

Patterns of Chromosomal Imbalances in Adenocarcinoma and Squamous Cell Carcinoma of the Lung¹

Iver Petersen,² Michael Bujard, Simone Petersen, Günter Wolf, Almut Goeze, Anke Schwendel, Holger Langreck, Klaus Gellert, Martin Reichel, Kelly Just, Stanislas du Manoir, Thomas Cremer, Manfred Dietel, and Thomas Ried

Institute of Pathology, University Hospital Charité, Schumannstrasse 20-21, D-10098 Berlin, Germany [I. P., S. P., G. W., A. G., A. S., H. L., M. D.]; Institute of Human Genetics, University of Heidelberg, D-69120 Heidelberg, Germany [M. B., T. C.]; Department of Surgery, University Hospital Charité, D-10098 Berlin, Germany [K. G.]; Institute of Neuropathology, Department of Pathology, University Hospital, CH-8091 Zürich, Switzerland [M. R.]; and National Human Genome Research Institute, NIH, Bethesda, Maryland 20892 [K. J., S. d. M., T. R.]

Abstract

Comparative genomic hybridization was used to screen 25 adenocarcinomas and 25 squamous cell carcinomas of the lung for chromosomal imbalances. DNA copy number decreases common to both entities were observed on chromosomes 1p, 3p, 4q, 5q, 6q, 8p, 9p, 13q, 18q, and 21q. Similarly, DNA gains were observed for chromosomes 5p, 8q, 11q13, 16p, 17q, and 19q. Adenocarcinomas showed more frequently DNA overrepresentations of chromosome 1q and DNA losses on chromosomes 3q, 9q, 10p, and 19, whereas squamous cell carcinomas were characterized by increased overrepresentations of chromosome 3q and 12p as well as deletions of 2q. For the first time, we used a histogram representation and statistical analysis to evaluate the differences between both tumor groups. In particular, the overrepresentation of the chromosomal band 1q23 and the deletion at 9q22 were significantly associated with adenoid differentiation, whereas the DNA loss of chromosomal band 2q36-37 and the overrepresentations at 3q21-22 and 3q24-qter were statistically significant markers for the squamous cell type. The study strengthens the notion that different tumor subgroups of the respiratory tract are characterized by distinct patterns of chromosomal alterations.

Introduction

Adenocarcinoma and SCC³ account for about 70% of all lung tumors. Whereas adenocarcinomas tend to be located in the periphery of the lung, SCCs arise preferentially from bronchi near the hilus with potential involvement of the trachea. This pattern parallels the distribution of putative precursor cells of each tumor entity. SCC derives from stem cells of a dysplastic multilayer epithelium that underwent squamous metaplasia, whereas adenocarcinoma originates preferentially from precursor cells of the mono- or bilayer surface epithelium of the lung periphery.

Up to now only few specific chromosomal aberrations could be assigned to the different histologies. This is partly due to the fact that mixed tumor types may occur. Using molecular genetic studies with polymorphic markers, the balance of evidence suggests that loss of DNA sequences are more frequent in SCCs than in adenocarcinomas. Particularly, a higher incidence of loss of heterozygosity on chromosomes 3p and 17p has been reported to occur in SCCs (1, 2). Karyotype analysis, however, has failed thus far to identify typical cytogenetic changes in either tumor entity except for an isochromosome 8q

formation, which was observed exclusively in a subset of adenocarcinomas (3-5).

To screen the DNA from adenocarcinomas and SCCs of the lung for specific, nonrandom genetic alterations, we used CGH to investigate 25 tumors of each tumor entity. CGH is a powerful molecular cytogenetic method of surveying a tumor genome for genetic imbalances (6, 7). Test and reference DNA are differentially labeled and simultaneously hybridized to normal chromosome spreads. After detection with two different fluorochromes, the ratio profiles of the fluorescence intensities along single chromosomes allow the mapping of DNA gains or losses.

In our previous studies, we observed distinct patterns of chromosomal imbalances in SCLC and HNSCC (8-11). The present study indicates that adenocarcinoma and SCC are characterized by recurrent patterns of chromosomal changes.

