Two Distinct Regions of Loss on Chromosome Arm 4q in Primary Head and Neck Squamous Cell Carcinoma

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Objective: To more clearly define the frequency and the regions of chromosome arm 4q loss in head and neck squamous cell carcinoma.

Design: A retrospective microsatellite analysis of DNA from previously microdissected primary tumor samples.

Setting: Academic medical center.

Patients and Methods: One hundred primary tumor samples from patients with head and neck squamous cell carcinoma were analyzed for loss of heterozygosity on the long arm of chromosome 4. The Kaplan-Meier method was used to estimate survival for 97 patients for whom clinical data were available. The Cox proportional hazards model was used to compare survival, and logistic regression was used to search for associations between clinical tumor characteristics and 4q status.

Results: Analysis of 33 polymorphic microsatellite markers identified 51 samples (51%) exhibiting loss of heterozygosity of 4q in at least 1 locus. Eighteen tumors revealed loss at all informative markers, indicating monosomy or complete deletion of 4q. Thirty-three tumors displayed partial loss of heterozygosity and delineated 2 minimal areas of loss at 4q2324 and 4q2829. Eleven tumors displayed loss solely at the 4q2324 region, 13 tumors displayed deletions confined to the 4q2829 region, and 9 tumors displayed selective loss at both regions. A separate analysis in a subset of 94 primary head and neck tumors was done to further delineate the minimal area of chromosomal loss at 4q2324. Analysis of 8 markers in this region allowed us to identify the smallest region of loss between markers D4S2986 and D4S1564 (a distance of 2 centimorgans). Review of the clinical records of 97 patients revealed no statistically significant association between 4q status and any clinical variable, including survival.

Conclusion: These results confirm a high frequency of chromosome arm 4q loss in primary head and neck squamous cell carcinoma and might demarcate 2 novel putative suppressor loci involved in progression of this carcinoma.

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HEAD AND neck squamous cell carcinomas (HNSCCs) compose 5% of the more than 1 million new cancers affecting Americans each year.1 However, little is known about the molecular events associated with oncogenesis. Elucidation of the precise nature of these changes will enhance our understanding of the biologic features of HNSCC and development of new diagnostic and therapeutic tools. An initial allelotype of primary HNSCC identified chromosome arm 4q as the fifth most common site of loss after 9p, 3p, 11q, and 14q.2 Recently, Califano et al3 developed a molecular progression model of HNSCC based on the temporal accumulation of specific genetic changes during histopathologic progression. In this model, losses on 4q are among the latest in HNSCC progression, rising in frequency only in the invasive stages of cancer. However, initial screening for allelic loss in a given tumor type must be followed by fine mapping of many tumors to identify an area of minimal loss. These small regions of chromosomal deletion serve to demarcate the borders of potential tumor suppressor gene loci.4,5

The objective of this study was to analyze partial losses of chromosome 4 to better define putative tumor suppressor loci. We analyzed 100 primary HNSCC samples for loss of heterozygosity (LOH) to construct a deletion map aimed at identification of putative tumor suppressor gene loci. Our results identify a high frequency of loss on chromosome arm 4q, a chromosome that has been implicated in other neoplasms, including colorectal, hepatocellular, bladder, ovarian, cervical, and Hodgkin disease. Moreover, we identi-
PATIENTS, MATERIALS, AND METHODS

PATIENT POPULATION

The study population comprised 100 patients with HNSCC. Clinical information was available from medical records for 97 patients. All clinical statistical analyses were performed on this subset. All patients had been treated with aggressive curative intent and had been followed up for at least 18 months at the time of data analysis.

SAMPLE COLLECTION AND DNA EXTRACTION

Primary HNSCC tumors were obtained fresh at the time of surgical resection with prior consent from patients at the Johns Hopkins Hospital, Baltimore, Md, as described previously. Samples were fresh frozen in liquid nitrogen or at 80°C and carefully microdissected on a cryostat to separate out nonneoplastic cells. Samples with greater than 70% tumor tissue were digested in sodium dodecyl sulfate/proteinase K at 60°C for 6 hours, followed by phenol-chloroform extraction and ethanol precipitation. Blood samples were obtained by venipuncture from these patients with prior consent, and DNA samples from lymphocytes were obtained as described previously.

POLYMERASE CHAIN REACTION AMPLIFICATION AND DATA ANALYSIS

Normal and tumor DNA samples were analyzed for LOH after polymerase chain reaction (PCR) amplification of polymorphic dinucleotide repeat sequences. Oligonucleotide primers for microsatellite PCR analysis were obtained from Research Genetics (Huntsville, Ala.). The primers were labeled with γ[32P]adenosine triphosphate using T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.). Fifty nanograms of genomic DNA was subjected to 30–35 cycles of PCR amplification, as described previously. Products from PCR analysis were separated by electrophoresis in denaturing 7% urea-polyacrylamide-formamide gels followed by autoradiography. For informative cases, allelic loss was scored and confirmed by 2 independent observers (S.I.S. and D.S.). Loss was scored if the intensity of 1 allele was at least 50% reduced in the tumor compared with normal DNA.

The medical records of 97 patients were reviewed to tabulate clinical variables, including age at diagnosis, sex, site and stage of disease, treatment, and clinical outcome. Follow-up for surviving patients ranged from 20 to 84 months (mean, 48 months). Disease-free survival and overall survival were calculated from the date of initial diagnosis. Stage was recorded as that at initial presentation, although some patients were entered into the study (biopsy specimen obtained) at the time of local recurrence after initial therapy. Survival was then calculated using the Kaplan-Meier method, censoring for death due to unrelated cause and loss to follow-up. Survival of patients with and without loss of any marker on 4q was compared using the Cox proportional hazards model. Associations between 4q status and patient age, tumor site, stage, sex, and cervical nodal status were sought using logistic regression.

