Visualization of the Timing of Gene Amplification during Multistep Head and Neck Tumorigenesis

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ABSTRACT

Head and neck tumorigenesis is thought to represent a multistep process whereby carcinogen exposure leads to genetic instability in the tissue and the accumulation of specific genetic events, which result in deregulation of proliferation, differentiation, and cell loss and the acquisition of invasive capacity. Chromosome 11q13 amplification is frequently observed in head and neck squamous cell carcinoma (HNSCC), and the amplified gene products are assumed to play important functional roles in the tumor phenotype. However, it is not well understood whether gene amplification precedes carcinoma development or results from the unstable nature of intact tumors. To determine the timing of gene amplification during tumorigenesis, tissue sections from amplified HNSCC specimens (containing a contiguous transition from normal epithelium to hyperplasia to dysplasia to carcinoma) were probed for INT2 gene copy number by chromosome in situ hybridization. In addition, representative epithelia were microdissected from the tissue sections, and the DNA was isolated and assessed for INT2 gene copy number by semiquantitative PCR. In those cases containing amplified INT2 in the carcinoma, gene amplification appeared to precede HNSCC development. In one case, INT2 gene amplification appeared in the hyperplasia to dysplasia transition, whereas in two other cases, gene amplification was apparent at dysplasia. These results suggest that gene amplification can occur early during head and neck tumorigenesis and that genetic instability is an important driving force in the tumorigenesis process.

INTRODUCTION

HNSCC has been hypothesized to represent a multistep evolutionary process in an anatomical field repeatedly exposed to carcinogenic insult. Induced genetic instability is then postulated to lead to the accumulation of specific genetic hits important for expression of the carcinoma phenotype (1–4). Reflective of this multistep process, histological sections from HNSCC resections often exhibit an apparently contiguous and continuous transition from normal to hyperplastic to dysplastic epithelium to carcinoma. Previously published studies using such informative tissue specimens demonstrated that this histological progression was marked by increasing genetic instability (5), increasingly deregulated proliferation (6), and increasingly abnormal expression of growth regulatory molecules, such as epidermal growth factor receptor (7) and p53 (8). Thus, this tissue model system provides a unique opportunity to map out genotype/phenotype interactions associated with head and neck tumorigenesis.

Over recent years, the specific genetic events important in the development of head and neck tumors have been increasingly elucidated at the chromosome (9–12) and molecular (13–15) levels. More recently, larger scale gains and losses in specific chromosome regions have been detected by comparative genomic hybridization (16–18). Several candidate genes associated with these genetic anomalies have now been identified (19–21), and in vitro studies and in vivo gene knock-out or knock-in studies are beginning to elucidate the in vivo consequences of gene alterations. Little is known about when these specific genetic events occur during multistep head and neck tumorigenesis, their specific pathobiological downstream consequences, or the in vivo interactions between different genetic events. However, recent studies have suggested that some molecular events can be detected as clonal outgrowths in premalignant epithelium (22–25).

Amplification of the chromosome 11q13 region is a frequently described event in HNSCC, detected in a reported 30–50% of cases (26). Several genes that have potential functional importance for head and neck tumorigenesis are frequently coamplified from this region, including INT2/FGF3 (a member of the fibroblast growth factor family), cyclin D1 (an important factor in the activation of cyclin-dependent kinases regulating cell cycle transit), EMS1 (a human homologue of chicken c-myc, an actin-binding protein putatively involved in cellular adhesion), FGF4 (another member of the fibroblast growth factor family), vascular endothelial growth factor β, PPP1CA, and glutathione S-transferase θ (a protein important in detoxification of carcinogens; Ref. 27). Although gene amplification is frequently reported in many epithelial tumors, it has generally been thought to be a very late event in tumorigenesis for several reasons: (a) random evaluations of premalignant lesions have not demonstrated gene amplification (28); and (b) permissiveness for gene amplification has been shown to require the prior inactivation of other cellular checkpoint mechanisms (e.g., p53 mutation) and/or the overexpression of factors that overcome checkpoint controls following cellular insult (29, 30). To determine the timing of gene amplification and its functional consequences during head and neck tumorigenesis, tissue sections of head and neck tumors and their contiguous premalignant lesions were assessed for INT2 gene copy number by chromosome in situ hybridization and semiquantitative PCR of dissected epithelium. The results suggest that gene amplification can be an early genetic event during the multistep evolution of HNSCC.