Materials and Methods

Tumor Samples. Tumor specimens were either collected from postmortem examinations at the pathology institutes of the Charité, the University Hospital of Kiel, and the University Hospital of Zurich 3-48 h after the patients died or were obtained from surgical resections at the Department of Surgery of the Charité at the Humboldt University Berlin within 1 h after operation. One aliquot was frozen in liquid nitrogen and kept at -80°C until DNA extraction. DNA was extracted from several 30-µm cryostat tissue sections by proteinase K and phenol-chloroform extraction, which was verified to consist of a minimum of 70% tumor cells in each case. A second aliquot was subjected to formalin fixation and paraffin embedding. The diagnosis of adenocarcinoma and SCC was established in every case according to the WHO guidelines based on H&E and periodic acid-Schiff-stained tissue sections (12). Five SCCs and three adenocarcinomas were derived from metastatic lesions. The remaining specimens originated from primary tumors.

CGH Preparation. Chromosome metaphase spreads, DNA labeling, hybridization, and detection were performed essentially as described previously (8, 9). Detailed protocols are available on the World Wide Web.⁴ Briefly, normal genomic DNA from blood leukocytes of healthy donors and tumor DNA were labeled in a standard nick translation with digoxigenin-11-dUTP and biotin-16-dUTP (both from Boehringer Mannheim), respectively. For CGH, 1 µg each of tumor and normal DNA were ethanol precipitated in the presence of 10 µg of salmon sperm DNA and 30 µg of human Cot1 DNA. The probe mixture was dried and resuspended in a 15-µl hybridization solution (33% formamide, 13.3% dextran sulfate, 2.7× SSC, (1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7), which was applied to the denatured and dried metaphase spreads. In general, the chromosomes were not subjected to a pepsin or protease treatment. The hybridization was performed for 3 days at 37°C, after which the biotinylated tumor DNA was detected with FITC conjugated to avidin (Vector Laboratories), and the digoxigenin-labeled normal DNA was visualized with antidigoxigenin Fab fragments conjugated to rhodamine (Boehringer Mannheim). Chromosomes were counterstained with

Received 2/18/97; accepted 4/26/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported by the German Research Foundation (Deutsche Forschungsgemeinschaft Grant Pe 602/1) and the Berlin Cancer Society.

² To whom requests for reprints should be addressed. Phone: 49-30-2802-2611; Fax: 49-30-2802-3407.

³ The abbreviations used are: SCC, squamous cell carcinoma; CGH, comparative genomic hybridization; HNSCC head and neck squamous cell carcinoma; SCLC, small cell lung carcinoma.

⁴ Address: <http://amba.rz.charite.hu-berlin.de/cgh/>.

4,6-diamidino-2-phenylindole dihydrochloride and embedded in 90% glycerol containing 2.3% 4,6-diazabicyclo[2.2.2]octane (Sigma Chemical Co.).

Image Acquisition and Analysis. Fluorescence images were obtained either by a Zeiss Axiophot or a Leica DMRBE epifluorescence microscope, which was mounted by a 12-bit cooled charge-coupled device camera (Photometrics, Tucson, AZ). Three images per metaphase spread were acquired, quantitated as eight-bit gray level images, and stored in a tagged image file format (TIFF). The 4,6-diamidino-2-phenylindole dihydrochloride image was used for chromosome identification. FITC and rhodamine fluorescence, specific for the tumor and reference genome, respectively, were used to compute fluorescence ratio images and ratio profiles. For every case, at least five metaphases were analyzed and average ratio profiles were calculated to suppress random changes. The digital image analysis was performed by a CGH software developed by Wolf *et al.* (13) and Roth *et al.* (14, 15) in all cases. Thirteen cases were additionally analyzed by the CGH program of du Manoir *et al.* (7, 16). The same fluorescence images were analyzed by both programs in seven cases, yielding consistent results.

The alterations were determined by the average ratio profiles according to fluorescence ratio thresholds as described (9) and are represented in Fig. 1 as lines. Details of the digital image analysis are published (15) and are also available at the Web site.⁴ The alterations depicted in Fig. 1 were drawn manually by use of a graphic program. This is the classical form of the representation of CGH results.

In Fig. 2, we have introduced a new form of representation of CGH results

that enables the statistical analysis of differences between tumor groups. For every case, the deviations of the average ratio profiles from the normal ratio value, 1.0, were tested for significance by the Student's *t* test. The relative number of significant alterations of a tumor group were then represented by histograms; *e.g.*, the maximum value of 100% is reached if all tumors of the tumor group carry a change at a specific chromosomal region. Fig. 2 shows the difference histogram of the adenocarcinomas and SCCs. The percentage of changes occurring only in the adenocarcinoma group is represented by the green color, whereas an excess of changes in the SCC group is shown in red. The differences between the tumor groups were analyzed for significance by the χ^2 test. Areas with 95% significance ($0.01 < P < 0.05$) are depicted in bright gray, and areas with 99% significance ($P < 0.01$) are depicted in dark gray.

