Significance of p63 Amplification and Overexpression in Lung Cancer Development and Prognosis


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ABSTRACT

The fight against lung cancer is greatly compromised by the lack of effective early detection strategies. Genomic abnormalities and specifically the amplification of chromosomal region 3q26-3qter in lung cancer represent a major signature of neoplastic transformation. Here, we address the significance of p53 homologue p63 mapping to 3q27 in lung tumorigenesis. We analyzed p63 gene copy number (CN) by fluorescence in situ hybridization and expression by immunohistochemistry in tissue microarrays of 217 non-small cell lung cancers (NSCLCs) and correlated them with survival. We additionally characterized our findings in a subset of 24 NSCLCs by reverse transcription-PCR and Western blotting. We analyzed p63 CN and protein expression in 41 preinvasive squamous lesions. The p63 genomic sequence was amplified in 88% of squamous carcinomas, in 42% of large cell carcinomas, and in 11% of adenocarcinomas of the lung. The predominant splice variant of p63 expressed was ΔNp63α. Western analyses revealed ΔNp63α expression in normal bronchi and squamous carcinomas but not in normal lung or in adenocarcinomas. Furthermore, p63 genomic amplification and protein staining intensity associated with better survival. We found a significant increase in CN in preinvasive lesions graded severe dysplasia or higher. Our data demonstrate that there is early and frequent genomic amplification of p63 in the development of squamous carcinoma of the lung and that patients with NSCLC showing amplification and overexpression of p63 have prolonged survival. These observations suggest that p63 genomic amplification has an early role in lung tumorigenesis and deserves additional evaluation as a biomarker for lung cancer progression.

INTRODUCTION

The fight against lung cancer is greatly compromised by a lack of effective early detection strategies. Genomic abnormalities represent one major signature of neoplastic transformation and tumor progression (1). Using array comparative genomic hybridization (CGH), we recently identified genetic amplification on chromosome 3q26-3qter as the most common genomic abnormality in squamous carcinoma of the lung (2). The PIK3CA gene, also known as p110α, is responsible for phosphatidylinositol-3′-kinase activity, maps to this region (3q26). We have shown that increase in PIK3CA gene CN correlates with increased protein kinase B activity, implicating the phosphatidylinositol-3′-kinase pathway in lung tumorigenesis. This amplicon also contains a number of other important genes, including p63, hTERT, neutral endopeptidase, and somatostatin.

The p63 genomic sequence maps to the peak of the 3q amplicon (chromosome 3q27). p63 is a transcription factor that transactivates p53 target genes (3) and induces apoptosis when expressed in cells (4). Although p63 was recently discovered, it is the most ancient homologue that encodes six splice variants. These splice variants possessing a p53-like NH2-terminal transactivating domain are known as TA/p63 and have properties similar to p53 (3). In contrast, splice variants lacking the NH2-terminal transactivating domain, known as ΔNp63, are thought to be inactive and function as dominant negatives, promoting growth and survival by competing for p53 binding sites (3). Interest in p63 stems from this “two genes in one” concept with agonist and antagonist properties that may be involved in tumor development. The ΔNp63α splice variant is known to be the most commonly expressed isoform in squamous epithelia (3) and is often found in head and neck and lung tumors with amplification of p63 (6).

The function of p63-specific splice variants in normal and malignant epithelial cells is subject to active investigation. In contrast to p53, loss-of-function mutations of the p63 gene are rarely found in human tumors (7). Some evidence suggests that ΔNp63α may function as an oncogene. ΔNp63α has been shown to inhibit transcription when transiently transfected into a p53 luciferase reporter assay (3). Overexpression of ΔNp63α in Rat 1a cells increased tumor size in mice (6). ΔNp63α was found to cause accumulation and signaling of β-catenin, additionally supporting the oncogenic function of p63 (8). In addition, ΔNp63α overexpression in keratinocytes may have a dominant negative effect on the endogenous p53 transcriptional activity required for UV-B-induced apoptosis in vivo (9). Finally, ΔNp63α can function as a potent transcriptional repressor and dissociates from promoter binding sites of key growth inhibitory genes (p21 and 14-3-3σ) during normal human keratinocyte differentiation (10). For these reasons, p63 gene amplification and overexpression may have important implications in tumorigenesis. The prevalence of p63 amplification in NSCLCs is unknown. Moreover, whether amplification correlates with overexpression of p63 and whether either abnormality has any role as a biomarker for the early diagnosis or for the prognosis of lung cancer has not yet been determined.