MATERIALS AND METHODS

Cell Lines. HNSCC cell lines 886, 1386, 1986, and 1186, kindly provided by Dr. Peter Sacks (Memorial-Sloan Kettering Cancer Center), and normal human fibroblast cell line1529 (American Type Culture Collection, Rockville, MD) were cultivated in Hsu’s modified McCoy’s 5A medium (Life Technologies, Inc., Grand Island, NY) with 15 or 20% FCS (Flow, Costa Mesa, CA), respectively. Cell pellets of trypsinized populations were fixed in 10% buffered formalin and embedded in paraffin.

DNA Probes and Probe Labeling. The cosmid probe for human INT2, a 40-kb genomic DNA fragment in a pCOS2 EMBl vector, was isolated by alkaline lysis, biotin-labeled by nick translation using the BioNick Labeling System (Life Technologies, Inc., Gaithersburg, MD), and purified using G50 DNA purification spin columns (Worthington Biochemical Co., Freehold, NJ). The digoxigenin-labeled total chromosome 11 painting probe was obtained from Oncor, Inc. (Gaithersburg, MD).
FISH. Metaphase spreads of cultured cells were treated with 100 μl of 1 mg/ml RNase in 2× SSC at 37°C for 60 min, washed twice in 2× SSC, dehydrated through an ethanol series, and air dried. After warming the slides at 37°C, they were denatured for 2 min at 72°C in 70% formamide/2× SSC and then dehydrated through an ethanol series. The biotinylated INT2 probe was mixed with cot-1 DNA (Life Technologies, Inc.) at a ratio of 1:30, dried by speed vacuum, and dissolved in hybridization solution (50% formamide/2× SSC/10% dextran sulfate/1 mg/ml salmon sperm DNA). After denaturing the probe solution at 70°C for 5 min, hybridization was carried out at 37°C for 16 h in a humidified chamber. Posthybridization washing included 65% formamide/2× SSC (pH 7.0) at 43°C for 20 min, followed by two 15-min washes at 37°C in 2× SSC. The digoxigenin-labeled total chromosome 11 painting probe was denatured at 70°C for 10 min, reannealed at 37°C for 2.5 h, and hybridized to the INT2-labeled slides for 20 h at 37°C. The posthybridization wash consisted of 50% formamide/2× SSC (pH 7.0) at 43°C for 15 min and 0.1× SSC at 60°C for 15 min. The slides were then transferred to 1× PBD (0.2% Tween 20 in 1× PBS). The slides were double labeled with fluorescein-labeled avidin and rhodamine-labeled anti-digoxigenin (Oncor, Inc.,) per the manufacturer’s recommendations and counterstained with 4',6-diamidino-2-phenylindole/antifade solution (Oncor, Inc.).

Tissue sections from paraffin blocks were processed as described previously (6), with the following exceptions: the slides were denatured in 70% formamide/2× SSC (pH 7.0) at 85°C for 12 min and dehydrated through an ethanol series, and the posthybridization wash conditions were 65% formamide/2× SSC (pH 7.0) at 42°C for 10 min twice, followed by two washes in 0.1× SSC at 37°C. The slides were counterstained with 20 μl of propidium iodide/antifade (Oncor, Inc.,). 