Results

The summary of genetic changes of the adenocarcinomas and SCCs is displayed in Fig. 1, A and B, respectively. Vertical lines to the left of each chromosome ideogram represent loss of genetic material in the tumor, whereas those on the right side correspond to a gain. Amplification sites are represented by solid squares or bars.

Differences between both tumor subgroups can be deduced from the histogram representation of Fig. 2. Adenocarcinomas carried sig-

A

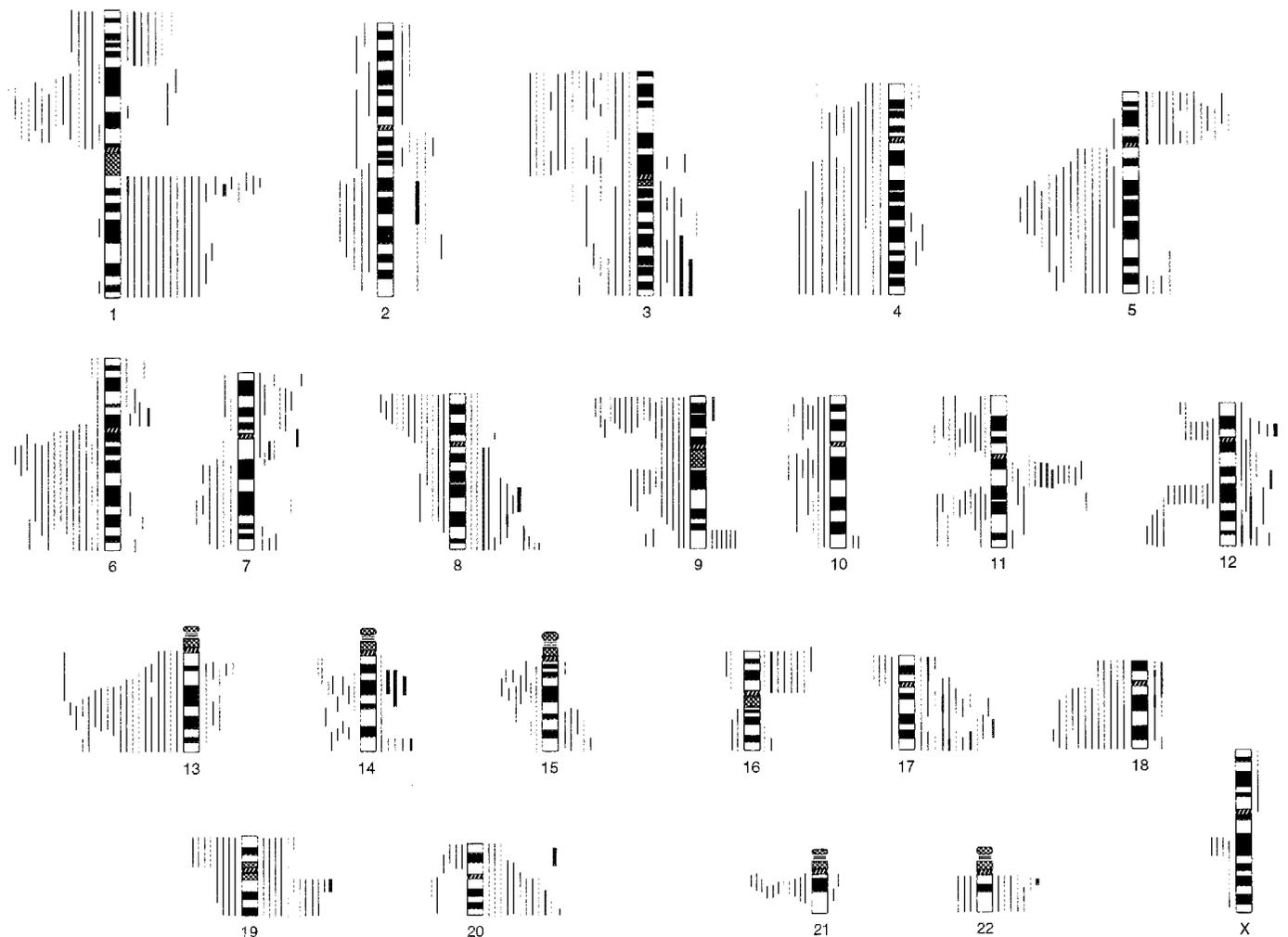


Fig. 1. Summary of genetic imbalances detected in the 25 adenocarcinomas (A) and 25 SCCs (B). Vertical lines to the left of each chromosome ideogram represent loss of genetic material in the tumor; vertical lines to the right correspond to a gain. High copy overrepresentations are represented semiquantitatively (bold lines). Similar patterns regarding DNA underrepresentations were observed for chromosomes 1p, 3p, 4q, 5q, 6q, 8p, 9p, 13q, 18q, and 21q. DNA gains were detected frequently in both entities on chromosomes 5p, 8q, 11q13, 16p, 17q, and 19q.