This study addressed the following questions: (a) what is the prevalence of p63 amplification in NSCLC? (b) is amplification associated with p63 protein expression, and if so, which p63 splice variant is preferentially expressed? (c) at what stage of lung tumor development is p63 amplified? (d) what is the prognostic value of p63 CN or protein expression in NSCLCs? and (e) are there significant relationships between p63 CN, p63 expression, p53 expression, and proliferation marker Ki-67 expression profiles?

MATERIALS AND METHODS

Tissue Samples. Fresh tissues, including lung tumors and normal lung or bronchus, were obtained from surgical specimens through the Specialized Program of Research Excellence in Lung cancer at Vanderbilt University. Paraffin-embedded, formalin-fixed tissues (including preinvasive lesions) were obtained from the archives of the pathology department at Vanderbilt Universi-
ity and the Department of Veterans Affairs Medical Center in Nashville, Tennessee. Additional preinvasive lesions were selected from the archives of the pathology department at the University of California, San Francisco. The study was approved by the local Institutional Review Board for all institutions involved.

**NSCLC Tissue Microarrays.** Tissue microarrays of NSCLC were prepared from paraffin blocks and an equal number of normal lung tissues (11). There were 94 squamous carcinomas, 93 adenocarcinomas (including 16 bronchioalveolar carcinomas), and 30 large cell carcinomas represented on the tissue microarrays. Archived tissue blocks from 1989 to 2002 were retrieved from the files of Vanderbilt University and the Nashville Veterans Affairs Medical Center pathology departments. For all tissue blocks, H&E-stained sections were reviewed by our pathologist (A. L. G.). Areas to be punched for array production were carefully marked. All 217 tumors were punched in triplicate, normal samples only once. Cores 0.6 mm in diameter were taken from the selected area of each specimen and inserted into a recipient paraffin block. Five mm sections were cut from the arrays and mounted onto charged slides. Every fifteenth section was stained by H&E to confirm the presence of the histological feature of interest (tumor or normal histology).

**FISH.** BAC clones for specific genes were selected from different libraries: p63 (RP03B2743; FHIT (2175D15); and centromeric chromosome 3 (CEP3) probe was purchased from (Vysis, Inc., Downers Grove, IL). Dual color FISH was performed on interphase nuclei in tissue sections as described earlier (2). Briefly, 2 μg of BAC DNA were labeled by nick-translation with either digoxigenin-dUTP or Cy3-dUTP. Labeled probes were separated from the reaction using spin columns and denatured. Tissue sections were deparaffinized and treated with sodium thiocyanate for 10 min followed by pepsin (4 mg/ml) digestion in 0.2N HCl for 10 min at 37°C. Slides were then denatured in 70% formamide/2× SSC for 5 min at 72°C and incubated with a hybridization mixture consisting of 50% formamide, 2× SSC, Cot-1 DNA, and 100 μg of both digoxigenin-labeled and Cy3-labeled BAC DNA. After 2 nights of incubation at 37°C, the slides were washed and counterstained with antifade solution containing 4′,6-diamidino-2-phenylindole. Test and reference hybridization signals were scored in 50 nuclei for each tumor core under a 100× immersion objective. Nuclei in which the nuclear boundaries were broken were excluded from the analysis. For invasive tumors, the average number of spots counted in 50 nuclei was reported as CN for the test gene. To address the variability in FISH spot counting. Two observers were asked to count fluorescent spots in 50 nuclei for both test (red) and control genes (green) for 10 separate tumor biopsies represented on a tissue microarray (observers blinded to histological subtype). We found an excellent inter-observer correlation with r² of 0.64 (r = 0.8032, P < 0.0001).

