# Short Communication

## Amplification of the 3q26.3 Locus Is Associated with Progression to Invasive Cancer and Is a Negative Prognostic Factor in Head and Neck Squamous Cell Carcinomas

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**Amplification of the 3q26-q27 has a high prevalence in squamous cell carcinomas of mucosal origin, including those originating in the head and neck region. To elucidate its role as a prognostic tool in head and neck squamous cell carcinoma, a yeast artificial chromosome (YAC) contig spanning the entire 3q26-27 region was constructed. The minimal region of amplification was refined within a 1- to 2-Mb genomic segment contained within three overlapping, nonchimeric YAC clones using sequential fluorescent *in situ* hybridization analysis. These YAC clones containing the apex of amplification were used to develop a two-color fluorescence *in situ* hybridization assay and applied to the detection of 3q copy numbers in interphase nuclei on archival tumor tissue from 29 cases of normal mucosa, 20 of premalignant mucosa, and 50 of invasive head and neck squamous cell carcinomas. The presence of 3q amplification increased from 3% in normal mucosa to 25% in premalignant mucosa and 56% in invasive cancers (*P* < 0.01). In invasive tumors, low-level 3q amplification (3 to 4 X copy number) was identified in 18 of 50 primary head and neck cancers and high-level amplification (>4 X copy number) in 10 of 50 cases. With a median follow-up of 82.5 months, an increas- ing proportion of recurrences (32%, 72%, and 90%; *P* = 0.003) and cancer-related deaths (14%, 44%, and 70%; *P* = 0.006) were seen in patients with normal 3q copy number, low-level amplification, and high-level amplification, respectively. The 3-year disease-free (69%, 56%, and 10%; *P* = 0.001) and cause-specific (94%, 83%, and 40%; *P* = 0.01) survivals also de- creased from normal copy number to low-level and high-level amplification. Only high-level amplifica- tion at 3q remained a significant prognostic variable on multivariate analysis including common prognos- tic predictors for both disease-free (relative risk, 5.1; 95% confidence interval = 1.9 to 13.9) and cause- specific survival (relative risk, 7.6; 95% confidence interval = 1.9 to 29.6). The findings suggest that the 3q copy number status is an important marker for tumor progression and prognostication in patients with head and neck squamous cell carcinoma.   (*Am J Pathol 2002, 161:365–371*)**

Amplification of genetic material can confer a more ag- gressive phenotype to affected cancers through diverse mechanisms that impart growth advantages. Identifica- tion of amplified chromosomal regions in solid tumors has been enhanced by the advent of comparative genomic hybridization (CGH).[1–3] With the application of CGH, am- plification involving the 3q locus has been identified in more than 15 different tumor types, with a prevalence as high as 92%.[2–6] Amplification at 3q has a strong predi- lection for squamous cell carcinomas of mucosal origin, with lung, cervix, esophageal, and head and neck can-

cers having this alteration in 37 to 92% of cases.[1–4,6] The specificity for squamous cell carcinomas can be appreciated from CGH analyses of lung cancer showing a significantly higher prevalence of 3q amplification in squamous cell carcinomas in contrast to adenocarcinomas.[7]

Several CGH studies have suggested that the presence of 3q amplification has biological and clinical significance.[2,5,8] The identification of 3q amplification in 25% of dysplasias, in contrast to 75% of invasive squamous cell carcinomas of the cervix, suggests that 3q amplification may be a marker for transition to cancer.[2,8] However, the inaccuracy of CGH analysis in precisely defining the amplified region and its limitations in assessing paraffin-embedded tissue have restricted attempts at direct correlation with tumor progression and outcome.

In this study a yeast artificial chromosome (YAC) contig spanning the 3q26-27 chromosomal region of amplification in head and neck tumors was constructed. Subsequent fluorescence *in situ* hybridization (FISH) analysis enabled the refinement of the amplification region within three overlapping YAC genomic clones of 1 to 2 Mb. These clones were used to develop a two-color FISH assay that detected copy number changes in interphase nuclei on archival tumor tissue from 29 cases of normal mucosa, 20 premalignant mucosa, and 50 invasive head and neck squamous cell carcinomas (HNSCCs). Moreover, high-level amplification of 3q26 was shown to be a significant parameter in both tumor progression and survival. The application of this assay in the detection of gain/amplification in head and neck tumors provides an important tool for prognostication.