nificantly more DNA overrepresentations at the chromosomal band 1q23 and deletions on chromosomes 3q, 9q, 10p13, 15q21-22, 19p13.3, and 19q13.2. In contrast, SCCs showed significantly more deletions on chromosome 2q36-37 and DNA gains on chromosome 3qter and 12p13.

The CGH superkaryograms (9-11) of both entities are available on our web site.⁴

Discussion

Since the introduction of CGH (6), the technique has been used to screen a variety of solid tumors for genetic imbalances. In carcinomas of the respiratory tract, SCLCs and SCCs of the head and neck have been investigated in particular (8-10, 17-19). The aim of the present study was to detect nonrandom chromosomal imbalances in adenocarcinomas and SCCs of the lung that might correlate to the histogenesis of these tumor types.

Chromosomal regions frequently affected by DNA gains in adenocarcinomas and SCCs were observed on chromosomes 5p, 8q, 11q13, 16p, 17q, and 19q. Known oncogenes on these chromosomal arms are the *c-myc* gene at 8q24, the *cycD1* at 11q13 and the *erbB2* at 17q12-21. These genes have been shown to be altered in at least one of both subtypes (20-23). In general, the pattern of overrepresentation is similar to that in HNSCC. The fact that the gain of chromosome 3q was particularly prevalent in the SCCs is consistent with previous

reports in HNSCC and SCC of the uterine cervix (11, 18, 19, 24). This chromosome arm showed frequently overrepresentations and amplifications of the telomeric subregion 3q26-qter, which seem to contribute a growth advantage to carcinomas of the squamous cell type as well as SCLC. Putative oncogenes on this chromosome arm have been discussed previously (10). Together with the overrepresentation of the chromosomal band 12p13, it carried the highest degree of significance in comparison to the adenocarcinomas.

Adenocarcinomas showed frequently an overrepresentation of the entire chromosomal arm 1q. This is consistent with findings in adenocarcinomas of the breast (25-27), the colon (28), and the prostate (29). In particular, the overrepresentation of chromosomal band 1q23 was significantly different to SCC. The fact that we mapped high copy amplifications to the chromosomal bands 1q21-23 suggests that this region harbors a gene that contributes to adenoid differentiation in a variety of tumors.

Adenocarcinomas carried a higher incidence of DNA gains on chromosome 20q. This correlates to the finding that invasive adenocarcinomas of the breast show an increased frequency of amplifications within the chromosomal subregion 20q13, which is implicated in tumor progression (30).

Deletions occurred frequently in both entities on chromosomes 1p, 3p, 4q, 5q, 6q, 8p, 9p, 13q, 18q, and 21q. This pattern is again similar to that in HNSCC (11, 18, 19). Significant differences between both