We selected 33 well-spaced markers from informative microsatellite loci previously identified on chromosome arm 4q. We then amplified these microsatellite markers by PCR from primary HNSCC tumor and matched control DNA to screen for allelic loss. Fifty-one (51%) of the 100 tumors displayed loss of at least 1 marker on 4q. Eighteen of these samples demonstrated loss at all informative markers, indicating the probable presence of monosomy. The 33 remaining samples displayed only partial loss and helped define the 2 minimal regions of loss on 4q. We further analyzed the proximal region with 8 additional markers in a subset of 94 tumors to define the smallest region of loss.

Figure 1 denotes representative tumors defining the minimal areas of deletion on 4q. A small area of loss confined to 4q2324, between markers D4S2986 and D4S1564, is seen in tumor sample 5. Another small region of loss confined to 4q2829, between markers D4S247 and D4S2989, is seen in tumor sample 1072. A scarcity of markers in this region did not allow further mapping. These regions span physical distances of 2 and 11 centimorgans (cM), respectively. Figure 2 shows the pattern of chromosome arm 4q loss for 7 of 9 HNSCC tumors in which both regions were lost. These samples add evidence for the presence of 2 distinct regions of loss and potentially 2 novel tumor suppressor loci.

The Table provides data on the clinical characteristics of the patient population. The status of chromosome 4q was not found to correlate with any clinical variable, including patient sex, age at presentation, tumor stage, site, or the status of the cervical lymph nodes. Advanced age at presentation, the presence of cervical nodal metastases, and advanced tumor stage were each weakly associated with decreased overall survival. However, the status of chromosome arm 4q in the tumors had no impact on disease-free or overall survival. Chromosome arm 4q LOH conferred a hazard ratio of 1.3 for death by any cause, but \( P = .46 \) for the Cox proportional hazards survival analysis.

It has been established that cancer arises from a series of genetic changes. These alterations in DNA potentially lead to clonal outgrowth of cells, all of which will have a growth advantage initially provided by the parent cell. Discerning the timing and the nature of these alterations in HNSCC is crucial to biological and clinical comprehension of disease. Through analysis of the relative rate of molecular alterations in premalignant and invasive tu-
mors, it seems that deletion of chromosome arm 4q occurs late in HNSCC tumor progression.4

Initial allelotyping of HNSCC revealed LOH of 4q in many primary tumors tested at one microsatellite locus.3 We confirmed that 4q loss is a frequent genetic event in HNSCC, and our mapping with 33 dinucleotide markers reveals 2 minimal areas of loss on 4q. Although chromosome arm 4q loss has been described previously in other major tumor types, high-density mapping in primary HNSCC has allowed us to delineate 2 distinct loci at 4q23-24 and 4q28-29. These observations are consistent with the finding of more than 1 suppressor locus in many cases in which monosomy is present.12

Recently, investigators13 performed an allelotype of chromosome arm 4q, finding one large region of loss spanning 7 cM on 4q. Our study served to further extend their findings by defining a smaller minimal region of loss in the same region (2 cM) and identifying another distinct area of loss on 4q. One of the 2 minimal regions defined on chromosome arm 4q in our study (4q23-24) has also been implicated in the progression of Hodgkin disease and cervical cancer.15,16 Polascik et al16 recently defined loss at 2 regions in chromosome 4, one at 4p and another at 4q22-23, in a large number of primary bladder cancers. It is of particular interest that chromosome arm 4q is now implicated in 2 types of squamous cell carcinoma (cervical and head and neck cancer). Our findings in head and neck and bladder cancers strengthen the possibility that a putative tumor suppressor gene located at 4q22-23 plays a role in the progression of multiple forms of cancer.

It is now well established that tumor suppressor genes are a critical part of cancer progression. For example, it is known that TP53 and CDKN2A are 2 major tumor suppressor genes involved in HNSCC tumor progression.7,12 The study of these tumor suppressor genes has had a direct impact on patient treatment by augmenting diagnostic and prognostic tools.17 For patients with
HNSCC, clinical staging often fails to predict how an individual neoplasm will respond to therapy, and it cannot be relied on to elucidate the eventual outcome for a patient with HNSCC. Currently, these patients' treatment options include radiation therapy, surgery, and/or chemotherapy, and the choice is usually based on the site and stage of disease. However, gross staging cannot predict which patients will fare well with which treatment modality.

Molecular markers have shown promise toward optimizing patient therapy and predicting patient response. TP53 suppresses the outgrowth of genetically damaged cells in 2 primary ways: by apoptosis or cell cycle arrest to allow for DNA repair. When TP53 function is lost, damaged cells can proliferate rather than be terminated by antineoplastic therapies. Koch et al had hoped that patients with invasive HNSCC who had mutations of the TP53 gene had an increased risk of locoregional failure when treated with primary radiation given with curative intent. Because the tumor progression model places 4q LOH late in the process of malignant transformation, Koch et al had hoped that 4q status might be associated with clinical outcome. However, the results of the present study indicate no association of 4q status with more advanced clinical disease or decreased survival. These results might seem to be contradictory to earlier assertions of the position of 4q alteration in HNSCC tumor progression. However, for the purposes of the tumor progression model, all invasive carcinomas are regarded as advanced disease with a full spectrum of molecular changes. Molecular alterations that correlate with poor outcome or metastatic potential are actively being sought, but these results suggest that 4q status will not be a useful marker for aggressive disease.

Identification of the putative tumor suppressor genes that lie within these regions of minimal loss might allow us to develop better diagnostic and therapeutic targets for patients with HNSCC.

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