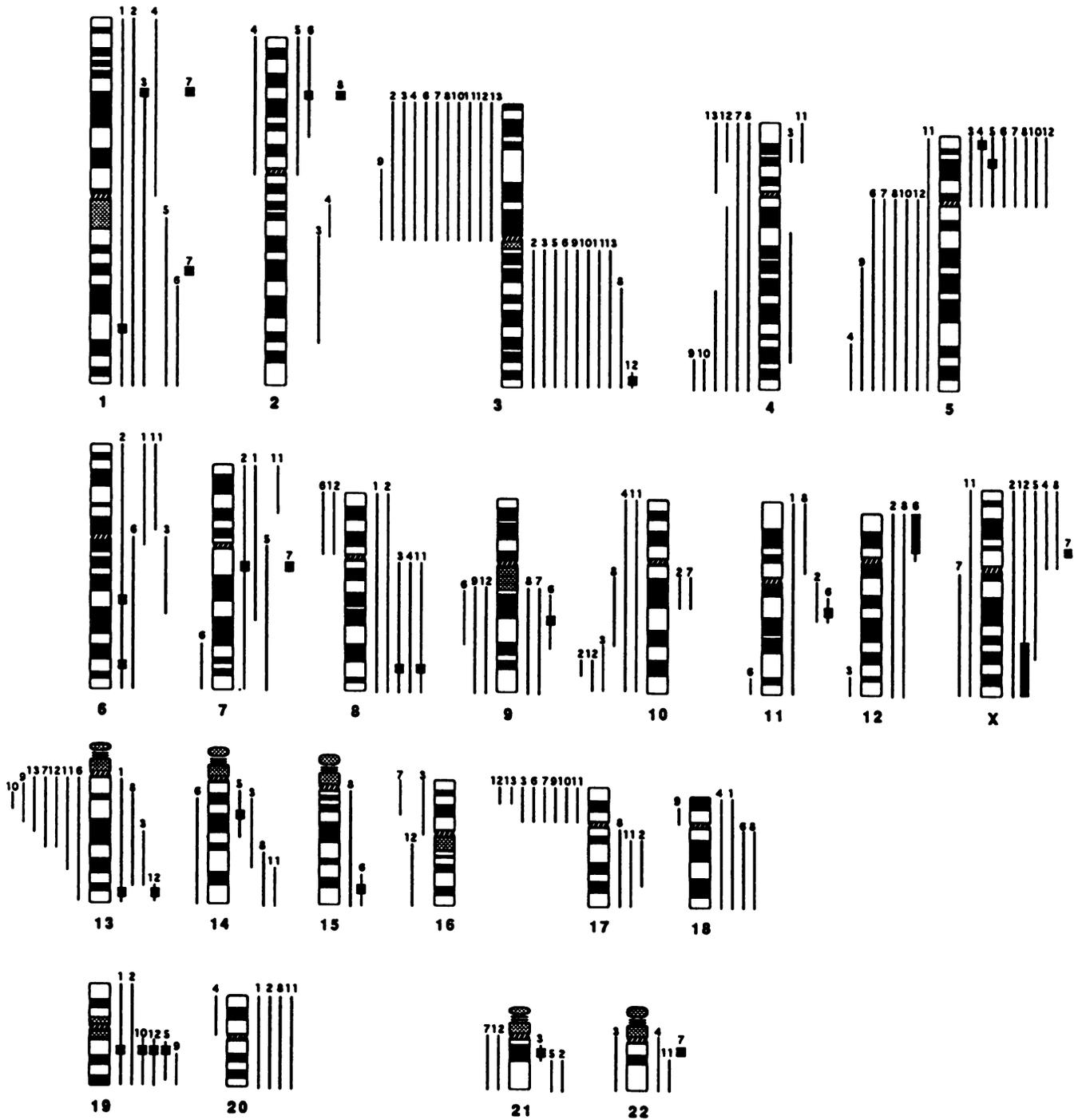


Fig. 3. Summary of genetic imbalances detected in 13 primary SCLC. Vertical lines on the left side of each chromosome ideogram represent loss of genetic material in the tumor, whereas those on the right side correspond to a gain. Changes in individual cases can be identified by the case number provided on the top of each line. Amplification sites are represented as solid squares or bars.

studies involving RFLP markers (25). In one case, the deleted region was restricted to chromosomal bands 3p12–21.2. However, in the majority of cases the entire short arm was underrepresented. In 10 cases, we observed a simultaneous overrepresentation of the long arm. Such a result would be expected in case of an isochromosome 3q formation. Isochromosomes are the cytogenetic correlate of low level amplification of many genes which might induce a growth advantage important for tumor initiation (26). Chromosome 5 frequently showed a similar pattern, *i.e.*, the gain of the short arm was accompanied by the loss of 5q in six cases. This result is suggestive for an isochromosome 5p. Both an isochromosome 3q and an isochromosome 5p

were reported previously in cytogenetic analysis of SCLC, although less frequently than in our series of SCLC (27). The fact that, in addition to values reflecting a gain of 3q and 5p, amplification sites were mapped to these chromosome arms might favor the hypothesis that not only the loss of heterozygosity on 3p and 5q but also the low level amplification of certain genes on 3q and 5p are important events in tumor initiation.

