[CANCER RESEARCH 55, 1010–1013, March 1, 1995]

Advances in Brief

Comparative Genomic Hybridization Detects Novel Deletions and Amplifications in Head and Neck Squamous Cell Carcinomas

Michael R. Speicher,[1] Chris Howe, Paul Crotty, Stanislas du Manoir, José Costa, and David C. Ward

Departments of Genetics [M. R. S., D. C. W.] and Pathology [C. H., P. C., J. C.], Yale University School of Medicine, New Haven, Connecticut 06510, and National Center for Human Genome Research, NIH, Bethesda, Maryland 20892 [S. d. M.]

Abstract

To gain a better understanding of genetic changes in squamous cell carcinomas of the head and neck we used comparative genomic hybridization for the analysis of 13 primary tumors. Copy number increases were most frequently observed on chromosomes 3q (10 cases) and 5p (8 cases) and less frequently on 1q (4 cases), 2 (1 case), 7 (2 cases), 8q (2 cases), 9 (1 case), 10p (2 cases), 13q (2 cases), 14q (1 case), 16 (1 case), 17 (2 cases), 20p (2 cases), 21q (1 case) and 22q (1 case). Copy number decreases occurred most frequently at 3p (5 cases), 5q (4 cases), 19p (6 cases), and 19q (5 cases). Copy number decreases also were observed on 1p (2 cases), 2q (2 cases), 4p (2 cases), 4q (2 cases), 7q (2 cases), 8p (1 case), 10q (1 case), 11p (2 cases), 11q (3 cases), 13q (3 cases), 14q (1 case), 16p (1 case), 17p (3 cases), 17q (1 case), 18q (1 case), and 22 (2 cases). Eight sites exhibiting significant sequence amplification were mapped to 3q26→qter (3 cases), 11q13 (2 cases), 12p (2 cases), 2q33–36 (1 case), 7q21–22 (1 case), 7q33→qter (1 case), 9p (1 case), and 13q32→qter (1 case). Our data suggest that the regions 3q26→qter and 5p may harbor oncogenes important for initiation or progression of squamous cell carcinomas of the head and neck. In addition, comparative genomic hybridization defines a subgroup of tumors with 11q13 involvement, the location of the *PRAD1/(CCND1)/cyclin D1* gene.

Introduction

HNSCC[2] occur in more than 50,000 new cases annually (1) yet little is known about genetic changes in this disease. Cytogenetic analysis identified only a limited number of clonal structural abnormalities with loss of segments from chromosomes 3p, 7q, 8p, and 11q and gain of genetic material of chromosomes 1q, 3q, 8q, and 15q (for recent papers see Refs. 2 and 3). Allelotyping indicated losses in chromosomes 3, 4, and 8 and chromosome segments 6p, 9p, 11q, 13q, 14q, 17p, and 19q (4, 5). To further define the genetic changes in squamous cell carcinomas of the head and neck we used CGH (6, 7). In CGH, differentially labeled tumor and normal DNAs are mixed in equimolar amounts and hybridized simultaneously to normal metaphase chromosomes. Regions within the tumor DNA that are overrepresented or underrepresented in the probe mixture can be identified by an increased or decreased color ratio of the two fluorochromes used to detect hybridized DNA sequences on the reference chromosomes. Consensus deletion regions were identified for chromosomes 3p, 5q, and 19, whereas frequently occurring overrepresentations were observed for 3q and 5p. Several DNA segments revealed a high level of amplification. Three high level amplifications were mapped to 3q26→qter. These findings may narrow down the region on 3q important for tumor initiation or progression in HNSCC. Four tumors demonstrated a relative overrepresentation at 11q13, the region that harbors the *PRAD1/(CCND1)/cyclin D1* gene.

Materials and Methods

Tumor Samples. Clinical and histological data on the primary tumors from 13 different patients are summarized in Table 1. All tumor samples were frozen within 30 min after operation. Samples were verified for the percentage of tumor cells present in the tissue used for DNA extraction and in all cases a minimum of 50% tumor cells were present. DNA was extracted from 20-μm tissue sections by proteinase K digestion and phenol-chloroform extraction according to standard protocols. The diagnosis of HNSCC was established in every case on hematoxylin and eosin-stained sections. Staging followed International Union Against Cancer criteria.

