[CANCER RESEARCH 63, 8582–8585, December 15, 2003]

Advances in Brief

Use of Complete Coverage Array Comparative Genomic Hybridization to Define Copy Number Alterations on Chromosome 3p in Oral Squamous Cell Carcinomas

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Abstract

Loss of 3p has been associated with oral cancer progression and is common in many cancers. However, regions of alteration on 3p are poorly defined. We have constructed a high-resolution chromosomal array using a tiling set of 535 human bacterial artificial chromosomes that provides near complete coverage of 3p. Array comparative genomic hybridization analysis of 20 microdissected oral squamous cell carcinomas showed multiple and recurrent segments of copy number changes. These include a deletion containing the *FHIT* gene; novel segments of copy decrease at 3p22, 3p24, and 3p26; and an unexpected ~0.7 Mbp segmental increase at 3p21. These data strongly support the value of using chromosomal array comparative genomic hybridization for detailed profiling of oral squamous cell carcinomas.

Introduction

Deletion on 3p is a frequent event that is likely to play a significant role in the pathogenesis of many cancer types, including oral squamous cell carcinomas [OSCCs (1, 2)]. The current literature suggests that there are multiple regions of chromosomal loss on this arm. However, these regions are poorly defined and their prognostic significance is unknown, with the exception of one region (3p14) where allelic loss has been associated with risk of progression of oral premalignant lesions to cancer (3–6). The recent development of array comparative genomic hybridization (CGH) technology and its application to microdissected archival tissue support the feasibility of high-resolution profiling of copy number changes on an entire chromosome arm in a single experiment (7). However, the only report of array CGH analysis of an entire chromosome arm involves the use of a full coverage array of chromosome 22 [~450 bacterial artificial chromosome (BAC) clones (8)]. The present study represents the first use of this approach to demarcate regions of copy number alterations in OSCCs.

Materials and Methods

Tissue Samples. Formalin-fixed paraffin-embedded tissue blocks were obtained from the British Columbia Oral Biopsy Service and diagnoses were confirmed by an oral pathologist. Tumor cells were microdissected from H&E-stained sections and DNA was isolated and quantified as described previously (9). The clinical features of the 20 cases are shown in Table 1.

BAC Array Construction. A minimal, near-overlapping tiling set of 535 human BAC clones spanning chromosome arm 3p (3p12.3 to 3p26.3) was selected from the RPCI-11 library. The choice of these clones was based on their location on the human physical map[4] and their map location was verified using the University of California at Santa Cruz Biotechnology Human Genome Browser.[5] This clone list is available publicly online.[6] The identity of each BAC DNA sample was confirmed by its *Hin*dIII fingerprint. To generate sufficient quantities of DNA for array construction, we amplified each BAC DNA sample by linker-mediated PCR. As described previously (10), *Mse*I-digested BAC DNA was ligated to linkers (5′-AGTGGGATTCCGCATGCTAGT-3′ and 5′-TAACTAGCATGC-3′) and amplified by two rounds of PCR. The amplified DNA was dissolved in a 20% DMSO solution, boiled for 10 min, and re-arrayed for robotic printing. Each clone was spotted in triplicate with Stealth Micro Spotting Pins onto amine-coated slides (Telechem/ArrayIT SMP2.5, Sunnyvale, CA) using a VersArray ChipWriter Pro system (Bio-Rad, Mississauga, Ontario, Canada). The clones were arranged to ensure that adjacent BACs on the tiling set were not placed next to each other on the array. To allow normalization of the hybridization signal intensities between dyes, linker-mediated PCR-amplified normal male human genomic DNA samples (Novagen, Madison, WI) were spotted on the array 48 times. In addition, 96 randomly selected BACs distributed throughout the genome were included on this array as internal control spots to detect hybridization artifacts such as hybridization signal gradients. The DNA was then covalently bonded to the slides by baking and UV cross-linking. Slides were washed to remove unbound DNA.