## Materials and Methods

### Tissue Samples

Paraffin-embedded samples were randomly selected from available archival tissue resources at Memorial Sloan-Kettering Cancer Center. Invasive cancers were derived from previously untreated patients with HNSCC who underwent treatment with curative intent. Noncancerous samples were obtained in two categories: smokers without any cancer history and histologically noncancerous mucosal margins from patients undergoing resection for head and neck cancer. All samples were coded to maintain patient anonymity.

### Histological Evaluation

Paraffin-embedded tissue from each case was cut into 5- to 7-$\mu$m-thick sections and fixed onto glass slides. Representative sections were stained with hematoxylin and eosin (H&E) and categorized as normal mucosa ($n = 30$)-, premalignant mucosa (dysplasia/*in situ* carcinoma, $n = 30$)-, or invasive cancer ($n = 50$)-based microscopic analysis. Dysplastic mucosa was identified by the presence of cellular atypia, keratosis, and parakeratosis. Carcinoma *in situ* was defined as atypia of the entire thickness of the epithelium, loss of polarity, and frequent mitosis, but without penetration of the basement mem-

brane. Invasive carcinoma was defined by invasion of the basement membrane. The pathological differentiation of cases of invasive cancer was determined by review of the slides, with the poorest grade used to define differentiation in cases with mixed differentiation. For the purposes of this study, the premalignant group included cases showing either dysplasia or *in situ* carcinoma, as reliable segregation with sufficient nuclear numbers of these histopathological entities proved difficult after slide treatment for FISH. The presence and location of the target regions were mapped on the slide. Sequential sections were used for FISH analysis to allow identification of target regions.

### Generation of Physical Map

YAC clones were identified by screening computerized resources at The Whitehead Institute for Biomedical Research/MIT Center for Genome Research (*www-genome.wi.mit.edu*) and the San Antonio Genome Center (*http://apollo.utscsa.edu*) as described.[9] Individual YAC clones were purchased from Research Genetics (Huntsville, AL). YAC clones were mapped to the 3q region and tested for chimerism by FISH on metaphase spreads from normal lymphocytes. The relative position of the YAC clones was accomplished by sequence tagged site (STS) content polymerase chain reaction screening and sequential dual-color FISH analysis as described.[9] Pulsed-field gel electrophoresis was used to determine the size of the genomic insert according to manufacturer's protocol (Bio-Rad Laboratories, Richmond, CA).

### FISH

FISH was performed by investigators blinded to the clinical data as described previously.[10] To determine the amplification status, 400 individual nuclei were analyzed for each case based on localization in corresponding H&E-stained sections, as described above. The number of analyzed nuclei allows compensation for the issues related to tangential sectioning, as has been detailed elsewhere.[11] The presence of amplification was accepted if more than 10% of tumor nuclei displayed increased copy number relative to the chromosome 3 centromeric probe signals and/or tumor ploidy. In cases in which there was a discrepancy between the number of centromeric signals and ploidy, the ploidy number was used to estimate the relative copy number at 3q26.3. Cases with amplification detected were further subdivided into low-level amplification for 3 to 4 copy numbers and high-level amplification for greater than four copy numbers.

### Clinical Data

Study data were collected for each case by review of the hospital's and physician's office records by investigators blinded to the origin of the tissue specimens. The clinical and laboratory data were merged based on coding information to avoid bias and maintain patient anonymity.

Demographic data (age, gender), tumor data (location, TNM stage), treatment information, and pathological information (tumor grade, margin status, presence of lymph node metastasis, and extracapsular spread) were collected for all cases. Tumor stage was determined by the criteria established by the fifth edition of the American Joint Committee on Cancer staging system.[12] The staging information was confirmed by review of tumor maps, operative records, and physicians' notes.

Outcome of treatment was evaluated on the date of last patient contact by determining the presence or absence of cancer, and the vital status (alive, dead, or unknown). For patients who died, the cause of death was determined from review of the death certificate, physician's death summary, and/or tumor registry records. The date and location of the first recurrence was also ascertained. Disease-free survival was determined as the differences between time 0 (date of first treatment) and the date of first recurrence. Cause-specific survival was the difference between time 0 and the date of death because of cancer.