**RT-PCR.** Total RNA was isolated from tissue specimens using the TRIzol protocol (Invitrogen, Carlsbad, CA). Reverse transcription reactions were carried out for 1 h at 42°C-44°C and contained 1 μg of total RNA, 250 ng of oligo(dT), 1× deoxynucleotide triphosphate mix, RNase inhibitor (Promega), 1× RT buffer, and 200 units of SuperScript II RT (Invitrogen) in a total volume of 20 μl. Amplification of TAp63 and ΔNp63 splice variants was performed in 50 μl of reaction mixture consisting of sense and antisense primers for TAp63, ΔNp63α, and ΔNp63γ; 2 μg of cDNA, 1× deoxynucleotide triphosphate mixture, 1× PCR buffer, 2.5 mM MgCl₂, and 2.5 units of AmpliTaq Gold DNA Polymerase (PerkinElmer, Wessley, MA). PCR conditions were: 3 min at 95°C; followed by 30 cycles of 94°C for 30 s; 54°C for 1 min; and 72°C for 1 min, with a final extension at 72°C for 5 min. The following primers were used: TAp63, 5′-ATGTTCCAGACAGCAGACAG-3′ (sense) and 5′-AGCCTATGTTGGGGCGC-3′ (antisense); ΔNp63α, 5′-CCTGGACTATTTCCAGGACC-3′ (sense) and 5′-TACCTCCCCCTCTTGG-3′ (antisense); and ΔNp63γ, 5′-TACAGCGACACAGCAAGCA (sense) and 5′-TATGGTGTACACTGAGG-3′ (antisense).

**Western Blotting.** Surgically resected specimens of squamous cell carcinomas and adenocarcinomas of the lung, as well as adjacent histologically normal tissue, were obtained from the Vanderbilt University Medical Center. Samples were snap-frozen and stored at −80°C until protein lysates were prepared. Specimens were homogenized and lysed in buffer containing 50 mM HEPES, 250 mM NaCl, 10% glycerol, 1 mM EDTA, 0.1% NP40, 50 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 1 tablet of protease inhibitors (Roche, Indianapolis, IN)/10 ml of buffer. Lysates were centrifuged at 14,000 K for 15 min at 4°C, and the supernatant was removed. Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA), and 100 μg of total protein from each sample were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane (Amer sham, Buckinghamshire, United Kingdom) at 100 V for 1 h at room temperature. Blocking was performed in 5% milk + 1X Tris-Buffered Saline/0.1% Tween-20 (TBST) overnight at 4°C. Primary antibody against p63 (4A4; Oncogene Research Products, San Diego, CA) was diluted 1:500 in PBS. Secondary antibody was horseradish peroxidase-conjugated mouse anti-human immunoglobulin (Promega, Madison, WI) diluted 1:2500 in PBS. Detection was done by enhanced chemiluminescence (Pierce, Rockford, IL). Equal loading was confirmed by Pon ceau Red staining and by probing the blot with antibody against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

**IHC.** IHC staining was performed by the avidin-biotin complex method using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) as described previously. Slides were deparaffinized in xylene and hydrated through a graded alcohol series before being placed in 3% H2O2-PBS blocking solution for 5 min to inhibit endogenous peroxidase activity. Slides were then digested with 0.01% trypsin in PBS at 37°C for 15 min, followed by rinsing with plain PBS for 5 min at room temperature. Antigen unmasking was done in 10 mM citrate buffer (pH 6.0) for 10 min, and slides were then allowed to cool at room temperature for 30 min. Blocking serum consisted of 50% of normal horse serum in 5 ml of PBS and was applied for 30 min at room temperature, followed by washing in PBS. Slides were incubated with either p63 4A4 primary antibody (Oncogene Research Products) diluted 1:500 in PBS, p53 DO-7 antibody diluted 1:100 (Dako, Carpinteria, CA), or Ki-67 MB-1 antibody diluted 1:50 (Immunotech, Marseilles, France) for 1 h at 4°C, washed in PBS, and treated with the corresponding biotinylated secondary antibody for 30 min at room temperature. After washing with PBS, sections were exposed to the ABC Elite reagent for 5–10 min at room temperature. Reactions were developed with 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and counterstained with hematoxylin. In invasive carcinomas, p63 and p53 staining was scored 0–4 on intensity. In preinvasive lesions, p63 was scored based as follows: 0 for no staining; 1 for basal layer; 2 for basal and parabasal layer staining; and 3 for full thickness staining. Ki-67-labeling index was defined as the percentage of nuclei immunohistochemically positive for MB-1 among a minimum of 500 cancer nuclei (12). One of the main concerns associated with the 0.6-mm biopsy is that the 0.6-mm biopsy may not represent the whole tumor specimen because of tumor heterogeneity. To address this question, we arrayed triplicates of tumors as described previously (13). We determined intercore variation by assessing variability for three biomarkers (p53, p63, and Ki-67) across triplicates tested by IHC and found low coefficients of variation (coefficient of variation, SD/mean) 0.34, 0.22, and 0.21, respectively, attesting for the low level of variability while sampling the same tumor multiple times.