Array CGH. Probe labeling was as described previously (10). Test and reference DNA (100 ng each) were labeled separately using Cyanine 3 and Cyanine 5 dCTPs, respectively. The DNA probes were combined, denatured, and annealed in a solution containing 100 μg of human Cot-1 in 25 μl of DIG Easy hybridization solution (Roche, Quebec, Canada), 50 μg of sheared herring sperm DNA (Sigma-Aldrich, Oakville, Ontario, Canada), and 250 μg of yeast tRNA (Calbiochem). The arrays were prehybridized with DIG Easy hybridization buffer (Roche) containing 1% BSA and 2 μg/μl sheared herring sperm DNA at 42°C for 1 h. The probe mixture was applied to the slide surface and hybridized for 36 h at 42°C. Arrays were washed five times with 0.1× SSC and 0.1% SDS (5 min each at room temperature), rinsed five times with 0.1× SSC, and then dried by centrifugation.

A charge-coupled device-based imaging system (Arrayworx eAuto; API, Issaquah, WA) was used to determine signal intensities of the Cyanine 5/Cyanine 3 channels. Images were analyzed with Softworx array analysis software. A scale factor based on the signal intensities of the aforementioned 48 human genomic DNA control spots on the array was used to normalize spot signal data for each channel. SDs for each triplicate spot set were calculated. The array CGH profile was presented as a graph plotting normalized Cyanine 5/Cyanine 3 log2 signal ratios *versus* the relative tiling path position of the BAC clones. A log2 signal ratio of 0 at a spot represents equivalent copy number between the sample and reference DNA. Quality control was applied to each batch of arrays synthesized. Normal DNA *versus* normal DNA hybridizations revealed spots with aberrant signal intensity, and these were removed from the analysis. Spots that exhibited signal ratios outside of 3 SDs

Received 9/21/03; accepted 10/14/03.

Grant support: Funds from the Canadian Institute of Health Research, National Cancer Institute of Canada, Genome British Columbia, National Institute of Dental and Craniofacial Research, and scholarships to Cathie Garnis from National Science Engineering Research Council.

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[4] http://genome.wustl.edu.
[5] http://www.genome.ucsc.edu.
[6] http://www.bccrc.ca/cg/ArrayCGH_Group.html.

Table 1 *Clinicopathological features of patients*

Case	Age (yrs)	Gender	Tobacco usage	Histological grade[a]	TNM[b] stage
114T	75	F	S	2	1
166T	82	F	N	2	2
199T	89	F	N/A[c]	2	2
211T	72	M	S	1	3
24T1	63	F	N	1	1
2T	58	F	S	2	2
43T	67	M	S	3	1
451T	67	M	S	1	3
453T	48	F	N	1	1
469T	75	M	S	1	N/A
478T	62	F	N/A	1	4A
528T	74	M	C	3	4A
539T	64	F	N	1	4A
566T	78	F	N/A	N/A	N/A
569T	76	F	N	1	3
573T	69	M	S	2	3
574T	78	F	N/A	1	N/A
587T	68	M	S	1	0
620T	55	M	S	2	1
628T	41	M	N	2	4A

[a] Histological grade of tumors: 1, well differentiated; 2, moderately well differentiated; and 3, poorly differentiated.
[b] TNM, tumor-node-metastasis.
[c] N/A, not currently available.

from 0 in these experiments were discarded, resulting in the establishment of a ±0.2 log2 ratio threshold for defining regions of copy number increase and decrease. In addition, alterations had to have more than one consecutive BAC with copy number change to be scored as a region of alteration.

Results and Discussion

Multiple Segmental Changes on 3p in OSCCs. We have constructed a high-resolution BAC array for CGH analysis of chromosome 3p. This array consists of 535 overlapping BACs, providing near complete coverage of chromosome 3p from telomere (3p26.3) to centromere (3p12.3).

Twenty oral tumors were microdissected and the DNA was analyzed for segmental copy number change using this array. Nineteen of these 20 tumors had profiles where the internal control DNA spots gave consistent signal ratios. Of these 19 tumors, 2 had no apparent copy number alteration (Fig. 1*A*), 3 showed a whole arm deletion (Fig. 1*B*), and the remaining 14 showed multiple segmental copy number alterations (Fig. 1*C*). As shown in Fig. 2, the pattern of alteration in these tumors was complex, showing discontinuous copy number change with either several regions of deletion or combinations of decreases and increases in copy number.