## Statistical Analysis

Descriptive statistics were used to summarize study data. Statistical significance was accepted at a two-tailed *P* value of less than or equal to 0.05. Nonparametric qualitative and quantitative comparisons were performed using Fisher's exact test and Kruskal-Wallis analysis of variance, respectively. The chi-square test was used for multigroup, qualitative comparisons. Endpoints of interest were time to first recurrence and time to death as a consequence of cancer. Survival data were censored for patients lost to follow-up, surviving to the end to the study, or dying of causes unrelated to cancer. Survival curves were generated using the Kaplan-Meier (product-limit) method to allow maximum use of censored observations. Survival comparisons were performed using the generalized Wilcoxon test, a distribution-free method for comparing product limit survival curves. Multivariate analysis was performed using the Cox proportional hazards model, to determine the relative risk between each independent variable and survival.

## Results

Our previous work demonstrated that amplification of the 3q26-27 chromosomal locus is a common and crucial event in head and neck tumors.[10] To further characterize the amplification region and assess its importance as a prognostic tool in HNSCC tumorigenesis, we constructed a YAC contig spanning ~38 cM within chromosomal bands 3q26-27. Seventy-three YAC clones were isolated using simple tandem repeat DNA polymorphisms and STSs genetically mapped to chromosomal bands 3q26-27 (Figure 1). To verify the exact cytogenetic location and to test for possible chimerism all 73 YACs were mapped to normal metaphase chromosomes by FISH. Sequential dual-color FISH was used to narrow the amplification boundaries in the cell line MDA886, which
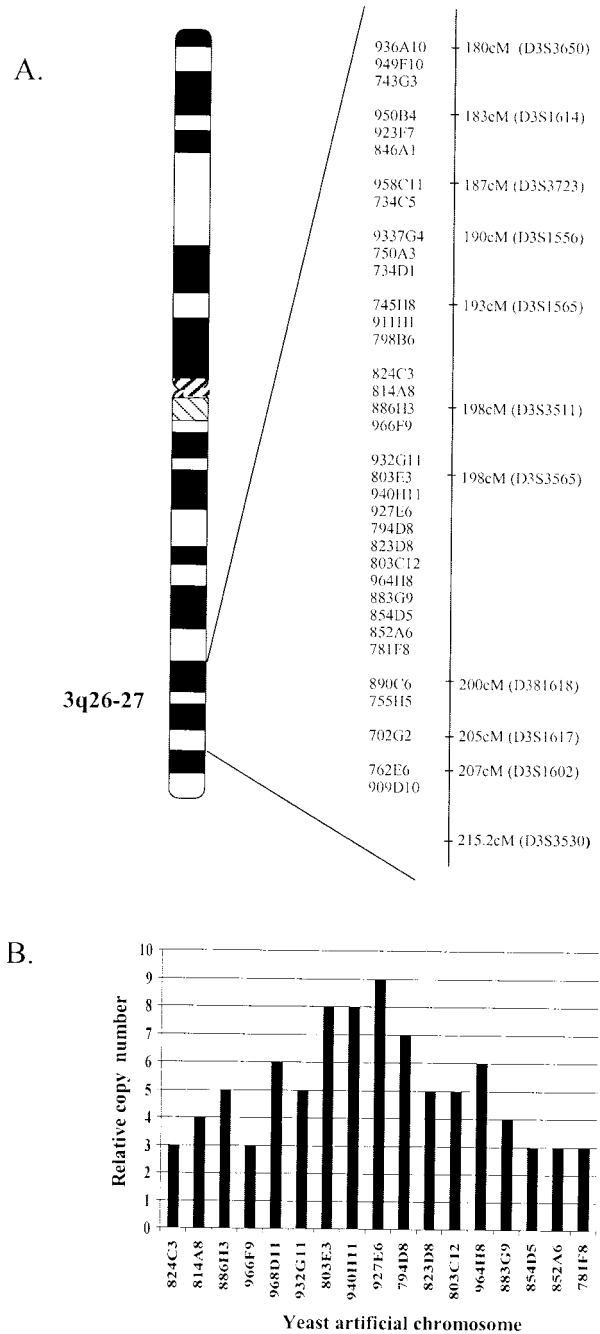


**Figure 1.** Analysis of 3q amplification by FISH. **A:** Yeast artificial chromosome (YAC) coverage of the 3q26-27 region. **B:** Relative copy numbers based on sequential two-color fluorescent analysis (using YAC clones) of MDA886, the cell line with the smallest minimal common region of amplification identified by CGH.

contains the minimal amplified region identified by CGH. This was performed using YAC clones at the most telomeric and centromeric boundaries and proceeding centrally. YAC clones overlapping or peripheral to those without amplification in MDA886 were excluded from the analysis. Based on analysis including 43 YAC clones, the apex of amplification was restricted to within three highly amplified, overlapping YAC clones (803E3, 940H11, 927E6) at 3q26.3, with copy numbers ranging from 8 to

10. The size of the genomic segment contained within these overlapping clones was determined by pulse-field gel electrophoresis analysis to span ca.1.5–2.0Mb.