CGH and Digital Image Analysis. CGH, image acquisition, and image processing were done as described previously (7–9). Briefly, 200–500 ng of biotinylated tumor DNA were mixed with the same amount of normal male reference DNA, labeled with digoxigenin, and hybridized to normal metaphase spreads (46,XY) in the presence of 50 μg of Cot1-DNA and 20 μg of sonicated salmon DNA. Hybridization was allowed to proceed for 2–3 days. Wash conditions and probe detection were carried out as described (7–9).

Gray level images were taken separately for each fluorochrome using an epifluorescence microscope (Zeiss Axioskop) equipped with a cooled charged coupled device camera (Photometrics, Tucson, AZ). Chromosomes were identified using the fluorescence banding pattern obtained by 4,6-diamidino-2-phenylindole dihydrochloride staining. Fluorescein (FITC) and rhodamine (TRITC) fluorescence were specific for the tumor and the control genome, respectively.

The over- and underrepresented DNA segments were determined by calculating FITC:TRITC average ratio profiles (Figs. 2 and 3). Average FITC:TRITC ratio images were calculated from at least 10 metaphases and have fixed thresholds which were tested by control experiments using normal DNA as well as DNA from cell lines with known numerical aberrations. The central line in the profiles represents the modal fluorescence ratio value measured for all reference metaphase spreads. The left and the right lines correspond to the theoretical ratio value for a monosomy or trisomy, respectively, in 50% of the cell population (9). DNA gains and losses of the individual tumors, summarized in Fig. 1, were determined from these ratio profiles.

Table 1 *Clinical and histological data on primary tumors of 13 patients*

Case	Age (yr)	Sex	Site	TNM[a]	Stage	Histological grade
1	69	M	Pharynx	$T_3N_0M_X$	3	1
2	43	M	Tongue	$T_3N_2M_X$	4	2
3	80	M	Pharynx	$T_2N_2M_X$	4	1
4	78	M	Neck	$T_XN_2M_X$	4	2
5	63	F	Epiglottis	$T_2N_2M_X$	4	2
6	65	M	Tongue	$T_XN_2M_1$	4	2
7	72	M	Pharynx/esophagus	$T_3N_XM_X$	3	3
8	85	F	Lip	$T_2N_1M_X$	3	2
9	80	M	Tongue	$T_2N_1M_X$	3	1
10	69	F	Larynx	$T_3N_XM_X$	3	2
11	60	M	Pharynx	$T_4N_XM_X$	4	2
12	75	M	Pharynx	$T_3N_2M_X$	4	2
13	66	M	Tonsil	$T_4N_2M_X$	4	2

[a] TNM, tumor-nodes-metastases classification.

Received 1/25/95, accepted 1/27/95.

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] Recipient of a grant from the Deutsche Forschungsgemeinschaft (SP 460/1-1). To whom requests for reprints should be addressed, at Department of Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510.

[2] The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; CGH, comparative genomic hybridization; TRITC, tetramethylrhodamine isothiocyanate.

Results

To determine DNA copy number changes in HNSCC we used DNA extracted from 13 frozen tissue sections. A schematic summary of all copy number changes observed in each tumor using CGH is shown in Fig. 1.