Alteration Containing *FHIT*. A large region of copy number decrease (~27 Mbp) occurred at 3p14 (Fig. 2, *Region 1*). This alteration is present in 7 of the 15 tumors with segmental alterations. This region contains the fragile histidine triad gene, or *FHIT*, which has been shown to be altered in many tumor types as well as in oral premalignant lesions (11). Loss of *FHIT* expression is a predictor of poor outcome in oral cancer (11). The wide extent of alteration in Region 1 supports the possibility of other tumor suppressor genes in addition to *FHIT*.

Novel Regions of Copy Number Decrease. Although there were numerous regions of copy number change in the 14 cases with segmental alterations, three regions (Fig. 2, *Regions 3–5*) were present at frequencies equal to or greater than that of the *FHIT*-containing Region 1. These regions were small (<2 Mbp each; Table 2) and together contain only two known genes, *RBMS3* and *GRM7*, neither of which has previously been linked to oral cancer. *GRM7*, in Region 5, is a metabotropic glutamate receptor and *RBMS3*, in region 4, is a *MYC* gene single strand-binding protein. *MYC* gene single strand-binding proteins are thought to cooperate with *MYC* to regulate DNA replication, gene transcription, apoptosis, and cell cycle progression (12).

A Region of Copy Number Increase. To date, only deletions have been associated with 3p in oral cancer. The final region identified at 3p21.31 (Region 2) is unique in that it contains an increase in copy number. This alteration is present in 6 of 14 cases, with 5 cases defining a ~0.7 Mbp segmental copy number increase. For the sixth case (474T), the region covers only 0.2 Mbp. Based on the University of California at Santa Cruz Biotechnology Human Genome Browser (April 2003 version), there are eight known genes within the 0.7 Mbp region. Although none have been implicated in oral cancer, two are

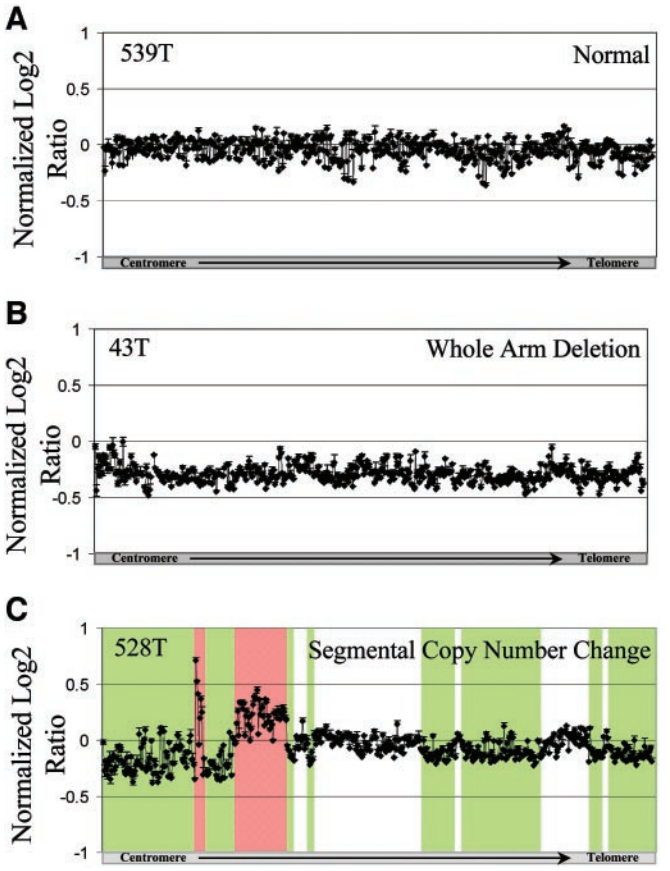

Fig. 1. 3p Array comparative genomic hybridization (CGH) profiles of oral tumors. *A*, tumor 539T has no significant copy number alterations across the entire arm. *B*, tumor 43T has a copy number decrease of the entire 3p arm. *C*, tumor 528T shows numerous segmental copy number increases and decreases. *Green shading* represents area of copy number decrease; *red shading* represents regions of copy number increase.