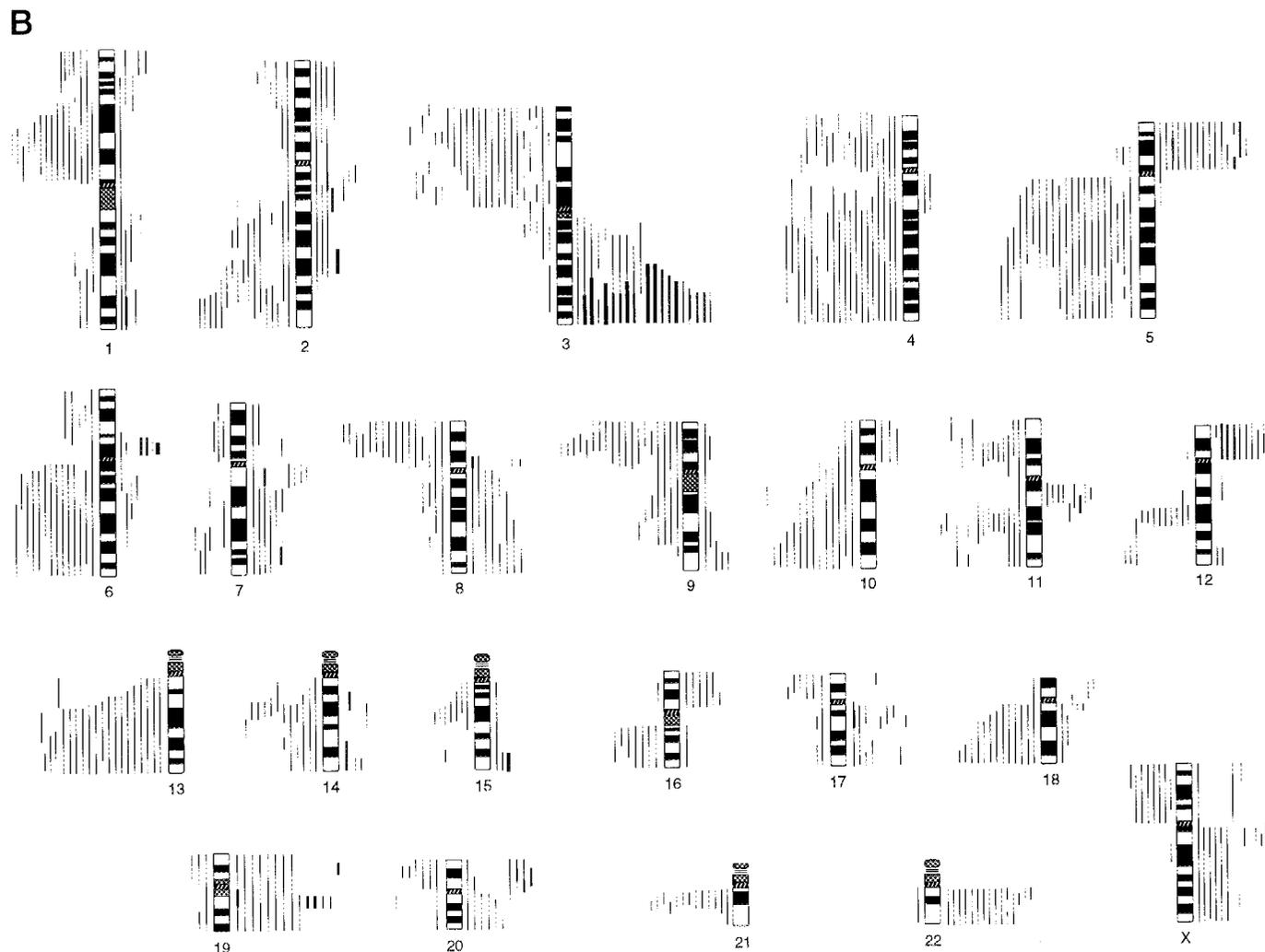


Fig. 1. continued.

