Delineating Genetic Pathways of Disease Progression in Head and Neck Squamous Cell Carcinoma

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Objective: To identify altered gene targets that characterize disease progression in squamous cell carcinoma (SCC) of the head and neck (HNSCC). Genetic alterations in HNSCC cell lines reflect the tumor in vivo and can serve as valuable tools to study the development and progression of HNSCC. Identification of key molecular events may be useful for more accurate distinction of prognostic groups for selection and targeting of therapy.

Design: Individual gene loci were analyzed for genetic alterations using a novel genomewide strategy.

Subjects: Head and neck squamous cell carcinoma primary (A) and recurrent or metastatic (B) cell lines UMSCC-11A/11B, UMSCC-17A/17B (previously karyotyped), and UMSCC-81A/81B are described.

Results: At the genome level, loss and gain of genetic loci concurred with tumor karyotypes. Several abnormal gene loci not apparent by cytogenetics were also identified. All except 11B indicated loss of CDKN2A (encodes p14 and p16), with concomitant loss of CDKN2B (encodes p15) in 11A, 17B, and 81A. All 6 cell lines showed gain of PIK3CA (encodes a PI3 kinase) located at 3q26.3.

Conclusions: We provide evidence for the role of 3 critical pathways in the development and progression of HNSCC. The CDKN2A/B genes encode various components of the Rb and p53 pathways, and the PIK3CA gene makes a catalytic subunit of the protein phosphatidylinositol 3-OH kinase (PI3K), which is known to be involved in the PI3K/ATK signaling pathways. Molecular events may ultimately serve to achieve genomic alterations that set off an interplay among key gene loci along discrete genetic pathways used by tumor cells in HNSCC.


SQUAMOUS CELL carcinoma (SCC) of the head and neck (HNSCC) is the sixth most common malignant disease worldwide. Despite advances in chemotherapy and radiation therapies, HNSCC carries a high mortality rate. The American Joint Committee on Cancer clinical tumor/node/metastasis (TNM) staging system is the most important prognosticator of survival at present. Nevertheless, problems with detection of occult metastases in the neck and the fact that tumors with clinically equivalent stages may behave biologically very differently, suggest that additional prognostic markers should be used to supplement TNM staging. Because of the advances of the Human Genome Project, there is presently a unique opportunity to identify new molecular biological markers for this purpose. In addition, study of gene alterations in cancer has led recently to the development of targeted therapies in head and neck cancer, for example, to the use of monoclonal antibodies against overexpressed EGFR (C225). Tumor repopulation after treatment, with the regrowth of clones resistant to apoptosis also represents an important challenge to effective long-term therapy for head and neck cancer.

This can be problematic owing to tumor heterogeneity and the fact that standard histological methods can only allow examination of limited cross sections of tumor and surrounding tissue. Identification and analysis of gene changes in cancer may additionally address each of these problems.

Genetic alterations in HNSCC cell lines reflect the tumor in vivo and can serve as valuable tools to study the development and progression of HNSCC. The HNSCC karyotype is typically very complex, but common features in SCC at one anatomic site are often very similar to SCC at other anatomic sites such as the esophagus, skin, and vulva, irrespective of the initiating changes (eg, tobacco and alcohol, pan, or human papillomavirus). These...
common changes strongly suggest that initiation, development, and progression of squamous epithelial neoplasia evoke common genetic pathways irrespective of anatomic site.

Chromosome aberrations have been landmarks to identify cancer genes in many tumor types; however, individual gene loci altered in tumors cannot be deduced solely from the type of chromosome rearrangement. We developed the multiplex ligation–dependent probe amplification (MLPA) assay (Figure 1), a novel genome–wide strategy to identify specific gene loci for amplification and loss. We interrogated 6 HNSCC cell lines against a selected panel of 96 unique gene loci implicated in cancer and distributed throughout the genome.12 Molecular fingerprints identified from our genomewide studies concur with chromosomal aberrations and provide a novel index to estimate the extent of genomic abnormality with disease progression.