FISH analysis using the combination of these three nonchimeric YAC clones on interphase nuclei and metaphase spreads from normal lymphocytes from healthy donors yielded two signals in both interphase nuclei and metaphase chromosomes. To validate the use of this probe in paraffin-embedded tissues, FISH analysis was performed in triplicate on touch preps and corresponding paraffin-embedded tissue from two cases of normal oral mucosa. This analysis consistently yielded two signals in 92.7% of analyzed nuclei with good correlation present between the touch preps and paraffin-embedded specimens. Next, the YAC probe was used for FISH analysis of three paraffin-embedded cases derived from invasive squamous cell cancer in which the 3q copy number status was known based on CGH analysis. Two of these cases contained normal 3q copy number and one had a gain of 3q. The copy number range of the combined analysis of the two cases with normal 3q copy number, performed in at least duplicate, was one (1.7% of nuclei), two (97.2% of nuclei), or three (1.4%) in analyzable nuclei. Less than 1% of cases displayed more than four signals. In the case with a gain of 3q by CGH analysis, three (34%) or four (37%) signals were seen in the majority of analyzable nuclei in replicate experiments. Two or fewer copies were seen in 28% and five or greater copies in less than 1% of analyzable nuclei. Although no differences in the number of signals detected was seen, the signal intensity was superior for the pooled YAC clone probe compared to individual YAC clones for the FISH analysis of paraffin-embedded tissues.

## 3q Copy Number Assessment by FISH Analysis

FISH analysis was successfully completed in 99 of the 110 samples (90%) included in the study. Technical problems and limitations in target cell numbers were the main causes for failure of FISH analysis occurring in one case of normal mucosa and 10 cases of premalignant mucosa. The main reason for failure of FISH analysis related to an inability to analyze the minimal number of nuclei required to validate the copy number changes, which was most pronounced in the premalignant group in which the number of representative nuclei was most restricted. Of the cases successfully analyzed, amplification at 3q was encountered in 1 of 29 cases (3%) of normal mucosa, 5 of 20 dysplasia/carcinoma *in situ* (25%), and 28 of 50 invasive carcinomas (56%; $P <$ 0.001) (Figure 2). Amplification at 3q26.3 exclusively occurred in tissues derived from the peritumoral milieu. Samples from the same patient for each differentiation category were available in 18 cases. Amplification at 3q was found in 1 of 18 (6%) cases of normal mucosa, 5 of 18 (26%) of dysplasia/*in situ* carcinoma, and 12 of 18 (67%) of invasive cancers ($P = 0.04$). Incidentally, local recurrences developed in four patients in this group, and all occurred in cases with 3q amplification identified in specimens derived from noncancerous mucosal margins ($P = 0.002$).
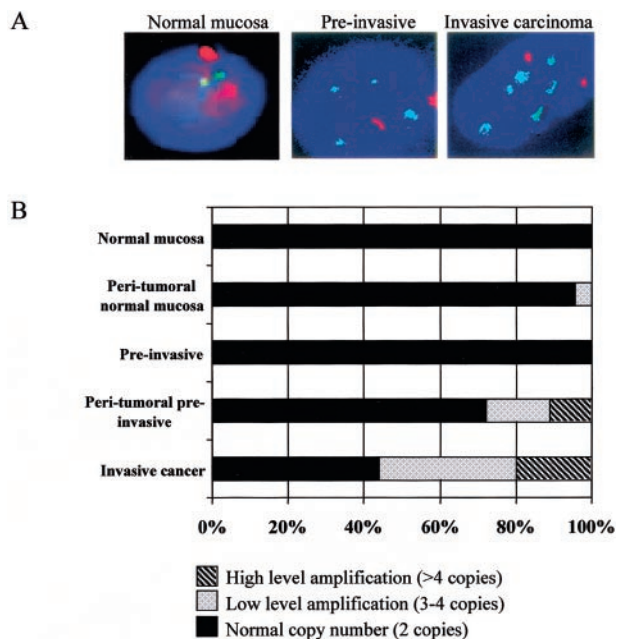