The most frequently observed chromosomal aberrations involved chromosome 3. Overrepresentation of the long arm of chromosome 3 was observed in 10 tumors (Fig. 2*a*). Three of these 10 tumors had a high level amplification ranging from 3q26→qter (Figs. 2*b* and 3). Five tumors demonstrated an underrepresentation of 3p; 4 of these 5 tumors also exhibited a gain of 3q DNA segments (Fig. 2*b*). Genetic

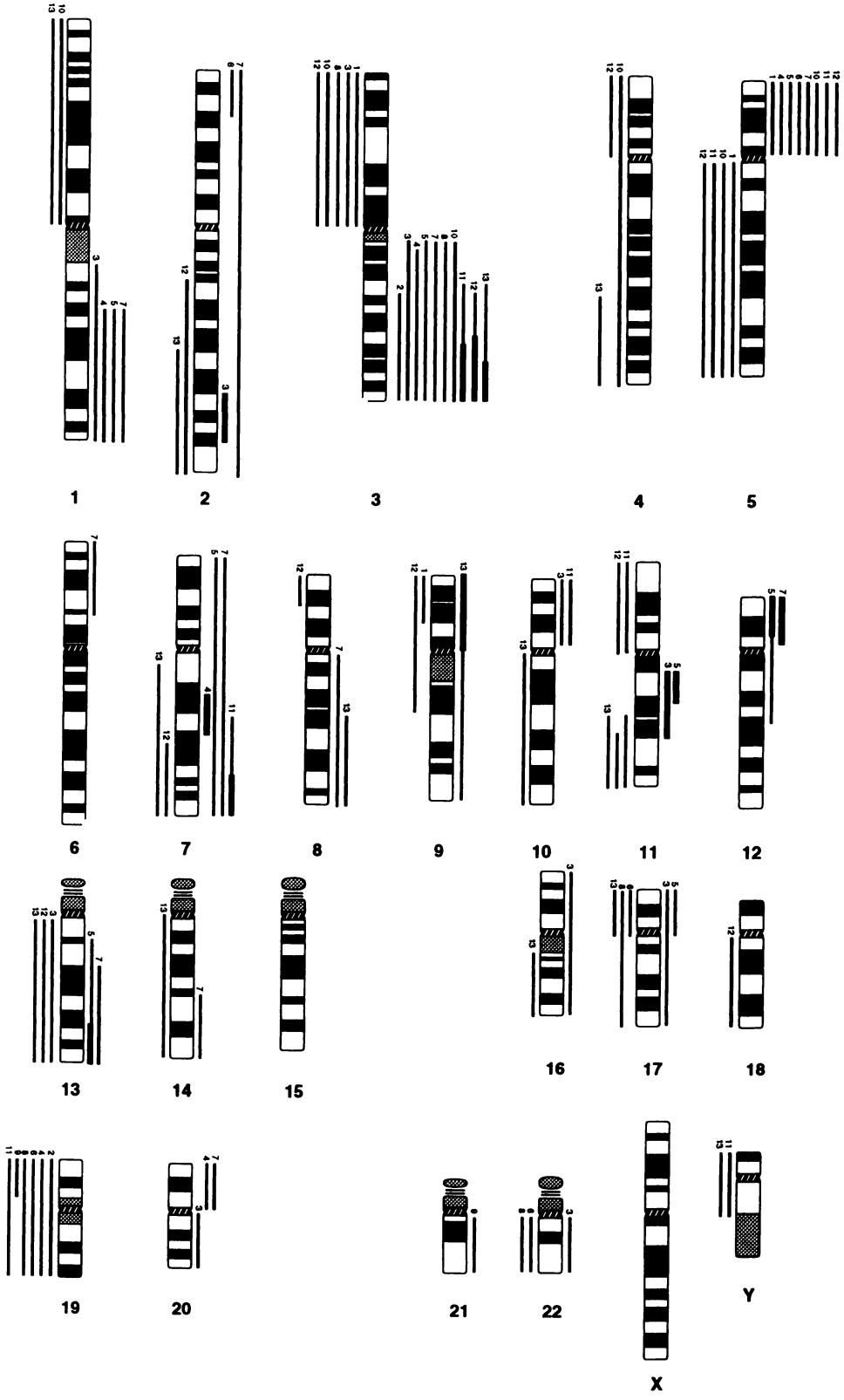

Fig. 1. Summary of all gains and losses found with CGH in HNSCC. *Vertical lines* on the *right side* of a chromosome, gain of genetic material; *vertical lines* on the *left side* losses; *thick lines*, high level amplifications. Case numbers are provided on the *top* of each line.