We provide evidence for the role of 3 critical pathways in the development and progression of HNSCC. We present data that substantiates loss of CDKN2A/B, which encode various components of the Rb and p53 pathways,15 against a background of apparently cytogenetically normal chromosome 9p21 regions. We also show gain of PIK3CA, a gene located at 3q26.3 that has been shown to predict clinical outcome for early disease HNSCC tumors12 and functions as part of a lipid-signaling pathway involved in multiple cancer-related functions.15,16

METHODS

HNSCC CELL LINES

Tumor sample acquisition, tissue culture, and karyotype analysis methods have been detailed elsewhere.17,18 The UMSCC-11A and -11B cell lines were derived from tumor tissue obtained from the primary tumor site (hypopharynx) before and after chemotherapy, respectively. The SCC cell lines 17A (supraglottis) and 17B (neck soft tissue) and UMSCC-81A (left false vocal cord) and -81B (right false vocal cord) were derived from tumor tissue obtained simultaneously from primary (A) and metastatic (B) sites. The UMSCC-81A and -81B cell lines have not been cytogenetically characterized.

DNA EXTRACTION

From the 6 cell lines, DNA was extracted using the QIAamp Kit (Qiagen Inc, Chatsworth, Calif) at passages 83 and 90 for 11A and 11B, respectively; 138 and 184 for 17A and 17B, respectively; and 24 and 129 for 81A and 81B, respectively.

THE MLPA TECHNIQUE

The MLPA assay is a new method for relative quantification of approximately 40 different DNA sequences in a single reaction requiring only 20 ng of human DNA. The MLPA assay has been successfully used for the detection of deletions and duplications of complete exons in the human BRCA1, MSH2, and MLH1 genes, detection of trisomies such as Down syndrome, characterization of chromosomal aberrations for gains and losses of genes in cell lines and tumor samples, and relative quantification of messenger RNAs.12 Probes added to the samples are amplified and quantified instead of target nucleic acids. Amplification of probes by polymerase chain reaction depends on the presence of probe target sequences in the sample. Each probe consists of 2 oligonucleotides (1 synthetic and 1 M13 derived), each hybridizing to adjacent sites of the target sequence. Such hybridized probe oligonucleotides are ligated, permitting subsequent amplification (Figure 1). All ligated probes have identical end sequences, permitting simultaneous polymerase chain reaction amplification using only 1 primer pair. Each probe gives rise to an amplification product of unique size between 130 and 480 base pairs. Probe target sequences are small (50-70 nucleotides). The prerequisite of a ligation reaction provides the opportunity to discriminate single nucleotide differences. The amplified fragments are separated on a DNA sequencer.

One of the applications of the MLPA method is the detection of chromosomal aberrations in DNA samples from tumors. This high throughput approach detects aberrant loci at 112 human genome sites.12 These gene loci were selected because of their reported involvement in cancer and span all 23 chromosomes including the X and the Y. The quantitative nature of the results allows the detection of loss of a gene copy (loss of heterozygosity) without the need for informative heterozygous markers.

INTERPRETATION

Normal tissue from each cancer subject serves as an internal reference when available. For cell lines, where normal DNA is not available, control (normal) male and female DNA samples are run with each probe set. Quantification and loss or gain of gene loci is determined through a process of normalization. The latter addresses variations in the surface area of a peak (intensity) encountered due to fluctuations in the assay run such as amount of DNA, ploidy variations, and polymerase chain reaction conditions. Briefly, the peak area for each probe is expressed as a percentage of the total surface area of all peaks of the same sample in an assay run (Figure 2 [1C mix] and Figure 3 [2C mix]). Relative copy number for each probe is obtained as a ratio of the normalized value for each locus (peak) of the sample to that of the normal control. A difference is significant only if the ratio is less than 0.7 (loss) or higher than 1.3 (gain). Complete loss or 0 copies is indicated by absence of a peak for that particular locus (illustrated for the loss of the CDKN2A at 9p21 [Figure 2] and CDKN2B at 9p21 and SRY at Yp11.2 [Figure 3]).
A relative copy number of 2 is considered normal, 1 or 0 copies is considered loss, and 3 copies or more is considered gain. The cytogenetic interpretation for gains and losses has been previously described. Briefly, loss of a chromosome or segment was defined as presence of only 1 copy against a near diploid background or presence of 1 or 2 copies against a 3N (triploid) or 4N background (near tetraploid). Gain was defined as presence of at least 3 copies against a diploid background, presence of at least 5 copies against a near triploid background, and presence of at least 6 copies against a near tetraploid background.