Table 2 *Description of regions of copy number alteration*

Region	Location	Centromeric BAC[a]	Telomeric BAC[a]	Extent (Mbp)	Known genes
1	3p12.2–3p21.1	603j22 (AC107030)	122d19 (AC018354)	27	750
2	3p21.3	447d11 (AC104447)	509i21 (AC104304)	0.7	8
3	3p22	56p22 (AC093557)	598j13 (AC073353)	0.7	0
4	3p24.1	35c18 (AC018359)	53912 (AC092503)	2	1
5	3p26.1	77i5 (AC011327)	7m24 (AC012136)	0.8	1

[a] GenBank accession number is given in parentheses. BAC, bacterial artificial chromosome.

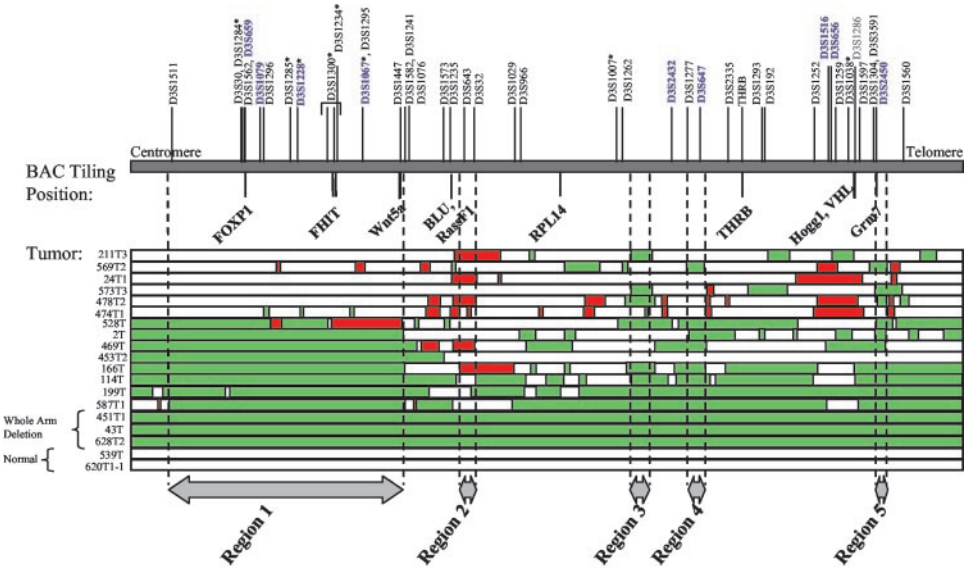

Fig. 2. Alignment of 19 oral squamous cell carcinoma (OSCC) array comparative genomic hybridization (CGH) profiles for chromosome arm 3p. Five common regions of copy number change are marked by *gray arrows*. *Red bars* denote regions of copy number increase, whereas *green bars* represent regions of copy number decrease. Tumor specimens are listed on the *left*. Microsatellite markers historically used in fine mapping studies have been mapped to the tiling set and are shown at the *top* of the figure. Markers in *blue* have been reported in previous studies (1, 2, 18, 19) to have loss of heterozygosity frequencies of >60%.

putative oncogenes. *Testes-Specific Protease 50* (*TSP50*) has been shown to be overexpressed in breast cancer (13) and *Teratocarcinoma-Derived Growth Factor 1* (*TDGF-1*) is overexpressed in a number of cancer cell lines (14). This region also contains a site recently reported to be sensitive to carcinogens present in tobacco (15).

Interestingly, genetic alterations near or within Region 2 have been described previously. Immediately centromeric to Region 2 is *RASSF1A*, which is frequently silenced by methylation in many cancers (16), although it is infrequently silenced by methylation in head and neck cancers (17).

Comparison with Known Alterations. We compared the known and novel regions of copy number alterations with previously published regions defined by fine mapping efforts using microsatellite markers and CGH analysis (1, 2, 5, 18–20). Historically, fine mapping by loss of heterozygosity in tumors is difficult due to the paucity of microsatellite markers as well as the constant revision of the human genome map. We have positioned the microsatellite markers used in the OSCC 3p fine mapping studies (1, 2, 5, 19) and highlighted those that showed allelic loss in >66% of cases in Fig. 2. Interestingly, these markers fall within Region 1 (which contains *FHIT*) at 3p14, Region 4 at 3p24, and Region 5 at 3p26.1 as defined by array CGH.

