Advances in Brief

Frequent Loss of Chromosome 9p21–22 Early in Head and Neck Cancer Progression[1]

Peter van der Riet, Homaira Nawroz, Ralph H. Hruban, Russell Corio, Kaori Tokino, Wayne Koch, and David Sidransky[2]

Department of Otolaryngology, Head and Neck Surgery, Division of Head and Neck Cancer Research [P. v. d. R., H. N., K. T., W. K., D. S.], Department of Pathology [R. H. H.], and Department of Oral Pathology [R. C.], The Johns Hopkins University, Baltimore, Maryland 21205-2196

Abstract

In order to define more clearly the role of chromosome 9 loss in head and neck squamous cell carcinoma (HNSCC), 29 invasive carcinomas and 17 preinvasive lesions were analyzed for loss of heterozygosity (LOH) on chromosome 9. We found LOH in 21 of 29 (72%) HNSCC tumors using highly polymorphic microsatellite markers. In 17 of 21, LOH was found at all informative sites on the p arm with no LOH of the q arm. Further mapping in tumors, with partial LOH of the 9p arm, localized a common region of loss between markers D9S165 and D9S156. Deletion of this region on chromosome 9 has been found in several other tumor types implying the presence of a tumor suppressor gene at this locus. The inactivation of a tumor suppressor gene on chromosome 9p may represent the most commonly described genetic alteration in HNSCC. A similar incidence of allelic loss on chromosome 9p was identified in 12 of 17 (71%) preinvasive lesions. The identical frequency of loss in preinvasive and invasive lesions suggests that loss of 9p is an early event in HNSCC progression.

Introduction

Malignant transformation in tumor progression results from a series of genetic changes (1). Determining the nature and timing of these changes will aid in understanding the biology of each tumor type and could facilitate the development of new diagnostic and therapeutic strategies.

The inactivation of tumor suppressor genes is among the most common molecular events that contribute to tumorigenesis. Although a variety of mechanisms exist for tumor suppressor gene inactivation, loss of chromosomal segments in tumor cells has proven useful in mapping regions containing putative tumor suppressor genes (2, 3). The relative importance of these genes to HNSCC[3] progression remains unclear. Cytogenetic studies have found no consistent chromosomal changes, and results apparently vary with tissue culture conditions (4). Immunohistochemical screening for *p53* showed intense staining in HNSCC tumors and cell lines and led to the identification of *p53* gene mutations in HNSCC (5–7). Analysis of over 100 lesions revealed that the incidence of *p53* alterations in preinvasive lesions was 19% and increased to 43% in invasive HNSCC (8).

To identify additional tumor suppressor gene loci, we screened HNSCC tumors for LOH, using markers on each autosomal arm (9). The highest frequency of allelic loss was found on chromosome 9p and was present in 72% of informative tumors. In this study, a panel of highly informative microsatellite markers was used to identify and

further characterize LOH for portions of chromosome 9 in both invasive and preinvasive lesions. Our results identify a distinct region of loss on chromosome 9p, which has previously been implicated in the progression of other neoplasms. Furthermore, loss of 9p occurs with the same frequency in preinvasive and invasive lesions suggesting loss of this putative tumor suppressor locus early in the progression of HNSCC.

Materials and Methods

Invasive HNSCCs obtained fresh from surgical resection were processed as described previously (8). Clinical characteristics of the patients and stage at the time of surgical resection are listed in Table 1. Tumor sections with greater then 60% tumor tissue were used to isolate DNA for further analysis.

Paraffin blocks of noninvasive lesions (4 severe dysplasias and 13 carcinomas *in situ*) were obtained and a hematoxylin/eosin-stained, formalin-fixed biopsy section was viewed under the microscope for each lesion. The corresponding neoplastic tissue from unstained 4-μm paraffin sections was microdissected away from nonneoplastic cells. The tumor tissue was deparaffinized in xylene, digested with sodium dodecyl sulfate/proteinase K and DNA was extracted with phenol/chloroform followed by ethanol precipitation (10).

Oligonucleotide primers for microsatellite PCR analysis were obtained from Research Genetics (Huntsville, AL) and listed in Fig. 2. One of the primers was labeled with [γ-^{32}P]ATP using T4-polynucleotide kinase (New England Biolabs). Fifty ng of genomic DNA were subjected to 30 cycles of PCR amplification as described previously (10). For amplification of *D9S165*, Taq polymerase (Boehringer Mannheim) was added only after preheating samples to 95° for 3 min. PCR products were separated by electrophoresis in denaturing 8% urea-polyacrylamide-formamide gels (11) followed by autoradiography. For informative cases allelic loss was scored if the intensity of one allele was at least 50% reduced in the tumor DNA as compared with the normal.