**Figure 2.** 3q copy number status determined by FISH for normal, premalignant, and invasive cancer tissue. **A:** 3q copy number status determined by FISH on normal (two copies from 3q26.3 and 3q centromeric probe), *in situ* carcinoma (three copies from 3q26.3 and two copies from 3q centromeric probe), and invasive squamous cell carcinoma (six copies from 3q26.3 and two copies from 3q centromeric probe). DAPI-stained interphase nuclei are shown. Red signals represent a 3q centromeric probe and green signals represent the pooled YAC probe. **B:** Results of FISH analysis of paraffin-embedded tissue. Overall, amplification at 3q26.3 was significantly more common in invasive cancers (56%), than preinvasive lesions (25%) or normal mucosa (4%; $P < 0.001$). Note that amplification at 3q26.3 was only detected in normal and preinvasive mucosal samples obtained for margins adjacent to the invasive cancer.

## Correlation with Clinical Behavior

The median age of patients with invasive cancer (64 years) and gender distribution (66% males) of the study cohort was representative of that seen in the general patient population with HNSCC. The majority of cases originated in the oral cavity (84%), with all but one of these being oral tongue lesions (Table 1). Overall, 27 patients (54%) had early stage (I or II) lesions, with the remaining cases being advanced stage (III or IV) cancers. The pathological differentiation of the tumors were well, moderate, and poor in 8%, 86%, and 6%, respectively. Treatment consisted of surgery alone in 64%, radiation therapy alone in 10%, and surgery with adjuvant radiation in 30% of cases. N1 or N2 nodal metastasis was present in 19 patients (38%), of which 8 (42%) had pathological evidence for extracapsular extension (Table 1).

In cases of invasive carcinoma, low-level (three to four copies) and high-level amplification (more than four) occurred in 18 and 10 patients, respectively. The median age, gender distribution, anatomical location, TNM stage, and treatment used did not vary by the 3q copy number status (Table 1). Similarly, the tumor differentiation, presence of extra-nodal spread, and primary treatment modality also did not vary by the 3q copy number status.

The median follow-up for the entire population was 82.5 months. The presence of locoregional recurrence increased with increasing 3q copy number, from 32% for

**Table 1.** Population, Treatment, and Outcome Characteristics Based on 3q Copy Number Status Assessed by Florescent *in Situ* Hybridization

| | 3q Copy number | | | |
| | Normal (2X) | Low-level amplification (3–4X copy number) | High-level amplification (>4X copy number) | P value |
|---|---|---|---|---|
| Sample size, n (%) | 22 (44%) | 18 (36%) | 10 (20%) | — |
| Male gender, n (%) | 59% | 67% | 80% | NS* |
| Median age | 61.5 years | 64 years | 70 years | NS† |
| TNM stage, n (%) | | | | NS‡ |
| I | 5 (23%) | 7 (39%) | 4 (40%) | |
| II | 6 (27%) | 3 (17%) | 2 (20%) | |
| III | 4 (18%) | 3 (17%) | 1 (10%) | |
| IV | 7 (32%) | 5 (28%) | 3 (30%) | |
| Tumor differentiation | | | | NS† |
| Well | 2 (9%) | 0 | 2 (20%) | |
| Moderate | 19 (86%) | 17 (94%) | 7 (70%) | |
| Poor | 1 (5%) | 1 (6%) | 1 (10%) | |
| Extra nodal spread, n (%) | 1 (17%) | 5 (63%) | 2 (40%) | NS* |
| Anatomic location, n (%) | | | | NS‡ |
| Oral cavity | 20 (91%) | 15 (83%) | 7 (70%) | |
| Larynx | 1 (4.5%) | 1 (6%) | 2 (20%) | |
| Oropharynx | 0 | 1 (6%) | 1 (20%) | |
| Hypopharynx | 1 (4.5%) | 1 (6%) | 0 | |
| Treatment, n (%) | | | | NS‡ |
| Surgery | 15 (68%) | 11 (61%) | 6 (60%) | |
| Surgery and radiation | 6 (27%) | 6 (33%) | 3 (30%) | |
| Radiation | 1 (5%) | 3 (17%) | 1 (10%) | |
| Median follow-up, months | 54 | 90 | 115 | NS† |
| Locoregional recurrence | 7 (32%) | 13 (72%) | 9 (90%) | 0.003* |
| Death due to cancer | 3 (14%) | 8 (44%) | 7 (70%) | 0.006* |
| 3-year disease-free survival | 69.3% | 55.6% | 10% | 0.006§ |
| 3-year cause-specific survival | 93.8% | 83.0% | 40.0% | 0.001§ |