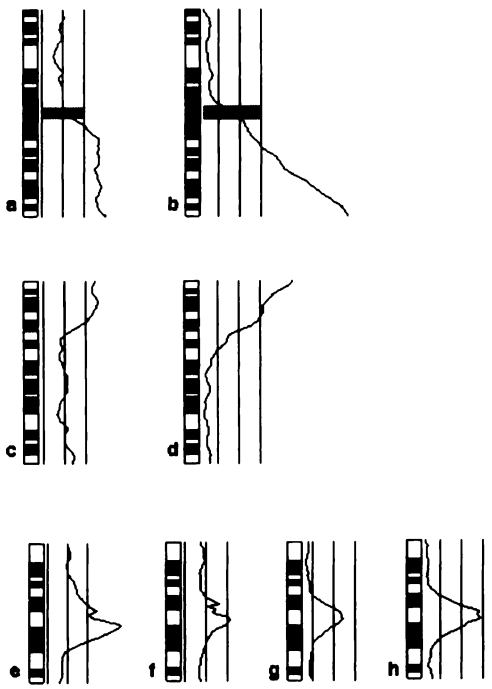

Fig. 2. Average ratio profiles of the most frequently involved chromosomes. For details see legend to Fig. 3. *a*, ratio profile of chromosome 3 (tumor 7) calculated from 13 chromosomes (n = 13) displaying an overrepresentation of 3q; *b*, ratio profile of chromosome 3 (tumor 12; n = 12) demonstrating loss of 3p and gain of 3q. The region 3q26→qter shows a high level amplification (compare right shift in *b* with right shift in *a*); *c*, chromosome 5 ratio profile (tumor 7; n = 14) with 5p gain; *d*, chromosome 5 ratio profile (tumor 11; n = 15) displaying 5p gain and 5q loss; *e* and *f*, tumor 3 (n = 10) and tumor 5 (n = 11), respectively, demonstrating 11g23 amplifications; *g* and *h*, tumor 11 (n = 14) and tumor 12 (n = 13) showing loss of chromosome 11 material but balanced ratio values for the region 11q13.

imbalances on chromosome 5 were seen in 8 tumors. These were an overrepresentation of 5p in 4 cases (Fig. 2*c*) or a combination of 5p gain and 5q loss (Figs. 2*d* and 3) observed in 4 cases. Loss of chromosome 19 material was seen in 6 tumors.

Chromosome 11 revealed interesting findings in 4 tumors. Two tumors showed high level amplifications spanning chromosome bands 11q13–11q22 (Fig. 2*e*) and 11q13, respectively (Fig. 2*f*). Two other tumors demonstrated balanced ratio values for the region 11q13 but loss of the rest of chromosome 11 material (Figs. 2, *g* and *h*; Fig. 3).

High level amplifications observed less frequently were mapped to 2q33–q36 (1 case) 7q21–22 (1 case), 7q33–qter (1 case), 12p (2 cases), and 13q32→qter (1 case).

Other less frequently occurring copy number increases were observed on the following chromosome: 1q (4 cases); 2p (2 cases); 2q (1 case); 6p (1 case); 7 (2 cases); 7q (2 cases); 8q (2 cases); 9 (1 case); 10p (2 cases); 13q (2 cases); 14q (1 case); 16 (1 case); 17p (2 cases); 17q (1 case); 20p (2 cases); 21q (1 case); 22q (1 case). Copy number decreases were mapped to the following locations: 1p (2 cases); 2q (2 cases); 4p (2 cases); 4q (2 cases); 7q (2 cases); 8p (2 cases); 9p (2 cases); 10q (1 case); 13q (3 cases); 14q (1 case); 16q (1 case); 17p (3 cases); 17q (1 case); 18q (1 case); 22q (2 cases), Y (2 cases).

Discussion

The CGH technique, first reported in 1992 (6), has already become a powerful tool in tumor cytogenetics and has been used to identify a variety of novel and nonrandomly occurring changes in tumor genomes (10–16). In this study we applied CGH to the analysis of HNSCC, where cytogenetic changes are not well characterized.