Results

We selected well spaced markers from the highly informative microsatellite loci that have been identified on chromosome 9 (12). Through PCR amplification of microsatellite markers, 29 invasive HNSCCs were examined for allelic loss of chromosome 9 and 72%

Table 1 *Clinical parameters of primary HNSCC*

Tumor	Site	Stage	Age/sex	Tumor	Site	Stage	Age/sex
H1	OC[a]	T_2N_0	78/F	H16	OP	T_3N_0	58/F
H2	OC	T_2N_{2C}	39/M	H17	OP	T_2N_0	46/M
H3	LN	T_4N_0	48/M	H18	OC	T_1N_2	73/F
H4	LN	T_3N_{2B}	50/M	H19	LN	T_4N_0	80/M
H5	OP	T_3N_{2B}	63/M	H20	OP	T_3N_2	50/M
H6	OC	T_2N_{2C}	73/F	H21	LN	T_4N_2	46/M
H7	OP	T_3N	61/M	H22	OC	T_4N_0	84/F
H8	LN	T_2N_0	66/F	H23	LN	T_3N_{2C}	58/M
H9	OC	T_2N_0	51/F	H24	OC	T_4N_0	70/F
H10	LN	T_2N_{2A}	45/F	H25	LN	T_3N_0	66/M
H11	HP	T_4N_0	71/F	H26	OC	T_2N_{2C}	67/M
H12	OC	T_3N_0	50/M	H27	OC	T_3N_{2B}	65/M
H13	OC	T_4N_{2C}	59/M	H28	OC	T_1N_1	61/F
H14	OP	T_2N_{2A}	41/M	H29	LN	T_2N_0	64/M
H15	OC	T_3N_3	56/M				

[a] OC, oral cavity; :LN, larynx; OP, oropharynx; HP, hypopharynx; T, tumor stage; N, node stage.

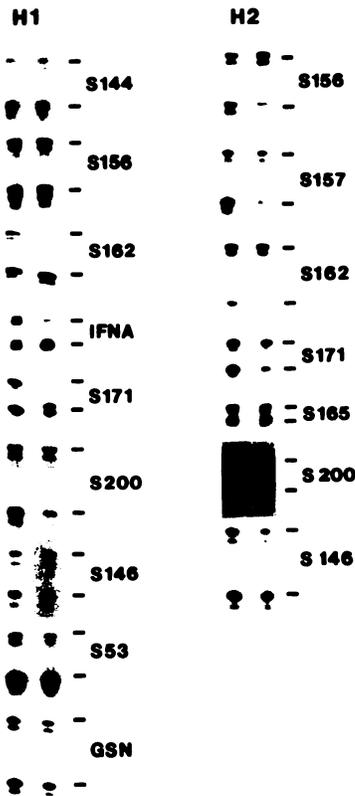(Further clinical data were not available for preinvasive lesions).

Fig. 1. Loss of heterozygosity analysis for two selected cases (H1, H2) of invasive HNSCC. Informative microsatellite markers were amplified from DNA derived from normal blood (*left lane*) or from tumor (*right lane*). Case H1 shows partial loss of 9p markers including *D9S200, D9S161, D9S171, IFNA*, and *D9S162*. Case H2 shows loss on 9p distal (telomeric) from *D9S165*. Relative position of markers on physical map is shown in Fig. 2.

(21 of 29) of invasive HNSCCs showed allelic loss of at least five markers on the p arm. Fourteen % (4 of 29) revealed allelic loss for all the markers analyzed on the chromosome. No partial allelic losses confined to the q arm were identified in these tumors.

Fine mapping is necessary for localization of putative tumor suppressor genes (2, 3). We further analyzed chromosome 9 with a total of 15 microsatellite markers. Several tumors showed partial LOH on 9p as illustrated in Fig. 1. Fig. 2 shows the distribution of allelic losses in 5 representative tumors of the 29 cases analyzed. The minimal area of allelic loss was confined to 9p21–22, defining a common region of loss between the markers *D9S156* and *D9S165*.