*Fisher's exact test.
†Mann-Whitney *U*-test.
‡Chi-square test.
§Wilcoxon test for the comparison of Kaplan-Meier survival curves.

patients with normal copy number, to 72% for low-level amplification, and 90% for high-level amplification (*P* = 0.003). The number of patients dying of cancer was also correlated with the 3q copy number, increasing from 14 to 44%, to 70%, for normal copy number, low-level amplification, and high-level amplification, respectively (0.006).

The 3-year disease-free and cause-specific survivals negatively correlated with 3q copy number status (Figure 3; Table 1). Multivariate analysis including the 3q status, TNM stage, and type of treatment showed that only the presence of high-level amplification at 3q was a significant predictor of disease-free survival (chi-square = 10.15; df = 3; *P* = 0.02) with a relative risk of 5.1 [95% confidence interval (CI) = 1.87 to 13.89; *P* = 0.001] and low-level amplification had a relative risk of 2.2 (95% CI = 0.87 to 5.55; *P* = 0.1). Similarly, multivariate analysis revealed only high-level amplification to be a significant predictor of cause-specific survival (chi-square = 10.03; df = 3; *P* = 0.02) with a relative risk of 7.6 (95% CI = 1.94 to 29.62; *P* = 0.004), whereas low-level amplification had a relative risk of 3.0 (95% CI = 0.80 to 11.32; *P* = 0.1).

## Discussion

HNSCC is the fifth most common malignancy worldwide, representing a major international health problem. In the United States, more than 40,300 cases and 11,700

deaths occur annually as a result of HNSCC.[13] Although these tumors can arise from diverse anatomical locations including the oral cavity, oropharynx, hypopharynx, larynx, and nasopharynx, they have in common an etiological association with tobacco and/or alcohol exposure. HNSCC represents a continuum of disease that progresses from normal mucosa, dysplasia, and ultimately invasive carcinoma. Several genetic aberrations have been investigated as markers of disease progression and/or outcome, but the overall genetic basis of these tumors remains ill defined.[14]