In summary, this is the first report of complete profiles of copy number changes on 3p in OSCCs. The data show the complex type of alterations that occur on this arm, many of which are small in size, requiring high-resolution assays to define critical regions of change relevant to oral cancer. Using this approach, we detected a large region containing the extensively studied *FHIT* gene, but we also detected three novel small regions of deletions (3p22, 3p24.1, and 3p26.1) at high frequency. We also report the first observation of a region showing copy number increase on 3p.

It is known that 3p alterations occur early in the development of oral cancer (3–6). Future studies will be targeted toward mapping early premalignant lesions through segmental copy number change and will begin to delineate minimal regions of alteration for gene discovery.

Acknowledgments

We thank Bradley Coe, Spencer Watson, and Chad Malloff for array synthesis and bioinformatics assistance and Michelle Morris for providing clinical information on the OSCC samples used in this study.

References

1. Rowley, H., Jones, A., Spandidos, D., and Field, J. Definition of a tumor suppressor gene locus on the short arm of chromosome 3 in squamous cell carcinoma of the head and neck by means of microsatellite markers. Arch. Otolaryngol., *122:* 497–501, 1996.
2. Roz, L., Wu, C. L., Porter, S., Scully, C., Speight, P., Read, A., Sloan, P., and Thakker, N. Allelic imbalance on chromosome 3p in oral dysplastic lesions: an early event in oral carcinogenesis. Cancer Res., *56:* 1228–1231, 1996.
3. Califano, J., van der Riet, P., Westra, W., Nawroz, H., Clayman, G., Piantadosi, S., Corio, R., Lee, D., Greenberg, B., Koch, W., and Sidransky, D. Genetic progression model for head and neck cancer: implications for field cancerization. Cancer Res., *56:* 2488–2492, 1996.
4. Rosin, M. P., Cheng, X., Poh, C., Lam, W. L., Huang, Y., Lovas, J., Berean, K., Epstein, J. B., Priddy, R., Le, N. D., and Zhang, L. Use of allelic loss to predict malignant risk for low-grade oral epithelial dysplasia. Clin. Cancer Res., *6:* 357–362, 2000.
5. Partridge, M., Emilion, G., Pateromichelakis, S., Phillips, E., and Langdon, J. Location of candidate tumour suppressor gene loci at chromosomes 3p, 8p and 9p for oral squamous cell carcinomas. Int. J. Cancer, *83:* 318–325, 1999.
6. Mao, L., Lee, J. S., Fan, Y. H., Ro, J. Y., Batsakis, J. G., Lippman, S., Hittelman, W., and Hong, W. K. Frequent microsatellite alterations at chromosomes 9p21 and 3p14 in oral premalignant lesions and their value in cancer risk assessment. Nat. Med., *2:* 682–685, 1996.
7. Pinkel, D., Segraves, R., Sudar, D., Clark, S., Poole, I., Kowbel, D., Collins, C., Kuo, W. L., Chen, C., Zhai, Y., Dairkee, S. H., Ljung, B. M., Gray, J. W., and Albertson, D. G. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nat. Genet., *20:* 207–211, 1998.
8. Buckley, P. G., Mantripragada, K. K., Benetkiewicz, M., Tapia-Paez, I., Diaz De Stahl, T., Rosenquist, M., Ali, H., Jarbo, C., De Bustos, C., Hirvela, C., Sinder Wilen, B., Fransson, I., Thyr, C., Johnsson, B. I., Bruder, C. E., Menzel, U., Hergersberg, M., Mandahl, N., Blennow, E., Wedell, A., Beare, D. M., Collins, J. E., Dunham, I., Albertson, D., Pinkel, D., Bastian, B. C., Faruqi, A. F., Lasken, R. S., Ichimura, K., Collins, V. P., and Dumanski, J. P. A full-coverage, high-resolution human chromosome 22 genomic microarray for clinical and research applications, Hum. Mol. Genet., *11:* 3221–3229, 2002.
9. Zhang, L., Michelsen, C., Cheng, X., Zeng, T., Priddy, R., and Rosin, M. P. Molecular analysis of oral lichen planus. A premalignant lesion? Am. J. Pathol., *151:* 323–327, 1997.
10. Garnis, C., Coe, B. P., Ishkanian, A., Zhang, L., Rosin, M. P. and Lam, W. L. Novel regions of amplification on 8q distinct from the MYC locus and frequently altered in oral dysplasia and cancer. Genes Chromosomes Cancer, *39:* 93–98, 2003.
11. Lee, J. I., Soria, J. C., Hassan, K., Liu, D., Tang, X., El-Naggar, A., Hong, W. K., and Mao, L. Loss of Fhit expression is a predictor of poor outcome in tongue cancer. Cancer Res., *61:* 837–841, 2001.
12. Penkov, D., Ni, R., Else, C., Pinol-Roma, S., Ramirez, F., and Tanaka, S. Cloning of a human gene closely related to the genes coding for the c-myc single-strand binding proteins. Gene (Amst.), *243:* 27–36, 2000.
13. Yuan, L., Shan, J., De Risi, D., Broome, J., Lovecchio, J., Gal, D., Vinciguerra, V., and Xu, H. P. Isolation of a novel gene, TSP50, by a hypomethylated DNA fragment in human breast cancer. Cancer Res., *59:* 3215–3221, 1999.
14. Baldassarre, G., Tucci, M., Lembo, G., Pacifico, F. M., Dono, R., Lago, C. T., Barra, A., Bianco, C., Viglietto, G., Salomon, D., and Persico, M. G. A truncated form of teratocarcinoma-derived growth factor-1 (cripto-1) mRNA expressed in human colon carcinoma cell lines and tumors. Tumour Biol., *22:* 286–293, 2001.