Semiquantitative PCR. Ten-μm tissue sections were stained with H&E, rehydrated, and air dried. After washing in 1× TE buffer (100 mM Tris-HCl, 3 mM sodium acetate, and 0.06–0.1 mM EDTA, pH 7.5), the slides were washed again in distilled water and air dried. The relevant epithelial regions were located under ×100 and ×400, lightly wetted, and microdissected with a 25–27 gauge needle tip. The dissected tissue was transferred to the bottom of an Eppendorf tube, mixed with 20 μl of GeneReleaser (Bioventure, Inc., Murfreesboro, TN), vortexed, and incubated with 2× GeneReleaser buffer (Bioventure, Inc., Murfreesboro, TN), vortexed, and incubated at 65°C for 10 min. The PCR mixture contained 100 μl of GeneReleaser buffer, 50 μM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 200 μM deoxynucleotide triphosphates, 0.06–0.1 μM of IFN-γ oligonucleotide primers, 0.5–0.6 μM of INT2 primers, 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer Corp., Norwalk, CT), 2.5 μCi [α-32P]dCTP (3000 Ci/mmol; ICN) in 50 μl of total volume. The primer and deoxynucleotide triphosphate concentrations were chosen from preliminary studies designed to ensure that the PCR reaction was not saturated and was stoichiometric for both the amplified and unamplified PCR products, 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer Corp., Norwalk, CT), 2.5 μCi [α-32P]dCTP (3000 Ci/mmol; ICN) in 50 μl of total volume. The primer and deoxynucleotide triphosphate concentrations were chosen from preliminary studies designed to ensure that the PCR reaction was not saturated and was stoichiometric for both the amplified and unamplified situations. The following primers were used for INT2 (9801–9945; 5′ end, TGCGTCCTGTGTGCGAAGAG; 3′ end, CTACAAAGCATCGACAGTAGA) and IFN-γ (5′ end, AGTGAAGCTGTAAGCTGGT; 3′ end, CTGGGATGCTCTTCGACT), yielding 145- and 85-bp products, respectively. The PCR reaction protocol included cycle 1, 94°C for 30 s; cycles 2–36, 94°C for 30 s, 57°C for 30 s, 72°C for 1 min; and cycle 37, 72°C for 7 min and held at 4°C. After PCR, 10 μl of each reaction were electrophoresed on a 12.5% polyacrylamide gel. The dried gel was subject to autoradiography for documentation and then analyzed using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The relative intensity of the INT2 target band to the IFN-γ reference band was measured after background subtraction.

RESULTS

Identification of HNSCC Cell Lines with INT2 Amplification. To identify suitable HNSCC resection specimens that were likely to carry INT2 gene amplification, several HNSCC cell lines (originally developed from tumors resected at M. D. Anderson Cancer Center by Peter Sacks [31]) were examined by Southern blot analyses using probes for INT2 and BCL1. Three HNSCC cell lines (886, 1386, and 1986) were identified that showed increased copy number for INT2 and BCL1. A fourth cell line (1186) was identified that did not show increased copy number in this region (Fig. 1). To characterize the nature of this increased copy number (e.g., double minutes versus homogeneous staining region), metaphase spreads of these four cell lines were subjected to FISH using an INT2 cosmide probe (labeled green with FITC) and a total chromosome 11 probe (labeled red with rhodamine). As shown in Fig. 2, the nonamplified cell line 1186 showed two normal chromosome 11s with a single INT2 signal on the q arm of each chromosome. In contrast, all three cell lines with increased INT2 gene copy numbers by Southern blot analysis demonstrated evidence for region-specific gene amplification. In the cases of cell lines 1386 and 1986, the amplified region was located telomeric to the residual single copy site on chromosome 11. In the case of cell line 886, the amplified regions were located on a chromosome other than chromosome 11, leaving behind the normal single copy of INT2 on the remnant chromosome 11q− fragment. The finding of red-labeled chromosome 11 sequences adjacent to one of the amplified sites support the possibility that a chromosome translocation preceded the amplification event. The finding of a single retained copy of INT2 on chromosome 11 in all cases is consistent with the bridge-breakage-fusion pathway for gene amplification (32).

FISH Analysis on HNSCC Resectons to Determine the Timing of Gene Amplification. Paraffin blocks of the HNSCC resection specimens corresponding to these four cell lines were identified and obtained from the M. D. Anderson Cancer Center Department of Pathology archives. Tissue sections were then stained with H&E and closely examined to identify regions exhibiting a contiguous and continuous histopathological transition from normal adjacent epithelium through hyperplastic and dysplastic morphology to invasive carcinoma (Fig. 3). To characterize the histological stage at which gene amplification occurred during head and neck tumor development in these cases, the adjacent tissue sections were subjected to FISH analysis using a labeled INT2 probe. FISH analysis of the tumor paraffin block corresponding to the nonamplified cell line 1186 showed no in situ evidence of INT2 amplification in the tumor region or in the adjacent premalignant regions (data not shown). In contrast, tissue sections from the HNSCC specimens associated with cell lines 886, 1386, and 1986 showed distinct in situ evidence for the presence of INT2 amplification in the carcinoma regions. Moreover, the HNSCC specimens associated with amplified cell lines 1386 and 1986 showed in situ evidence of increased INT2 copy number in the dysplastic region adjacent to the carcinoma, and that associated with cell line 886 demonstrated INT2 amplification at the hyperplastic to dysplastic transition (Fig. 4). The amplified signal was detected throughout the thickness of the premalignant epithelium, an intact basement membrane was apparent, and normal stromal cells and lymphocytes on the other side of the basement membrane exhibited...
single copy status. In addition, the amplified signal appeared region-
ally constrained within the nuclei from the first evidence of amplifi-
cation in the premalignant regions, and the size of the amplified signal
remained constant during subsequent histological evolution. Thus, in
these three head and neck tumors examined, the formation of double
minutes was not evident as an early event during in vivo gene
amplification, and the amplification event appeared in the tissue
sections as a quantum jump rather than as an evolving increase in gene
copy number.