The advent of CGH has significantly enhanced our ability to genetically screen solid tumors, including head and neck cancers. CGH analysis has identified gain of genetic information at 3q as the most common site of genetic overrepresentation in mucosal squamous cell carcinomas and has suggested it is a pivotal transition event in cancer pathogenesis.[2,5,8] However, given the limitations of CGH analysis, specifically the inability to assess individual cells, complementary analytic methods are needed to refine CGH findings. The value of FISH analysis is its ability to assess individual cells for copy number changes, allowing a more targeted approach. In this study we identified three YAC clones that contain the apex of the amplified region at 3q26.3 and can serve as probes for FISH analysis. We confirmed, optimized, and validated our YAC probe for assessing 3q copy number status in paraffin sections and showed significant corre-
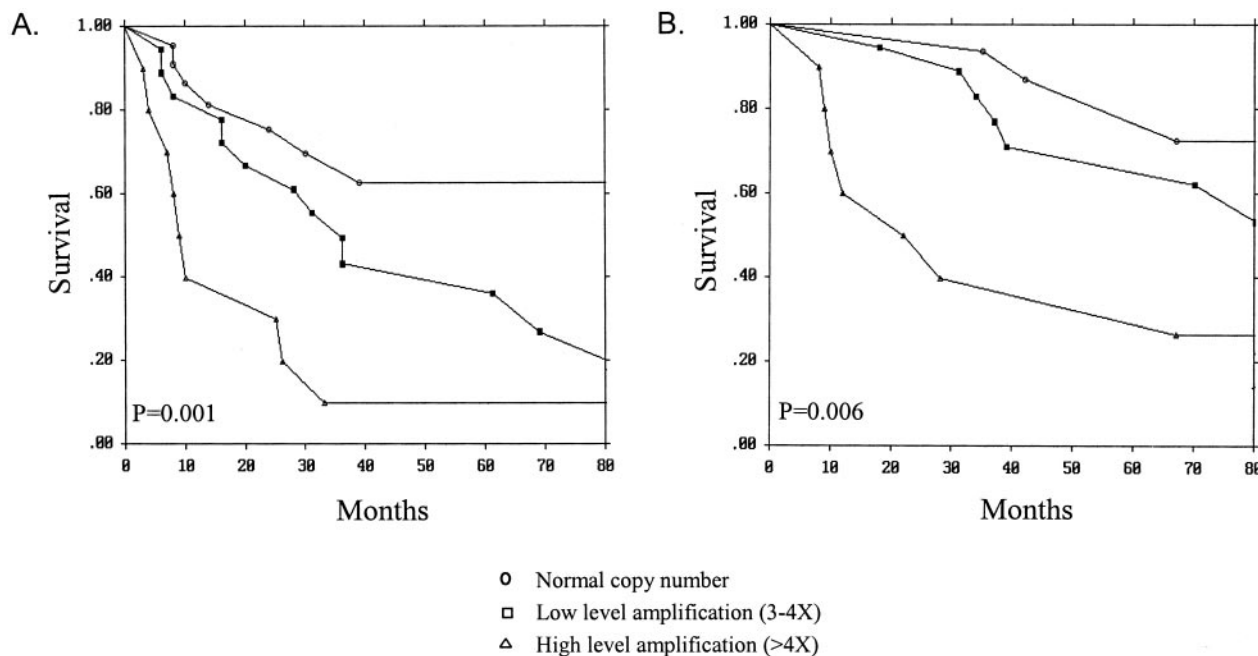
**Figure 3.** Survival outcomes based on 3q status. Curves were generated by the Kaplan-Meier method and compared using the log rank test. All *P* values are two-sided and considered statistically significant if less than or equal to 0.05. **A:** Probability of disease-free survival was significantly poorer in patients with 3q26.3 amplification identified by FISH than in those with normal copy number (*P* = 0.001). Multivariate analysis, including 3q copy number status, TNM stage, and treatment type, revealed that only high-level amplification (>4 X copy number) remained an independent predictor of disease-free survival with a relative risk of 5.1 (95% confidence interval, 1.9 to 13.9). **B:** Probability of cause-specific survival was significantly poorer for patients with 3q amplification (*P* = 0.006). Multivariate analysis, including 3q copy number status, TNM stage, and treatment type, revealed that only high-level amplification (>4 X copy number) remained an independent predictor of disease-free survival with a relative risk of 7.6 (95% confidence interval, 1.9 to 24.6).

lations between the presence of 3q amplification, tumor progression, and clinical outcome.

The detection of 3q amplification in 3% of normal mucosa, 25% of premalignant tissue, and 56% of invasive HNSCCs supports the contention of Heselmeyer and colleagues[2] that 3q amplification is a transition event in the progression to invasive squamous cell carcinoma. The incidence of 3q amplification in a subset of 18 matched cases of normal mucosa, premalignant tissue, and invasive carcinoma from the same patient were similar to those seen in the overall population. The significantly higher rate of local recurrence in five cases (80%) with 3q amplification in noncancerous tissues from surgical margins lends further support to the role of 3q amplification as a marker of cancer progression. The exact reason for the higher rate of local recurrence is difficult to define, but is similar to the findings of previous studies showing higher rates of local failure in cases with genetic aberrations detected in normal mucosal margins.[15] Amplification at 3q26.3 in the primary tumor is also associated with significantly higher rates of tumor recurrence and cancer-related death. In congruence, a negative correlation was observed between the presence of 3q amplification and disease-free and cause-specific survival. Only the presence of high-level 3q amplification remained a significant predictor of outcome when multivariate analysis was used to control for the treatment modality and the TNM stage at presentation. The risk for tumor recurrence was 5.1 times higher and death as a consequence of cancer 7.6 times higher in patients with high-level 3q amplification.

In summary, this study has validated the use of the pooled YAC clones as probes for FISH analysis. The presence of 3q amplification was shown to be an independent predictor of clinical tumor behavior and long-term outcome in patients with head and neck cancer. These data suggest that 3q amplification may represent an important biomarker for the assessment of patients with lesions in the upper aerodigestive tract.

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