The frequent deletions of chromosomes 3p and 5q in SCLC are indicative for a pivotal mutation in tumor formation. Both chromosomal arms harbor at least one tumor suppressor gene, *i.e.*, the von Hippel-Lindau gene on 3p25–26 and the *FAP* gene on 5q (28–30).



Fig. 4. Example of the chromosomal mapping position of an amplification site in SCLC case no. 3. The CGH experiment was performed on prometaphase chromosomes. Similar to the signals after hybridization with cosmid or YAC clones, two distinguished spots were detected, indicating the mapping position of amplified sequences in the tumor with high resolution. The signals are observed at chromosome band 8q24.1.

The contradictory results for loss of 3p material using CGH and PCR-RFLP analysis, which we observed in one case (no. 1), can be explained by several cytogenetic events: (a) small, submicroscopical deletions which are beyond the resolution of CGH on metaphase chromosomes; (b) mitotic recombination; and (c) chromosome loss that was followed by reduplication (31). Both mitotic recombination and reduplication would result in LOH and uniparental disomy that would not be accompanied by copy number changes detectable with CGH. We observed LOH with primers for chromosomal bands 3p14 and 3p24. A deletion of that size would be clearly detectable by CGH. Thus, mitotic recombination or chromosome reduplication appear to be the more likely mechanisms that account for the described discrepancy. In all other cases (nos. 3–5, 7–9, and 13) where LOH studies were informative (19), the results were consistent with the CGH analysis.

The tumor suppressor genes *p53* and *RB* are located on chromosomes 17p and 13q, respectively (32). We observed partial and complete DNA loss on these chromosomal arms in 8 of 13 (62%) and 7 of 13 (54%) cases. The chromosome 13 losses were invariably accompanied with underrepresentation for 17p. Both chromosomal loci are subject to allelic loss in SCLC as determined by LOH studies using RFLPs (33). An additional consensus region involved in losses was observed on the chromosomal band 10q24–25 in 5 of 13 cases. This region has not been reported previously to be deleted in lung cancer but is known to reveal LOH in *e.g.*, glioblastoma and renal cell carcinoma (12). Loss of 11p was not observed in our collective, in contrast to previous LOH studies (12). Additional DNA losses were found on chromosomes 2p, 4, 8p, 9q, 16, 11q, 12q, 18p, 20p, 21, and 22.

The pattern of chromosomal gains and losses in SCLCs is very complex and involves virtually every site in the genome. This indicates a high genomic instability. Some chromosomes (*e.g.*, 9, 13, 21, and 22) were involved in both gains and losses of the same subregions. This could possibly be explained as the result of random chromosomal changes which occur early in tumor development. Such random changes would be present in the predominant clone(s) and thus could be detected by CGH. However, some chromosomal sites were involved with much higher frequencies than others (see above), indicating their specific role in these tumors. It is a striking observation that chromosome arms 3p, 5q, 10q, and 17p and chromosome bands 4q31–35, which were commonly deleted in these tumors, were never involved in gains of genetic material. From these data, it appears that random gains of these chromosome segments would be prohibitive with regard to the formation of SCLCs. An interesting observation in this respect was reported by Satoh *et al.* (34); the

tumorigenic potential of a lung adenocarcinoma cell line was suppressed after the introduction of chromosomes 3 and 11 by means of cell fusion experiments.

Amplification of protooncogenes are a common feature of solid tumors (35, 36). A cytogenetic correlate is provided by double minute chromosomes or homogeneously staining regions. In contrast to deleted regions, amplifications usually reflect advanced and more aggressive tumor stages. In SCLC, amplification of the *myc* protooncogene family has been reported (37–39). We mapped the origin of amplicons to chromosome bands 1p31–32 (case nos. 3 and 7), 8q24 (case nos. 3 and 11), and 2p23 (case nos. 6 and 8). These sites harbour the *L-myc*, *N-myc*, and *c-myc* protooncogenes, respectively (40). Additionally, amplified regions were mapped to chromosomal sites containing genes of the *ras* family, *i.e.*, 12p (*v-Ki-ras2*). An additional site which showed overrepresentation in 4 of 13 cases was identified on chromosome 19q13.1. To our knowledge, no protooncogene has been mapped so far to this chromosome band. Candidate genes include the gene for the transforming growth factor (41) and a zinc finger gene (42). The possible involvement of these genes can now be tested by means of Southern blot analysis or PCR analysis.

Recent progress in solid tumor molecular biology, *e.g.*, the identification of the gene for the familiar tumor syndrome von Hippel-Lindau (30) and the *FAP* gene (28, 29), was achieved by positional cloning techniques. The identification of the chromosomal mapping position of commonly deleted or amplified loci in SCLC, as exemplified in this study, will streamline cloning efforts considerably. Future work will be focused on the identification of genetic changes specific for certain histologically defined lung cancers. Also, CGH of a higher case number, including primary tumors and metastatic sites, should contribute to identify those genetic aberrations that are responsible for a more aggressive clinical course, including the potential for metastasis.

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