**Survival Analysis.** **CN ratios (continuous variable) and IHC scores (parametric variables) were tested for correlations and for survival analysis. The average scores of triplicate biopsies were used for FISH analysis. Maximal immunostain scores from triplicates were used for IHC and data obtained from the tumor registry allowed survival analysis. Clinical data elements were obtained from the Bioinformatics Core of the Vanderbilt Ingram Cancer Center. Data analysis included Spearman correlation coefficients and Kaplan-Meier survival estimates with Cox proportional hazards regression models. Survival analysis was calculated from date of diagnosis to date of death or last date of contact for those alive at the time of the analysis. Curves were compared by the log-rank test. All analyses were carried out with SAS statistical software (SAS Institute, Inc., Cary, NC).

**RESULTS**

*p63 gene CN in NSCLC.* We analyzed p63 gene CN in NSCLCs by performing FISH analysis on tissue microarrays. Comparison of p63 CN with histological subtype produced a distinct pattern of p63...
amplification. Among 217 tumors tested, 88% of squamous carcinomas, 42% of large cell carcinomas, and 11% of adenocarcinomas of the lung exhibited p63 CN ratios > 3 (Fig. 1). The level of amplification showed internal consistency between the three punches taken from different regions of the same tumor. Among 10 tumors each punched in triplicates, the mean coefficient of variation for p63 CN was 32.38 ± 4.5 (standard deviation) while the coefficient of variation for FHIT CN was 37.5 ± 4.09. We also found a high correlation between p63 amplification and squamous histological subtype ($r = 0.65, P < 0.001$), yet the degree of p63 amplification was independent of stage and grade. Specifically, CN values varied from 3 to 11.5 copies (average for 50 nuclei). Although the CN values for p63 varied significantly between cells, every cell layer was affected. None of the nontumor cells showed 3q amplification. To determine whether p63 genomic amplification was attributable to locus amplification or aneusomy, we determined CN for p63 and for centromeric chromosome 3 by dual color FISH in a subset of 85 NSCLCs. We found that increased CN was due to aneusomy of chromosome 3 in 21% of the tumors.

**Expression of p63 Splice Variants in NSCLCs.** Because different splice variants of p63 have been shown to harbor distinct functional domains, we examined ΔNp63α, ΔNp63γ, and TAp63 transcripts by

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**Fig. 1. A.** p63 gene CN distribution for NSCLCs. Scatter graph representation of p63 CN/nucleus assessed by FISH in interphase nuclei of NSCLCs. Data represent the average number of fluorescent spots counted in 50 nuclei in tissue microarrays of NSCLCs. Comparison of these CNs with histological subtype produced a distinct pattern of p63 amplification. Bars, mean of each group of tumors. Ps were obtained by Bonferroni t-tests. B. dual color FISH of p63 gene (3q27, red spots) and a FHIT probe on the opposite arm of the same chromosome (3p14.2, green spots) on an adenocarcinoma (left) and a squamous carcinoma of the lung (right) from the same tissue microarray. Interphase nuclei of adenocarcinoma cells stained with 4’6-diamidino-2-phenylindole show equal p53 gene CN (2) of p63 and FHIT. However, in squamous carcinoma cells, interphase nuclei show amplification of the p63 gene.
RT-PCR in 24 NSCLCs, in 12 normal lung tissues, and in normal bronchial epithelium. \(\Delta Np63\alpha\) transcripts were found in bronchial epithelium and in squamous carcinomas but not in normal lung or in adenocarcinomas (Fig. 2 A). \(\Delta Np63\gamma\) transcripts were expressed in normal bronchial epithelium and in some squamous carcinomas but not in adenocarcinoma or in normal lung (Fig. 2, B and C). TaP63 transcripts were found in striated skeletal muscle, bronchial epithelium, and some squamous carcinomas at low levels but not in adenocarcinomas or normal alveolar tissue (Fig. 2, B and C).

**Correlation between p63 amplification and protein expression.**

We determined whether amplification of the p63 gene in squamous carcinoma affected protein expression by Western analysis and IHC.

We identified the presence of a \(M_r \approx 70,000\) protein in squamous carcinomas (Fig. 3 A, B) that was entirely absent in normal (Fig. 3 A) tissue and in adenocarcinomas (Fig. 3 A, C). This migration is similar to that previously reported for \(\Delta Np63\alpha\) (3, 10). The data shown in Fig. 3 are representative examples of 12 squamous, 12 adenocarcinomas and 12 matched normal lung tissue samples.