The most frequently observed findings were copy number changes on chromosomes 3 and 5. Loss of the entire short arm of chromosome 3 was accompanied by a gain of the whole long arm in three cases. This CGH pattern suggests the formation of an isochromosome i(3)(q10) in a subset of HNSCCs, an aberration also seen by karyotype analysis (2, 3). However, karyotyping suggests that complex rearrangements are more likely to lead to loss of segments from chromosome arm 3p and gain of genetic material of 3q, respectively (2, 3).

Three cases demonstrated a high level amplification of the region 3q26→qter, with 3q26.3→qter being the smallest common highly amplified region. This suggests that the telomeric region of 3q may contain one or more genes important for tumor initiation and/or progression. It is also of interest that 2 of the 3 well differentiated tumors lack the 3q alterations found in 10 of 13 cases. No candidate oncogenes are known in this region. The possible involvement of known zinc finger genes mapped to 3q26.3–qter, such as *LAZ3* (17) or *BCL-6* (18), must be elucidated by molecular genetics methods.

Chromosome 5 showed a reverse pattern of DNA change relative to chromosome 3 in four cases, *i.e.*, a gain of 5p occurring simultaneously with a loss of 5q, suggestive of an i(5)(p10) formation. In particular the gain of 5p, observed in 8 of 13 cases, is a nonrandom change in HNSCC. This is a new finding as involvement of chromosome 5 has been observed infrequently in karyotype analysis and thus has not been considered as a nonrandom change before (2, 3).

Gain of oncogenes on 3q and 5p may be pivotal for tumor formation. In addition, loss of 3p and 5q might be necessary events in the multistep genesis of HNSCC. Both chromosome arms harbor at least one tumor suppressor gene, the *VHL* gene on 3p25–p26 (19) and the *FAP* gene on 5q21 (20).

Our data also suggest an important role for the region 11q13 in a subset of HNSCC tumors. The candidate oncogene in this region is *PRAD-1 (CCND1)*, which encodes cyclin D1. Previous studies have

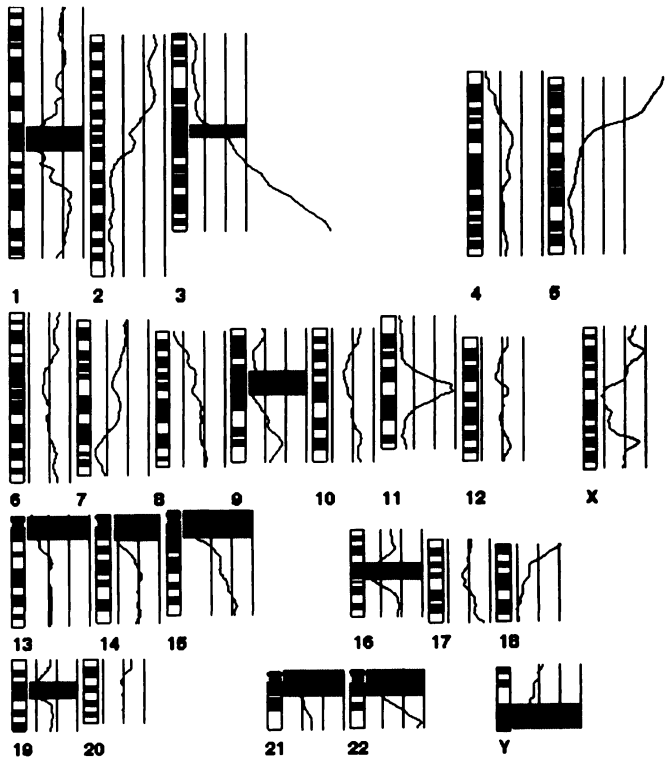

Fig. 3. Average ratio profile of tumor 12. Ratio profiles along the individual chromosomes are shown on the *right side* of each chromosome. *Left*, *middle*, and *right vertical lanes*, lower, middle, and upper thresholds of the normal range. Due to the suppression with Cot1 DNA fraction the heterochromatic blocks (in particular the centromeric or paracentromeric regions of chromosomes 1, 9, 16, and the p arms of all acrocentric chromosomes) yield unreliable ratio values and are excluded from evaluation. For details see text and Ref. 9.

shown that amplifications in this region and of this gene correlate with aggressive growth and high proliferation and probably occur as a late event in tumorigenesis (21, 22).