RESULTS

HNSCC CELL LINES

Complete karyotypes for UMSCC-11A/B and -17A/B have been previously published. The UMSCC-11A cell line had a consensus modal number of 88 chromosomes (hypotetraploid), and UMSCC-11B had a consensus modal number of 51 chromosomes (hyperdiploid). The UMSCC-17A and -17B cell lines were both hyperdiploid with consensus modal numbers of 47-49 and 47, respectively.

Overall, at the genome level, loss and gain of genetic loci concurred with tumor karyotypes. Chromosome 3 aberrations in 11A, 17A, and 17B showed gain of 3q, which was supported by increased copy number (3-4 copies) of PIK3CA at 3q26.3. The PIK3CA gene, located at 3q26.3, indicated gain of copy number in all of the cell lines analyzed (Figure 4). Gain of this region of 3q is also internally validated by gain of MME and BCL6 genes at 3q27 (Table). Loss of the SRY gene at Yp11.2 in 11A and 11B is consistent with the cytogenetic observation of lack of the Y chromosome. Loss of SRY was also observed in 81A; however, 81B had 2 SRY copies (Figure 1). The UMSCC-17A and -17B cell lines were female derived.

The BCL2 locus interrogated in separate probe mixes indicated loss (1 copy) of this gene in 11B, concordant with 11B tumor karyotypes (Table). The UMSCC-17A and -17B cell lines had 2 copies of chromosome 18, with loss of the BCL2 locus, which was also lost in 81A and 81B (Table).

Cytogenetics and MLPA results were discordant for CDKN2A/B genes at 9p21 (Figure 4). Both loci were completely lost in 11A, 17B, and 81A. This loss indicates a homozygous deletion of the 9p21 region harboring the CDKN2A/B genes in a background of 3 copies of chromosome 9 in 11B and 2 copies in 17B. The UMSCC-17A and -81B cell lines retained both copies of CDKN2B, but lost 1 copy of CDKN2A (Table).

COMMENT

Aberrant cancer genes rarely function independently, and evidence points to a carefully choreographed interplay among gene products in the regulation of normal growth. As an example, to progress through the cell cycle, mammalian cells must overcome the quiescent state of G0 and
enter the active G1 cell cycle phase. Entry into cell cycle phases is prudently orchestrated throughout. The G1/S cell cycle checkpoint is regulated mainly by the cyclin-dependent kinases (CDK4 or CDK6) bound to D-type cyclins. A number of negative regulators modulate CDK activity. The CDKN2A and CDKN2B genes map to 9p21 and are in tandem, spanning a region of approximately 80 kb, with CDKN2B located 25 kb centromeric to CDKN2A (Figure 5). The CDKN2A locus at 9p21 controls both the Rb pathway that regulates G1/S-phase transition and the p53 pathway that induces growth arrest or apoptosis in response to either DNA damage or inappropriate mitogenic stimuli by generating 2 gene products. The p16 protein product functions upstream of Rb, and the p14 protein blocks MDM2 inhibition of p53 activity. Inactivation of the CDKN2A gene, which includes mutations, homozygous deletion, and promoter methylation, have been reported at varying frequencies. Whereas CDKN2A/p16 gene mutations selectively inactivate the Rb pathway, deletion of the CDKN2A locus impairs both the Rb and p53 pathways. Deletion of the CDKN2A locus also frequently affects the CDKN2B locus, which encodes p15, an important mediator of the antiproliferative effect of transforming growth factor beta. Somatic alterations in the CDKN2A gene occur in many cancer types and germ-line mutation carriers are predisposed to a high risk of pancreatic and breast cancers. Inactivation of p16 is the most common genetic alteration in HNSCC, making it an ideal target for gene replacement. Recombinant adenovirus capable of directing a high level of p16 protein expression (Ad5-p16) demonstrated a significant antitumor effect of Ad5-p16 against human HNSCC in vivo. We found complete loss (homozygous deletion) of the CDKN2A/B locus in 11A, 17B, and 81A, contrary to
cytogenetic evidence indicating an intact 9p21 segment. The UMSCC-17A and -81B cell lines had loss of 1 copy of CDKN2A, but retained both copies of CDKN2B. Loss and retention of the CDKN2A locus genes p14 and p16 have been corroborated in another study (unpublished data, T.E.C., 2002). Cytogenetic deletion of 9p21-pter in early HNSCC has been documented28 and suggests abrogation of this locus as an early event in HNSCC. Loss of CDKN2A, which results in concurrent disruption of the p16-Rb and p14-p53 pathways in several cancer types, is associated with poor prognosis, and the dual inactivation is also shown to have an obligate role in tumor suppression in animal models.29,30