To identify the pattern of protein expression in normal lung and in invasive cancers, we performed IHC analysis on tissue microarrays prepared from paraffin blocks. Sections of tissue microarrays were incubated with the p63 4A4 monoclonal antibody (recognizing all six p63 splice variants). The distribution of scoring intensities across histological subtypes is presented in Table 1. In normal airway epithelium (Fig. 4A), p63 staining was nuclear and restricted to the basal cell layer. Alveolar epithelial cells did not stain with p63 (Fig. 4B). p63 expression was consistently found at high levels in squamous carcinoma (77 of 94) throughout the entire thickness of the tumor (Fig. 4E). The staining intensity was reduced within areas of additional differentiation and areas most distant from the vascular bed (Fig. 4E). p63 staining was present in only 17 of 93 adenocarcinomas (Fig. 4F) and in 7 of 30 large cell carcinomas. Staining intensity for p63 could be directly correlated with p63 amplification in NSCLCs overall (Spearman correlation coefficient, \(r = 0.502, P < 0.0001\)); however, this correlation did not hold for squamous carcinomas (\(r = 0.04, P = 0.717\)). This inconsistency points to an unknown mechanism of p63 gene expression that is independent of genomic amplification.

**p63 amplification as a candidate biomarker for squamous tumor development.**

To determine when p63 amplification is observed during the course of tumorigenesis, we performed FISH for p63 in 43 preinvasive lung squamous lesions from 39 patients. We were able to obtain clinical information on 14 patients and found that preinvasive lesions were synchronous to the invasive tumors in all 14. We found an increased CN for p63 in high-grade lesions [severe dysplasia and CIS] but not in lower grade lesions (\(P < 0.0001\); Fig. 5A). Severe dysplasia and CIS are almost invariably amplified for p63. Amplification of p63 correlated with p63 immunostaining (\(P < 0.001\)). In preinvasive tumors, p63 staining involved not only the basal but also the supra-basal layers and followed a pattern consistent with severity of histological grade (Figs. 4, C and D, and 5B). In invasive tumors, p63 was seen throughout the whole sheet of invasive tumor. All invasive tumors synchronous to high-grade preinvasive lesions exhibiting p63 CN...
amplification also showed p63 amplification. When invasive tumors did not show p63 amplification, their matched preinvasive tumors were also negative.

To determine the relationship between p63 expression and cell proliferation, 43 preinvasive lesions were stained with the Ki-67 antibody. In normal epithelium, Ki-67 was limited to the basal layer of the epithelium, whereas in neoplastic epithelia, Ki-67 was found in other layers, particularly in close proximity to vascular structures. However, the expression of Ki-67 did not colocalize or correlate with p63 (Fig. 6). This observation suggests that although p63 does not have a direct role in cell cycle regulation, p63 may allow a specific subpopulation of the epithelium to undergo either differentiation or proliferation given a specific signal.

**p63 genomic amplification and overexpression is a marker for better survival in squamous carcinoma.** In our series of 217 NSCLCs, patients with tumors displaying p63 genomic amplification (absolute CN ≥ 3) exhibited a survival advantage versus the overall population (P < 0.05) and also among patients with squamous carcinomas (P < 0.05; Fig. 7A). We also found a strong association between the intensity of p63 staining in squamous carcinomas and increased odds of surviving (P < 0.0174; Fig. 7B). After adjusting for stage, patients with tumors displaying increased p63 CN still had prolonged survival. Histological grade did not affect survival.

The p53 tumor suppressor gene is mutated in 35–50% of lung cancer (14, 15). When mutated, p53 can function as an oncogene and accumulate in the cytoplasm (16). p53 accumulation can be detected by IHC in the majority of mutations and is associated with reduced survival (17). Because p53 and p63 are partially homologous (3, 18) and because p63 has been proposed as an oncogene, we tested whether p63 CN or immunostaining pattern correlated with p53 immunostaining. We also tested the potential association of p63 with the proliferation marker Ki-67. There was no correlation between either p63 CN or p63 immunostaining (P = 0.5697) and p53 immunostaining in NSCLCs despite strong p63 and p53 staining (score ≥ 3) in 30% of the tumors (Fig. 8). We found that Ki-67-labeling index had no prognostic value (P = 0.2327) in the overall set of tumors or in any of the histological subgroups. Ki-67 index did correlate with p63 CN (P = 0.002) but not with IHC (P = 0.5347). These data suggest that
p63 is not a direct marker of proliferation and has a distinct role from p53 in tumor progression.