To determine the relative timing of chromosome 9p alterations in HNSCC progression, we analyzed allelic loss of chromosome 9p in preinvasive lesions. Four markers (*D9S144, D9S156, D9S162,* and *IFNA*) on chromosome 9p that spanned the common region of loss identified in invasive lesions were used. Our analysis revealed LOH in 12 of 17 (71%) informative preinvasive tumors (Fig. 3), essentially the same frequency as in invasive lesions. Despite the presence of inflammatory cells, preinvasive lesions could still be readily scored for allelic loss. There was no apparent difference in 9p loss between severe dysplasia (3 of 4) or carcinoma *in situ* (9 of 13).

Discussion

The progression of tumors from early noninvasive adenomatous lesions to invasive primary tumors has been well documented for colon cancer. Specific genetic changes have been correlated with histopathological lesions involved in neoplastic progression (1, 13). Although *p53* mutations have been found in a variety of tumors (14) including head and neck cancers (8), few specific genetic alterations have been described in HNSCC primary tumors. We discovered that loss of chromosome 9p appears to be involved in the majority (72%)
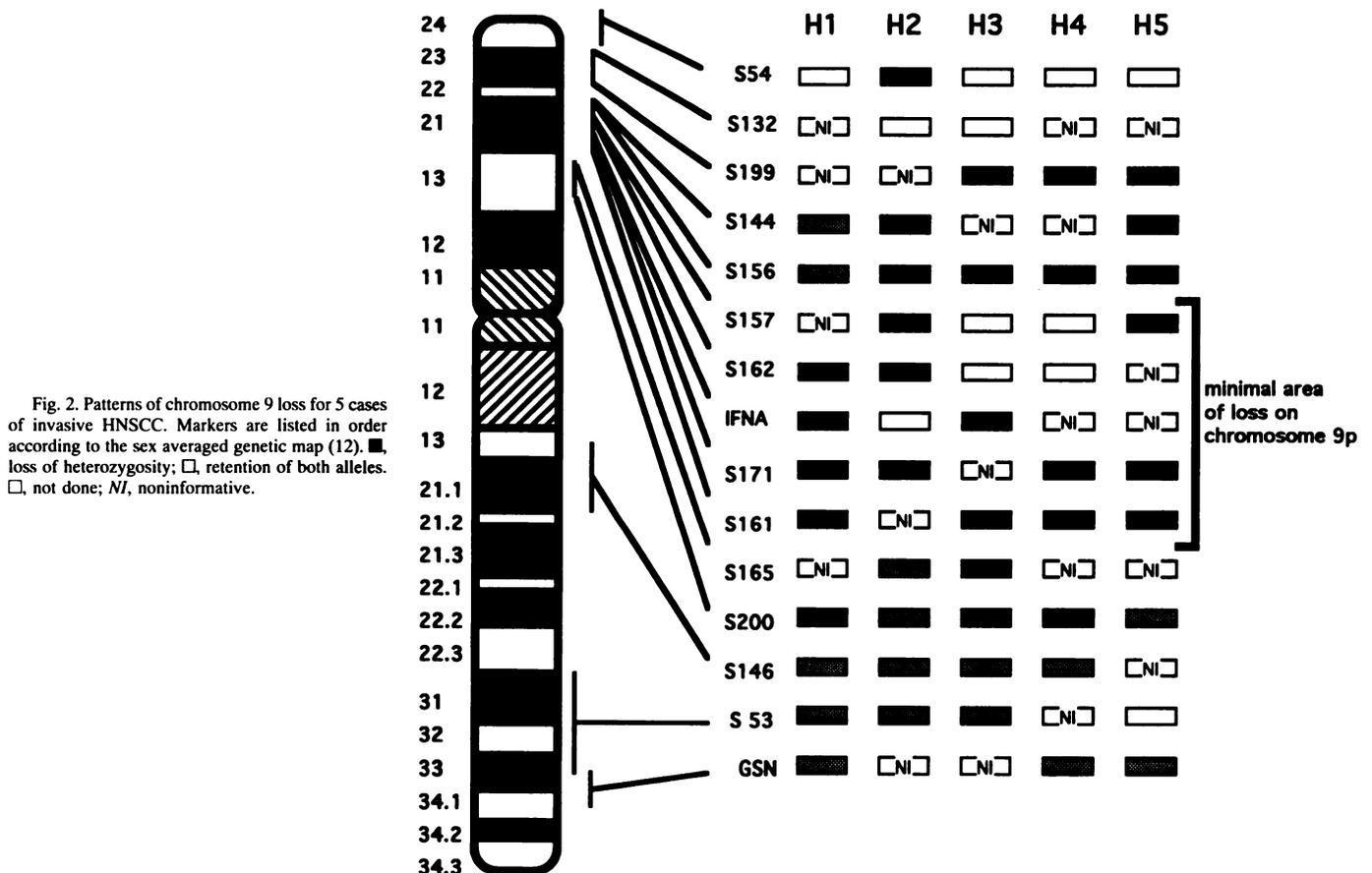