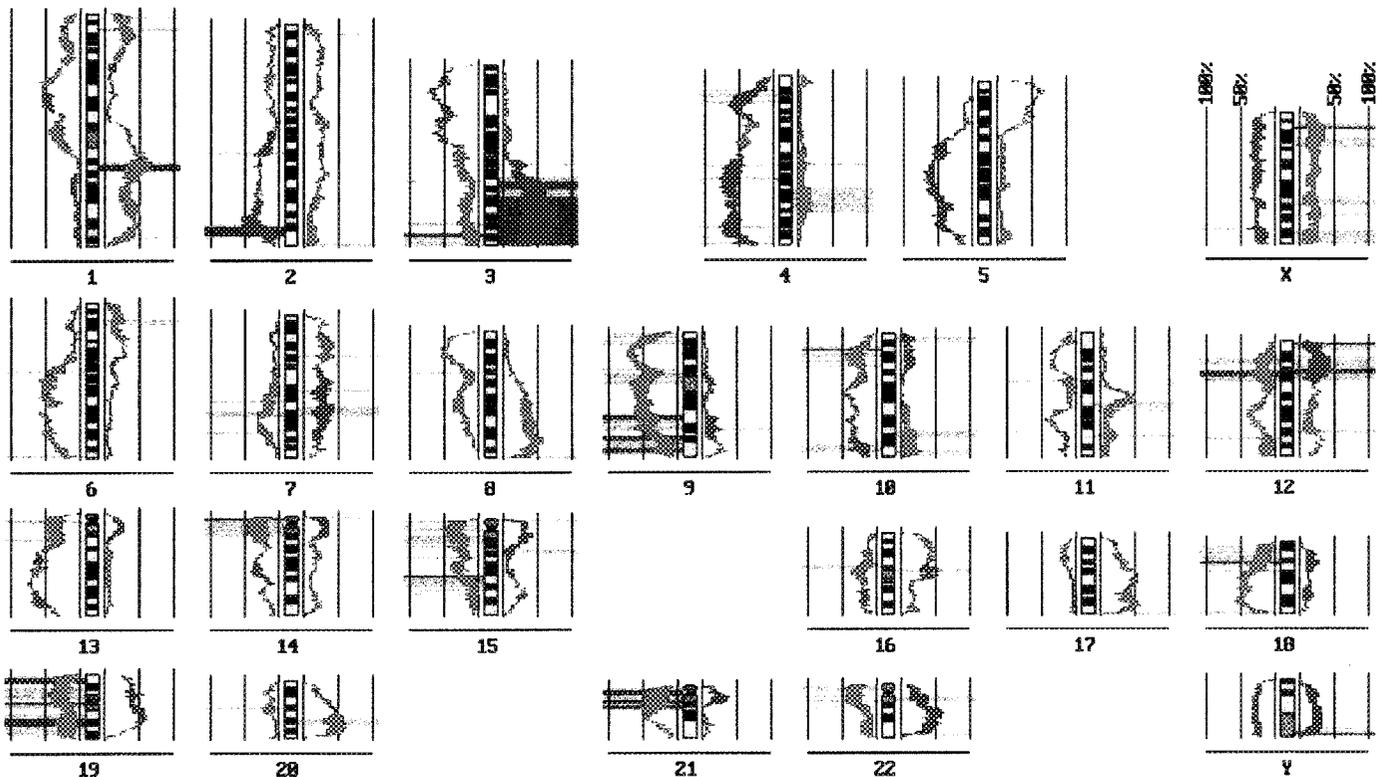


Fig. 2. Difference histogram of chromosomal imbalances between the adenocarcinomas and SCCs. *Green*, percentage of changes that are present only in adenocarcinomas. *Red*, excess of changes of the SCC tumor group. *White areas beneath the colored parts of each histogram*, percentage of changes that are present in both entities. *Gray horizontal lines*, statistically significant differences between the adenocarcinomas and SCCs. *Light gray lines*, regions with 95% significance; *dark gray lines*, 99% significance (χ^2 test). The overrepresentation of the chromosomal band 1q23 and the deletions at 2q36–37, 9q22, 9q32–33, 9q34, 10p13, 19p13.3, and 19q13.2 were associated significantly with adenoid differentiation. In contrast, the DNA loss of chromosomal band 2q36–37 and the overrepresentations at 3q21–22, 3q24–qter, and 12p13 are associated with the squamously cell type. Centromeric regions and heterochromatin-rich areas must be excluded due to the high variability of the fluorescence signal in these regions.

entities were observed for the chromosomal region 2q36–37, which was more frequently deleted in SCCs than adenocarcinomas. This is corroborated by findings in SCC of the uterine cervix and HNSCC and suggests that a yet-unknown tumor suppressor gene is located on the telomeric chromosome arm. In contrast, deletions on chromosome 3q, 9q, 10p, and 19 were more pronounced in adenocarcinomas with significant differences for the chromosomal bands 3q27–28, 9q22, 9q31–32, 9q34, 10p13, 19p13.3, and 19q13.2. We think that among these changes, particularly those that occur in more than 50% of the cases, deserve further investigation.

In general, the usefulness of the histogram representation can be seen by the fact that the peaks correspond well to chromosomal locations of known or suspected tumor-associated genes, *e.g.*, *Krev1/Rap1A* at 1p13.3, the *FHIT* gene at 3p14.2, *p16* at 9p21, and *cycD1* at 11q13. Furthermore, the approach can be used to compare any tumor subgroups, *e.g.*, metastasizing *versus* nonmetastasizing carcinomas.

In summary, the present paper shows that adenocarcinomas and SCCs of the lung are characterized by patterns of DNA gains and losses indicating regions with distinct and overlapping chromosomal imbalances. This correlates with the observation that non-small cell lung carcinomas represent a heterogeneous group of tumors that is frequently characterized by a morphological variability showing overlapping histological features within a single tumor. The fact that distinct chromosomal alterations can be identified is encouraging for our ongoing studies of strictly defined morphological and clinical tumor subgroups, which will help to dissect the genetic alterations underlying lung carcinogenesis.

Acknowledgments

The technical assistance of Heidi Holtgreve-Grez, Almut Richter, and Nicole Deutschmann is acknowledged gratefully.