The p53 protein, whose activity is modulated through its union to MDM2 (12q14.3-q15), also affects the G1 to S progression by increasing expression of another CDKI, p21WAF1/Cip1, which is able to inhibit any cyclin-CDK complexes, including D-type cyclin-CDK4/6 complexes.20 Molecular alterations in any of these genes affect the normal G1 to S transition and play an important role in human tumorigenesis. In this sense, mutations affecting p53 are the most common genetic alterations yet identified in sporadic human tumors,31 followed by genetic alterations of the 9p21 genes demonstrated in a wide variety of neoplasms.21-23 All 6 cell lines had a normal copy number of the MDM2 gene locus (12q14.3-q15), interrogated independently in separate probe mixes (Table).

Mutation analysis of the p53 gene32 indicated mutant p53 in 11A and 11B (TGC $\rightarrow$ TCC, Cys $\rightarrow$ Ser in codon 242), and normal wild-type protein in 17A and 17B (p53 status not known for 81). In 11A, 3 copies of distal 17p, encompassing p53 and CRK (Table), therefore, indicate gain of copy number in the presence of only mutant p53 protein. Note that cytogenetic and MLPA copy numbers were concordant for 2 copies of p53 and CRK in 11B, 17A, 17B, 81A and 81B (Table). The patient designated UMSCC-11 (T2 N2a M0) with mutant p53 had a much poorer survival outcome (14 months) compared with the patient designated UMSCC-17 (T1 N0 M0), whose tumor had only wild-type p53 and who lived for over 135 months. The latter suggests that the presence of mutant p53 in addition to loss of p14 and p16 may produce even more potent growth stimuli. In an esophageal SCC cohort (OSCC), inactivation of either the CDKN2A or p53
gene in most low-grade tumors suggested the possible important and independent role of these genes in the initiation process of OSCC, whereas tumor progression probably involved acquisition of the loss of both genes.33

Loss of the **RB1** locus was not seen in any of the 6 cell lines. In 17B, there was gain of **RB1**, concordant with 3 intact copies of chromosome 13. In the absence of loss of heterozygosity data for the **RB1** locus, loss of 1 **RB1** allele indicative of a tumor suppressor effect cannot be ruled out.

The **PIK3CA** gene encodes a catalytic subunit of the protein phosphatidylinositol 3-OH kinase (PI3K), which is known to be involved in the PI3K/ATK signaling pathway that plays a role in multiple cancer-related functions, such as cell survival, proliferation, cell migration, vesicle trafficking, and vesicle budding.34,35 In cervical SCC cell lines as in ovarian cancer cell lines, even low level increased **PIK3CA** copy number (ie, 3 copies) results in higher PI3K activity.36,37 Gain of 3 to 4 copies of **PIK3CA** was noted in all 6 cell lines. Gain of this locus was validated by gain in chromosome 3 copy number in 11B, 17A, and 17B.