15. Wu, X., Lippman, S. M., Lee, J. J., Zhu, Y., Wei, Q. V., Thomas, M., Hong, W. K., and Spitz, M. R. Chromosome instability in lymphocytes: a potential indicator of predisposition to oral premalignant lesions. Cancer Res., *62:* 2813–2818, 2002.
16. Burbee, D. G., Forgacs, E., Zochbauer-Muller, S., Shivakumar, L., Fong, K., Gao, B., Randle, D., Kondo, M., Virmani, A., Bader, S., Sekido, Y., Latif, F., Milchgrub, S., Toyooka, S., Gazdar, A. F., Lerman, M. I., Zabarovsky, E., White, M., and Minna, J. D. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J. Natl. Cancer Inst. (Bethesda), *93:* 691–699, 2001.
17. Hogg, R. P., Honorio, S., Martinez, A., Agathanggelou, A., Dallol, A., Fullwood, P., Weichselbaum, R., Kuo, M. J., Maher, E. R., and Latif, F. Frequent 3p allele loss and epigenetic inactivation of the RASSF1A tumour suppressor gene from region 3p21.3 in head and neck squamous cell carcinoma. Eur. J. Cancer, *38:* 1585–1592, 2002.
18. Beder, L. B., Gunduz, M., Ouchida, M., Fukushima, K., Gunduz, E., Ito, S., Sakai, A., Nagai, N., Nishizaki, K., and Shimizu, K. Genome-wide analyses on loss of heterozygosity in head and neck squamous cell carcinomas. Lab Investig., *83:* 99–105, 2003.
19. Kayahara, H., Yamagata, H., Tanioka, H., Miki, T., and Hamakawa, H. Frequent loss of heterozygosity at 3p25–p26 is associated with invasive oral squamous cell carcinoma, J. Hum. Genet., *46:* 335–341, 2001.
20. Huang, Q., Yu, G. P., McCormick, S. A., Mo, J., Datta, B., Mahimkar, M., Lazarus, P., Schaffer, A. A., Desper, R., and Schantz, S. P. Genetic differences detected by comparative genomic hybridization in head and neck squamous cell carcinomas from different tumor sites: construction of oncogenetic trees for tumor progression. Genes Chromosomes Cancer, *34:* 224–233, 2002.

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Cancer Res 2003;63:8582-8585.