References

1. Tsuchiya, E., Nakamura, Y., Weng, S.-Y., Nakagawa, K., Tsuchiya, S., Sugano, H., and Kitagawa, T. Allelotype of non-small cell lung carcinoma—comparison between loss of heterozygosity in squamous cell carcinoma and adenocarcinoma. *Cancer Res.*, 52: 2478–2481, 1992.
2. Sato, S., Nakamura, Y., and Tsuchiya, E. Differences of allelotype between squamous cell carcinoma and adenocarcinoma of the lung. *Cancer Res.*, 54: 5652–5655, 1994.
3. Miura, I., Siegfried, J. M., Resau, J., Keller, S. M., Zhou, J., and Testa, J. R. Chromosome alterations in 21 non-small cell lung carcinomas. *Genes Chromosomes & Cancer*, 2: 328–338, 1990.
4. Whang-Peng, J., Knutsen, T., Gazdar, A., Steinberg, S. M., Oie, H., Linnoila, I., Mulshine, J., Nau, M., and Minna, J. D. Nonrandom structural and numerical chromosome changes in non-small-cell lung cancer. *Genes Chromosomes & Cancer*, 3: 168–188, 1991.
5. Testa, J. R., and Siegfried, J. M. Chromosome abnormalities in human non-small cell lung cancer. *Cancer Res.*, 52: 2702s–2706s, 1992.
6. Kallioniemi, A., Kallioniemi, O.-P., Sudar, D., Rutovitz, D., Gray, J. W., Waldman, F., and Pinkel, D. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* (Washington DC), 258: 818–821, 1992.
7. du Manoir, S., Speicher, M. R., Joos, S., Schröck, E., Popp, S., Döhner, H., Kovacs, G., Robert-Nicoud, M., Lichter, P., and Cremer, T. Detection of complete and partial chromosome gain and losses by comparative genomic *in situ* hybridization. *Hum. Genet.*, 90: 590–610, 1993.
8. Ried, T., Petersen, I., Holtgreve-Grez, H., Speicher, M., Schröck, E., du Manoir, S., and Cremer, T. Mapping of multiple DNA gains and losses in primary small cell lung carcinomas by comparative genomic hybridization. *Cancer Res.*, 54: 1801–1806, 1994.
9. Petersen, I., Langreck, H., Wolf, G., Schwendel, A., Psille, R., Vogt, P., Reichel, M. B., Ried, T., and Dietel, M. Small cell lung cancer is characterized by a high incidence of deletions on chromosomes 3p, 4q, 5q, 10q, 13q and 17p. *Br. J. Cancer*, 75: 79–86, 1997.
10. Schwendel, A., Langreck, H., Reichel, M., Schröck, E., Ried, T., Dietel, M., and Petersen, I. Primary small cell lung carcinoma and their metastases are characterized