Recently, Redon et al14 combined comparative genomic hybridization and fluorescent in situ hybridization coupled with quantitative polymerase chain reaction to show that **PIK3CA** (3q26.3) rather than **p63** (3q28) is a likely target of 3p26-qter amplification in low-grade HNSCC.14 With treatment with PI3K already in place for cervical and ovarian cancer,36,37 it would be very useful to determine how early in the tumorigenesis process amplification of **PIK3CA** occurs. Based on data from our 6 cell lines, **PIK3CA** gain was observed in all primary (A) cell lines, suggesting that gain of this gene locus is an early event.

Loss of 18q21 is one of the consistent chromosomal regions lost in HNSCC.8 In HNSCC, loss of heterozygosity on 18q occurs frequently and has been linked to poor survival,38,39 suggesting that 1 or more genes on this chromosome are important in tumor behavior. In our study, interrogation of the **BCL2** gene at 18q21.2 showed loss of 1 copy in all cell lines except 11A, indicating that loss of **BCL2** at 18q21.2 may be affected by 18q loss of heterozygosity. **BCL2** encodes a mitochondrial membrane protein that blocks apoptosis.

Loss of the Y chromosome in HNSCC is not an uncommon event. In a study of 29 cases of HNSCC, the Y chromosome was lost from 10 of 19 tumors from male patients, and an Y rearrangement was observed in 4 others.8 Among female patients, loss of the short arm of the inactivated X was frequent in both HNSCC and in SCC of the female genital tract. The short arm of the X and the Y chromosome shares many genes, and loss of 1 or more of these might be advantageous to the tumor cells, but both also contain late replicating regions, the loss of which have implications for faster cell cycle rates.8 In prostate cancer, significant loss of Y chromosome–specific genes was reported, suggesting their role in pathogenesis of this disease. The loss of **SRY** and **BPY2** genes was more frequent in higher stages and grades of prostate cancer.40,41

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**Figure 5.** Genomic organization of the **CDKN2A** and **CDKN2B** genes on 9p21 (adapted from Smeds et al33). The p16 and p14 proteins encoded by **CDKN2A** are transcribed from separate promoters located at the 5′ side of unique exons 1α and 1β. The p15 protein is encoded by **CDKN2B**. The p16 and p15 proteins inhibit the cyclin D-CDK4/6 complex and prevent phosphorylation of Rb. The p14 protein inhibits MDM2-mediated degradation of p53. The latter functions in overlapping pathways and links Rb and p53. The stabilization of p53 mediated by p14 can lead to growth arrest via p21, apoptosis, and DNA repair.
Our genomewide search also indicated other changes not apparent by cytogenetics. Lack of concordance between the MLPA assay and cytogenetics, while uncommon, may be attributed in part to the ability of the MLPA assay to account for cytogenetically uncharacterized rearrangements such as marker chromosomes. For example 11A and 11B had loss of chromosome 7 accompanied by several markers chromosomes (Table) indicating that the assay can decipher and discriminate on the basis of DNA composition cytogenetically cryptic and unstable chromosomal rearrangements, especially submicroscopic deletions.

Historically, the molecular pathogenesis of cancer has been teased out 1 gene at a time. In the present study, adopting a comprehensive approach, we validated cytogenetic gains and losses uncovering specific gene loci as consistent molecular events in HNSCC.

We provide evidence for the role of 3 critical pathways in the development and progression of HNSCC. Losses of the CDKN2A/B genes at 9p21 encode various components of the Rb and p53 pathways. Gain of copy number of PIK3CA in all of the cell lines evaluated points to the involvement of the PI3K protein in lipid signaling pathways as key molecular events in both primary and metastatic disease progression. This information may be useful in molecular diagnosis, selection, and targeting of therapy in HNSCC.

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