Fig. 2. Patterns of chromosome 9 loss for 5 cases of invasive HNSCC. Markers are listed in order according to the sex averaged genetic map (12). ■, loss of heterozygosity; □, retention of both alleles. ▨, not done; *NI*, noninformative.
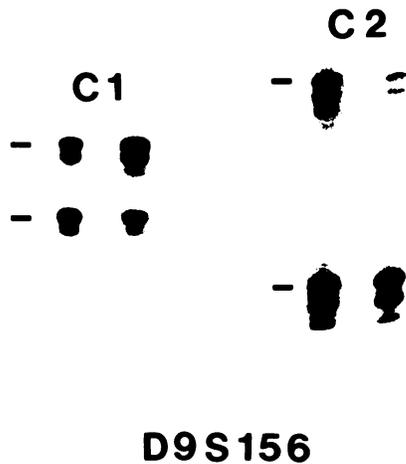
D9S156

Fig. 3. Loss of heterozygosity analysis for two selected preinvasive lesions (carcinoma in situ). C1 shows loss of the smaller allele and C2 loss of the upper allele for marker *D9S156* on chromosome 9p. Minimal residual signal is due to infiltration of the lesions with inflammatory cells.

of head and neck cancers. This frequency of loss is the highest reported in any chromosomal arm by either cytogenetic or molecular studies in head and neck cancer.

We found that preinvasive lesions, including severe dysplasia and carcinoma *in situ*, appear to lose chromosome 9p at a similar high frequency. This provides strong molecular evidence for early inactivation of a putative tumor suppressor gene on 9p leading to initiation or early progression of HNSCC. The high percentage of chromosome 9p allelic loss (71%) in these preinvasive lesions suggests that loss of this putative gene may be a critical early event in tumor formation. *p53* mutations occur in approximately 20% of preinvasive lesions and increase in frequency with invasion (8). This suggests that loss of chromosome 9p precedes loss of 17p and *p53* inactivation in HNSCC.

Recent studies have demonstrated 9p loss in a variety of neoplasms including leukemias, bladder cancer, gliomas, melanomas, and lung cancer (15–19). The minimal area of loss has been localized near the interferon cluster at 9p21–22 (20). HNSCC tumors appear to target a minimal region that also includes the interferon cluster. Although homozygous deletions of this region have been detected in other tumor types, there was insufficient DNA from our tumors to allow Southern blot analysis. Further mapping of primary HNSCC or cell lines should reveal if the same region is lost in all tumor types. Since many of these neoplasms are prevalent in the population, loss of a putative tumor suppressor gene at this locus may well rival the inactivation of *p53* as the most common genetic change in human tumors.

Furthermore, chromosome 9 loss has been shown to occur early in the progression of bladder cancer (21, 22) in concordance with our findings of 9p loss in preinvasive HNSCC. Although the relative timing of 9p loss in lung cancer has not been determined, HNSCC, bladder, and lung cancer all share certain epidemiological and clinical characteristics. These three cancer types are often linked to tobacco use and have a distinct propensity for multicentric presentation. Iden-

tification of this putative tumor suppressor gene on 9p should provide important clues to the initiation and progression of head and neck cancer and other tumor types.