CGH data are only suggestive for the involvement of particular genes in tumorigenesis. However, candidate genes revealed by CGH can be tested by Southern blot or PCR analysis, *e.g.*, to determine the possible role of *PRAD-1/CCND1/cyclin D1* in HNSCC. The identification of the chromosomal position of commonly deleted or amplified loci should also streamline cloning efforts considerably. Future work will be focused on the characterization of probes within such loci and identification of genetic changes specific for the anatomic site of the tumor. While correlation of genetic changes with histological grading, metastatic potential, etc., could contribute significantly to our understanding of tumors with a more aggressive clinical course, any meaningful clinical correlation will have to emerge from studies involving a larger number of patients.

CGH data from previous studies with breast cancer (10), small cell lung cancer (11, 15), uveal melanoma (12, 13), gliomas (14), and chromophobe renal cell carcinomas (16) showed a distinct CGH pattern for each tumor entity. In contrast, HNSCC show some interesting similarities to small cell lung cancer in terms of the most frequently occurring changes, *i.e.*, 3p loss, 3q gain, 5p gain, 5q loss. The significance of these findings is at this point unclear. Any possible relatedness between HNSCC and small cell lung cancer revealed by genetic alterations is worthy of further study.

Acknowledgments

We thank Tracy Davison for expert technical assistance.