- by a recurrent pattern of genetic alterations. *Int. J. Cancer Predict. Oncol.*, *74*: 86-93, 1997.
11. Bockmühl, U., Schwendel, A., Dietel, M., and Petersen, I. Distinct patterns of chromosomal alterations in high- and low-grade head and neck squamous cell carcinomas. *Cancer Res.*, *56*: 5325-5329, 1996.
 12. WHO. WHO Histological Typing of Lung Tumors. Geneva: WHO, 1981.
 13. Wolf, G., Petersen, I., Roth, K., and Dietel, M. Computergestütztes Programm für die molekulare zytogenetische Analyse von soliden Tumoren mittels Vergleichender Genomischer Hybridisierung (CGH) und digitaler Bildverarbeitung. *Verh. Dtsch. Ges. Pathol.*, *79*: 612, 1995.
 14. Roth, K., Wolf, G., Dietel, M., and Petersen, I. Analyseprogramm zur quantitativen Erfassung chromosomaler Aberrationen mittels Komparativer Genomischer Hybridisierung (CGH). *Pathologie*, *17*: 342-348, 1996.
 15. Roth, K., Wolf, G., Dietel, M., and Petersen, I. Image analysis for Comparative Genomic Hybridization (CGH) based on a karyotyping program for Windows. *Anal. Quant. Cytol. Histol.*, in press, 1997.
 16. du Manoir, S., Schröck, E., Bentz, M., Speicher, M. R., Joos, S., Ried, T., Lichter, P., and Cremer, T. Quantitative analysis of comparative genomic hybridization. *Cytometry*, *19*: 27-41, 1995.
 17. Levin, N. A., Brzoska, P., Gupta, N., Minna, J. D., Gray, J. W., and Christman, M. F. Identification of frequent novel genetic alterations in small cell lung carcinoma. *Cancer Res.*, *54*: 5086-5091, 1994.
 18. Speicher, M. R., Howe, C., Crotty, P., du Manoir, S., Costa, J., and Ward, D. C. Comparative genomic hybridization detects novel deletions and amplifications in head and neck squamous cell carcinomas. *Cancer Res.*, *55*: 1010-1013, 1995.
 19. Brzoska, P. M., Levin, N. A., Fu, K. K., Kaplan, M. J., Singer, M. I., Gray, J. W., and Christman, M. F. Frequent novel DNA copy number increases in squamous cell head and neck tumors. *Cancer Res.*, *55*: 3055-3059, 1995.
 20. Little, C. D., Nau, M. M., Carney, D. N., Gazdar, A. F., and Minna, J. D. Amplification and expression of the *c-myc* oncogene in human lung cancer cell lines. *Nature (Lond.)*, *306*: 194-196, 1983.
 21. Broers, J. L., Viallet, J., Jensen, S. M., Pass, H., Travis, W. D., Minna, J. D., and Linnoila, R. I. Expression of *c-myc* in progenitor cells of the bronchopulmonary epithelium and in a large number of non-small cell lung cancers. *Am. J. Respir. Cell Mol. Biol.*, *9*: 33-43, 1993.
 22. Betticher, D. C., Heighway, J., Hasleton, P. S., Altermatt, H. J., Ryder, W. D., Cerny, T., and Thatcher, N. Prognostic significance of *CCND1* (*cyclin D1*) overexpression in primary resected non-small-cell lung cancer. *Br. J. Cancer*, *73*: 294-300, 1996.
 23. Tsai, C. M., Chang, K. T., Perng, R. P., Mitsudomi, T., Chen, M. H., Kadoyama, C., Gazdar, A. F. Correlation of intrinsic chemoresistance of non-small-cell lung cancer cell lines with *HER-2/neu* gene expression but not with *ras* gene mutations. *J. Natl. Cancer Inst.*, *85*: 897-901, 1993.
 24. Heselmeyer, K., Schröck, E., du Manoir, S., Blegen, H., Shah, K., Steinbeck, R., Auer, G., and Ried, T. Gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma of the uterine cervix. *Proc. Natl. Acad. Sci. USA*, *93*: 479-484, 1996.
 25. Kallioniemi, A., Kallioniemi, O. P., Piper, J., Tanner, M., Stokke, T., Chen, L., Smith, H. S., Pinkel, D., Gray, J. W., and Waldman, F. M. Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. *Proc. Natl. Acad. Sci. USA*, *91*: 2156-2160, 1994.
 26. Ried, T., Just, K. E., Holtgreve-Grez, H., du Manoir, S., Speicher, M. R., Schröck, E., Latham, C., Blegen, H., Zetterberg, A., Cremer, T., and Auer, G. Comparative genomic hybridization of formalin-fixed, paraffin-embedded breast tumors reveals different patterns of chromosomal gains and losses in fibroadenomas and diploid and aneuploid carcinomas. *Cancer Res.*, *55*: 5415-5423, 1995.
 27. Isola, J. I., Kallioniemi, O. P., Chu, L. W., Fuqua, S. A. W., Hilsenbeck, S. G., Osborne, C. K., and Waldman, F. M. Genetic aberrations detected by Comparative Genomic Hybridization predict outcome in node-negative breast cancer. *Am. J. Pathol.*, *147*: 905-911, 1995.
 28. Ried, T., Knutzen, R., Steinbeck, R., Blegen, H., Schröck, E., Heselmeyer, K., du Manoir, S., Auer, G. Comparative genomic hybridization reveals a specific pattern of chromosomal gains and losses during the genesis of colorectal tumors. *Genes Chromosomes & Cancer*, *15*: 234-245, 1996.
 29. Cher, M. L., Bova, G. S., Moore, D. H., Small, E. J., Carroll, P. R., Pin, S. S., Epstein, J. I., Isaacs, W. B., and Jensen, R. H. Genetic alterations in untreated metastases and androgen-independent prostate cancer detected by comparative genomic hybridization and allelotyping. *Cancer Res.*, *56*: 3091-3102, 1996.
 30. Tanner, M. M., Tirkkonen, M., Kallioniemi, A., Collins, C., Stokke, T., Karhu, R., Kowbel, D., Shadravan, F., Hintz, M., Kuo, W. L., Waldman, F. M., Isola, J. J., Gray, G. W., and Kallioniemi, O-P. Increased copy number at 20q13 in breast cancer: defining the critical region and exclusion of candidate genes. *Cancer Res.*, *54*: 4257-4260, 1994.