References

1. Fearon, E. R., and Vogelstein, B. A genetic model for colorectal tumorigenesis. Cell, *61:* 759–767, 1990.
2. Stanbridge, E. J. Human tumor suppressor genes. Annu. Rev. Genet., *24:* 615–617, 1990.
3. Weinberg, R. A. Tumor suppressor genes. Science (Washington DC), *254:* 1138, 1991.
4. Jin, Y., Meltens, F., Mondahl, N., Heim, S., Olegard, C., Wennerberg J., Biorklund, A., and Mitelman, F. Chromosome abnormalities in eighty-three head and neck squamous cell carcinomas: influence of culture conditions on karyotipic pattern. Cancer Res., *53:* 2140–2146, 1993.
5. Ogden, G. K., Kiddie, R. A., Lunny, D. P., and Lane, D. P. Assesment of p53 protein expression in normal, benign and malignant mucosa. J. Pathol., *166:* 389–349, 1992.
6. Maestro, R., Dolcetti, R., Gasperatto, D., Doglioni, C., Pelvechi, S., Barzan, L., Grendi, E., and Boiocchi, M. High frequency of *p53* gene alterations associated with protein overexpression in human squamous cell carcinoma of the larynx. Oncogene, *7:* 1159–1166, 1992.
7. Somers, K., Merrick, M. A., Lopez, M. E., *et al.* Frequent *p53* mutations in head and neck cancer. Cancer Res., *52:* 5997–6000, 1992.
8. Boyle, J. O., Hakim, J., Koch, W., van der Riet, P., Hruban, R. H., Roa, R. A., Corio, R., Eby, Y. J., Ruppert, J. M., and Sidransky, D. The incidence of *p53* mutations increases with progression of head and neck cancer. Cancer Res., *53:* 4477–4480, 1993.
9. Nawroz, H., van der Riet, P., Hruban, R. H., Koch, W., and Sidransky, D. Allelotype of head and neck squamous cell carcinoma. Cancer Res., *54:* 1152–1155, 1994.
10. Van der Riet, P., Karp, D., Farmer, E., Wei, Q., Grossman, L., Tokino, K., Ruppert, J. M., and Sidransky, D. Progression of basal cell carcinoma through loss of chromosome 9q and inactivation of a single *p53* allele. Cancer Res., *54:* 25–27, 1994.
11. Litt, M., Hauge, X., and Sharma, V. Shadow bands seen when typing polymorphic dinucleotide repeats: some causes and cures. Biotechniques, *15:* 280–284, 1993.
12. Kwiatowski, D. J., Armour, J., Bale, A. E., Fountain, J. W., Goudie, D. R., Haines, J. L., Knowles, M. A., Pilz, A., Slaugenhaupt, S. A., and Povey, S. Report on the Second International Workshop on Human Chromosome 9. Cytogenet. Cell Genet., *64:* 93–121, 1993.
13. Baker, S. J., Preisinger, A. C., Jessup, J. M., Paraskeva, C., Markowitz, S., Willson, J. K. V., Hamilton, S., and Vogelstein, B. *p53* gene mutations occur in combination with 17p allelic deletion as late events in colorectal tumorigenesis. Cancer Res., *50:* 7717–7722, 1990.
14. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris C. C. *p53* mutations in human cancers. Science (Washington DC), *253:* 49–53, 1991.
15. Diaz, M. O., Rubin, C. M., Harden, A., Ziemin, S., R. A., Le Beau, M. M., and Rowley, J. D. Deletions of interferon genes in acute lymphoblastic leukemia. N. Engl. J. Med., *322:* 77–82, 1990.
16. Ruppert, J. M., Tokino, K., and Sidransky D. Evidence for two bladder cancer suppressor loci on human chromosome 9. Cancer Res., *53:* 5093–5095, 1993.
17. James, C. D., Collins, V. P., Allalunis-Turner, M. J., and Days, R. S. Localization of chromosome 9p homozygous deletions in glioma cell lines with markers constituting a continuous linkage map. Cancer Res., *53:* 3674–3676, 1993.
18. Fountain, J. W., Karayiorgou, M., Ernstoff, M. S., Kirkwood, J. M., Vlock, D. R., Titus-Ernstoff, L., Bouchard, B., Vijayasaradhi, S., Houghton, A. N., Lahti, J., Kidd, V. J., Housman, D. E., and Dracopoli, N. C. Homozygous deletions within human chromosome band 9p21 in melanoma. Proc. Natl. Acad. Sci. USA, *89:* 10557–10561, 1992.
19. Merlo, A., Gabrielson, E., Askin, F., Baylin, S., and Sidransky, D. Frequent loss of chromosome 9 in human primary non-small cell lung cancer. Cancer Res., *54:* 640–642, 1994.
20. Olopade, O. I., Bohlander, S. K., Pomykala, H., Maltepe, E., Van Melle, E., Le Beau, M. M., and Diaz, M. O. Mapping of the shortest region of overlap of deletions of the short arm of chromosome 9 associated with human neoplasia. Genomics, *14:* 437–443, 1992.
21. Sidransky, D., Frost, P., Von Eschenbach, A., Oyasu, R., Preisinger, A. C., and Vogelstein, B. Clonal origin of bladder cancer. N. Engl. J. Med., *326:* 737–740, 1992.
22. Cairns, P., Shaw, M. E., and Knowles, A. Preliminary mapping of the deleted region of chromosome 9 in bladder cancer. Cancer Res., *53:* 1230–1232, 1993.