References

1. Boring, C. C., Squires, T. S., and Tong, T. Cancer statistics, 1993. Cancer J. Clin., *51:* 7–26, 1993.
2. Rao, P. H., Sreekantaiah, C., Schantz, S. P., and Chaganti, R. S. K. Cytogenetic analysis of 11 squamous cell carcinomas of the head and neck. Cancer Genet. Cytogenet., *77:* 60–64, 1994.
3. Jin, Y., Mertens, F., Mandahl, N., Heim, S., Olegard, C., Wennerberg, J., Biörklund, A., and Mitelman, F. Chromosome abnormalities in eighty-three head and neck squamous cell carcinomas: influence of culture conditions on karyotypic pattern. Cancer Res., *53:* 2140–2146, 1993.
4. Nawroz, H., van der Riet, P., Hruban, R. H., Koch, W., Ruppert, J. M., and Sidransky, D. Allelotype of head and neck squamous cell carcinoma. Cancer Res., *54:* 1152–1155, 1994.
5. Li, X., Lee, N. K., Ye, Y. W., Waber, P. G., Schweitzer, C., Cheng, Q. C., and Nisen, P. D. Allelic loss at chromosomes 3p, 8p, 13q, and 17p associated with poor prognosis in head and neck cancer. J. Natl. Cancer Inst., *86:* 1524–1529, 1994.
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 gains and losses by comparative genomic in situ hybridization. Hum. Genet., *90:* 590–610, 1993.
8. Speicher, M. R., du Manoir, S., Schröck, E., Holtgreve-Grez, H., Schoell, B., Lengauer, C., Cremer, T., and Ried, T. Molecular cytogenetic analysis of formalin fixed, paraffin embedded solid tumors by comparative genomic hybridization after universal DNA-amplification. Hum. Mol. Genet., *2:* 1907–1914, 1993.
9. 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, 1994.
10. 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.
11. Ried, T., Petersen, I., Holtgreve-Grez, H., Speicher, M. R., 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.
12. Speicher, M. R., Prescher, G., du Manoir, S., Jauch, A., Horsthemke, B., Bornfeld, N., Becher, R., and Cremer, T. Chromosomal gains and losses in uveal melanomas detected by comparative genomic hybridization. Cancer Res., *54:* 3817–3823, 1994.
13. Gordon, K. B., Thompson, C. T., Char, D. H., O'Brien, J. M., Kroll, S., Ghazvini, S., and Gray, J. W. Comparative genomic hybridization in the detection of DNA copy number abnormalities in uveal melanoma. Cancer Res., *54:* 4764–4768, 1994.
14. Schröck, E., Thiel, G., Lozanova, T., du Manoir, S., Meffert, M. C., Jauch, A., Speicher, M. R., Nürnberg, P., Vogel, S., Jänisch, W., Donis-Keller, H., Ried, T., Witkowski, R., and Cremer, T. Comparative genomic hybridization of human malignant gliomas reveals multiple amplification sites and non-random chromosomal gains and losses. Am. J. Pathol., *144:* 1203–1218, 1994.
15. 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.
16. Speicher, M. R., Schoell, B., du Manoir, S., Schröck, E., Ried, T., Cremer, T., Störkel, S., Kovacs, A., and Kovacs, G. Specific loss of chromosomes 1, 2, 6, 10, 13, 17, and 21 in chromophobe renal cell carcinomas revealed by comparative genomic hybridisation. Am. J. Pathol., *145:* 356–364, 1994.
17. Kerckaert, J. P., Deweindt, C., Tilly, H., Quief, S., Lecocq, G., and Bastard, C. LAZ3, a novel zinc-finger encoding gene, is disrupted by recurring chromosome 3q27 translocations in human lymphomas. Nat. Genet., *5:* 66–70, 1993.
18. Ye, B. H., Lista, F., Lo, C. F., Knowles, D. M., Offit, K., Chaganti, R. S., and Dalla, F. R. Alterations of a zinc finger-encoding gene, BCL-6, in diffuse large-cell lymphoma. Science (Washington DC), *262:* 747–750, 1993.
19. Latif, F., Tory, K., Gnarra, J., Yao, M., Duh, F. M., Orcutt, M. L., Stackhouse, T., Kuzman, I., Modi, W., Geil, L., Schmidt, L., Zhou, F., Li, H., Wei, M. H., Chen, F., Glenn, G., Choyke, P., Walther, M. M., Weng, Y., Duan, D. S., Dean, M., Glavac, G., Richards, F. M., Crossey, P. A., Ferguson-Smith, M. A., Le Paslier, D., Chumakov, I., Cohen, D., Chinault, A. C., Maher, E. R., Linehan, W. M., Zbar, B., and Lerman, M. I. Identification of the von Hippel-Lindau disease tumor suppressor gene. Science (Washington DC), *260:* 1317–1320, 1993.
20. Kinzler, K. W., Nilbert, M. C., Su, L. K., Vogelstein, B., Bryan, T. M., Levy, D. B., Smith, K. J., Preislinger, A. C., Hedge, P., McKechnie, D., Finniear, R., Markham, A., Groffen, J., Boguski, M. S., Altschul, S. F., Horri, A., Ando, H., Miyoshi, Y., Miki, Y., Nishisho, I., and Nakamura, Y. Identification of FAP locus genes from chromosome 5q21. Science (Washington DC), *253:* 661–665, 1991.
21. Callender, T., el-Naggar, A. K., Lee, M. S., Frankenthaler, R., Luna, M. A., and Batsakis, J. G. PRAD-1 (CCND1)/cyclin D1 oncogene amplification in primary head and neck squamous cell carcinoma. Cancer (Phila.), *74:* 152–158, 1994.
22. Williams, M. E., Gaffey, M. J., Weiss, L. M., Wilczynski, S. P., Schuuring, E., and Levine, P. A. Chromosome 11q13 amplification in head and neck squamous cell carcinoma. Arch. Otolaryngol. Head Neck Surg., *119:* 1238–1243, 1993.

Comparative Genomic Hybridization Detects Novel Deletions and Amplifications in Head and Neck Squamous Cell Carcinomas

Michael R. Speicher, Chris Howe, Paul Crotty, et al.

Cancer Res 1995;55:1010-1013.