Molecular Confirmation of INT2 Amplification in Premalig-
nant Regions by Semiquantitative PCR. Although the FISH studies
using an INT2 cosmid probe provided strong evidence that gene
amplification could occur during the hyperplastic or dysplastic phases
of head and neck tumorigenesis, there was concern that differences in
chromatin texture between normal, premalignant, and tumor tissues
might influence the hybridization efficiency to yield the observed
results. To independently confirm the presence of gene amplification
in premalignant sites, epithelial regions corresponding to the normal,
hyperplastic, dysplastic, and tumor tissue were separately microdis-
sected from adjacent tissue sections, being careful to exclude stromal
regions. Extracted DNA from each region was then subjected to
multiplex, semiquantitative PCR analysis using INT2 primers as the
target and IFN-γ primers as a single copy reference. Paraffin blocks
of cell pellets of normal human fibroblast 1509 cells and 886 head and
neck tumor cells served as single copy and amplification controls,
respectively. After PCR amplification, the reaction products were run
on agarose gels, and the ratios of the INT2 and IFN-γ bands were
quantified by PhosphorImager analysis.

As shown in Fig. 5a, the INT2:IFN-γ band intensity ratio was
nearly 5-fold higher in the PCR reaction product of DNA from the 886
cell line block (Lane 1) than that from normal 1509 fibroblasts (Lane
2; Table 1). DNA isolated from normal adjacent epithelium of the
HNSCC surgical resection block associated with cell line 886 showed
a comparable band ratio to that observed for the normal control 1509
cells (Lane 3). In contrast, the INT2:IFN-γ band intensity ratio was
~2.7-fold higher in DNA derived from epithelium microdissected
from the hyperplastic region (Lane 4) and increased to ~5-fold in
DNA from the dysplastic and carcinoma regions (Lanes 5 and 6;
Table 1). Interestingly, the relative intensity of the IFN-γ band ap-
peared to be reduced between the hyperplastic and dysplastic regions,
suggesting a loss of one IFN-γ allele during this transition. In the head
and neck tumor block associated with cell line 1386, the relative band
intensity of the INT2:IFN-γ PCR reaction products showed a dramatic
increase at the transition from hyperplasia to dysplasia and continued
into the carcinoma (Fig. 5b). A similar but less profound transition
was observed for the HNSCC specimen associated with cell line 1986
(Table 1). This smaller amplification signal observed for the tumor
specimen was concordant with that found in the associated cell line.
In contrast to that found for the amplified HNSCCs associated with cell lines 886, 1386, and 1986, DNA obtained from the HNSCC specimen associated with nonamplified cell line 1186 showed no evidence of INT2 amplification by this semiquantitative PCR analysis (Fig. 5c). Thus, these semiquantitative PCR results independently confirm the FISH-based observation that INT2 gene amplification can precede HNSCC development, in these cases at the hyperplastic or dysplastic stage.

DISCUSSION

The results of this study suggest that the INT2 gene amplification can occur early during the multistep evolution of HNSCC. It is unlikely that the observed amplification was a result of tumor infiltration into adjacent premalignant regions for three reasons: (a) there was no histological evidence of tumor cell infiltration into the adjacent premalignant regions; (b) once amplification was detected by in situ hybridization, the majority of the cells throughout the full thickness of the premalignant epithelium showed evidence of amplification; and (c) the semiquantitative PCR analysis used to independently confirm the amplification event is a bulk assay, representing the average genetic state of all of the cells being analyzed. The observation reported here seemingly contradicts a previous report where premalignant lesions were sampled randomly from the tumor field (28). The present study, however, in an attempt to characterize multistep evolutionary genetic events during tumorigenesis, directed particular attention toward identifying histological regions that exhibited a contiguous and continuous transition from normal through premalignant lesions to tumor. This provided an increased chance for detecting successions of genetic events during tumor development if they occurred. Moreover, the present findings are consistent with other reported observations that allelic imbalance can be detected in premalignant lesions in the field of head and neck tumors (19).

The head and neck tumor model is particularly useful for functional studies of multistep tumorigenesis, because once contiguous tissue regions are located, specific genetic events purported to be important for tumorigenesis can be spatially correlated with specific downstream physiological consequences. Thus, this model provides a unique in vivo testing ground for paradigms discovered in the in vitro setting. At the initiation of this study, the target gene in the chromosome 11q13 region was postulated to be INT2. Although INT2 amplification has been observed in a number of human tumors, and although INT2 transfection into mammary cells can increase angiogenesis (33), the role of the INT2 gene itself in head and neck cancer has been questioned because of the low degree of INT2 gene expression found in tumor specimens (34). Subsequently, several other genes of potential functional importance for tumor development have been reported to be frequently coamplified with INT2, including cyclin D1, EMS, FGF4, SEA, vascular endothelial growth factor β, phosphatase 1α, and glutathione S-transferase π (27). For this reason, increased attention has been focused on the role of cyclin D1 and...
EMS expression in head and neck tumors. For example, high cyclin D1 expression has been found in head and neck tumors, where the cyclin D1 gene is amplified and has been shown to be associated with poor prognosis in laryngeal tumors (35).

The findings reported here are relevant to understanding the mechanisms of gene amplification in vivo. A number of models for gene amplification have been proposed based on studies carried out in in vitro systems, where it is difficult to follow the sequential set of events in an individual clone of cells. In one mechanistic model, gene amplification is proposed to be associated with the accumulation of double minutes, thought to be the product of dysregulated replication of genomic regions and preferential selection of cells containing the extra genomic copies. In another mechanistic model, gene amplification is proposed to evolve through repetitive chromosome bridge-breakage-fusion reactions or by a bridge-breakage-fusion mechanism, followed by an unknown amplification event(s) near the disrupted region of the genome (32). With regard to the first model, there was no evidence for the presence of minute chromosomes in the amplified head and neck tissue regions reported here because the in situ hybridization products within nuclei were always focal rather than diffuse.

Three findings reported here are of interest for the bridge-breakage-fusion model: (a) in all cases, the cell lines derived from the tumors appeared to retain the normal INT2 copy on chromosome 11q13; (b) the amplification event appeared either more telomeric on chromosome 11 than the INT2 chromosomal location or was translocated onto another chromosome. Double labeling studies on tissue sections using chromosome 11 centromere probes in combination with INT2, cyclin D1, and EMS1 probes suggest that the same held true in the original tumor (36); (c) the amplification reaction appeared to be a quantum event rather than a sequential set of events in vivo, because the degree of amplification remained constant once amplification was detected. However, this last observation must be taken with caution because the PCR assay was only semiquantitative, and gene amplification was characterized at only one point in actual time (at tumor resection). Thus, it is possible that cells with intermediate gene amplification events did not expand within the premalignant epithelium and that the cells with increased amplification were preferentially selected for clonal outgrowth in vivo.

Gene amplification has been traditionally presumed to be a late event in tumorigenesis, perhaps associated with intrinsic genetic instability in tumors (37). However, there is increasing evidence that genetic instability is an important driving force for the tumorigenesis process (38–40). The present report provides evidence that another type of genetic instability event, gene amplification, can also occur early in the tumorigenesis process. Previous in vitro studies have suggested that altered p53 gene function (29, 30), cyclin D1 gene overexpression (41), and/or disruption of the cyclin D1/cdk complex activity balance (42) makes cells unstable and permissive for gene amplification. Of interest, our previous studies indicated that dysregulated p53 expression can occur early in the multistep process (9) and

![Image](https://cancerres.aacrjournals.org)
is spatially associated with increased chromosome instability (43). Recent studies in this HNSCC model system indicate that cyclin D1 overexpression precedes cyclin D1 gene amplification and is spatially associated with several forms of genomic instability (44, 45). Thus, the HNSCC model system provides a unique opportunity to examine the spatial relationship between specific genetic alterations, changes in gene expression, and downstream phenotypic consequences associated with multistep tumorigenesis.

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