**DISCUSSION**

To study the role of p63 in tumorigenesis, we analyzed the frequency and timing of p63 genomic amplification by FISH and p63 protein expression by IHC on tissue microarrays and on formalin-fixed tissue sections of invasive and preinvasive NSCLCs. A series of 24 fresh-frozen tumors and 12 normal lung samples were used to determine the p63 splice variant expression profile in NSCLCs. Our results indicate that p63 gene amplification occurs in the majority of squamous cell carcinomas, rarely in adenocarcinomas, and in 42% of large cell carcinomas. We also found that p63 amplification is present in lesions exhibiting severe dysplasia and in more advanced stages of tumor progression.

/H9004/H9251, /H9004/H9253, and TA splice variants are expressed in NSCLCs, and /H9004/H9251 is the predominant splice variant expressed. In squamous cell carcinoma, p63 amplification and staining intensity are associated with better survival independently from stage and degree of differentiation of the tumor. We did not find a correlation between p63 immunostaining and p53 or Ki-67 expression.

Chromosome 3q26-ter amplification, which includes the p63 gene locus, is one of the most prevalent genomic abnormalities in solid tumors and is likely to play a critical role in tumorigenesis. Amplification of chromosome 3q has been described in squamous epithelial transformation from the lung (6), head and neck (19, 20), esophagus (21), bladder (22), cervix (23, 24), and stomach (25). We have previously observed that the size of the amplicon varies tremendously between tumors (15–70 Mb) and contains >300–600 known and unknown genes (2). The relevance of most of these genes to tumor progression remains to be determined. We recently demonstrated that the presence of 3q amplification alone allows the distinction between squamous and adenocarcinoma in >75% of cases (2). In the present study, we confirmed this in an independent tumor set and extended it

![Fig. 4. p63 IHC analysis with 4A4 antibody in lung tumorigenesis. A, bronchial epithelium showing immunostaining at the basal layer. B, epithelial cells of the alveolar space do not show immunostaining. C and D, mild and severe dysplasia of the bronchial epithelium, respectively, showing a progressive increase of p63 immunostaining from the basal layer to the surface of the epithelium. E, squamous carcinoma with strong staining for the majority of the tumor cells yet decreasing in most differentiated area. F, adenocarcinoma with no staining for p63.](image)

![Fig. 5. p63 gene CN and expression in preinvasive lung cancer. A, p63/FHIT gene CN ratio by FISH during tumorigenesis. Bar graph showing average of p63/FHIT CN ratio among normal preinvasive and invasive lung cancers. B, p63 IHC analysis with 4A4 antibody during tumorigenesis. n = number of independent observations; error bars represent SD.](image)
to a larger number of NSCLCs. Both increased p63 CN and p63 staining intensity were strongly associated with squamous histological subtype. Although p63 was amplified in 11% of adenocarcinomas, half of these tumors were aneuploid and some exhibited deletion of one p63 allele. Finally, 42% of large cell carcinomas also exhibited p63 amplification, yet these tumors are poorly differentiated and may actually represent poorly differentiated squamous carcinoma.

Although p63 CN did correlate with p63 staining intensity in NSCLCs overall, it did not correlate among 94 squamous cell carcinomas (P = 0.717). These observations are in agreement with data in head and neck cancer showing no correlation between genomic amplification and gene expression by comparative genomic amplification using quantitative RT-PCR in 10 tumors and 18 normal tissues (26). Absence of correlation between gene amplification and protein expression in squamous carcinoma suggests that the degree of amplification does not regulate protein expression directly. The functional repercussions of p63 amplification remain the subject of evaluation in the context of the large chromosome 3q amplicon.

p63 amplification occurs early in the development of lung cancer and may have important implications in early detection strategies. To evaluate CN in preinvasive lesions, we chose to determine CN ratio by dual color FISH, taking advantage of the frequent 3p deletion on 3p14 in NSCLC. This CN ratio (p63/FHT) increases the sensitivity of our assay to detect small genomic abnormalities in tissue sections. FHT is deleted in some NSCLCs (27–30), which we confirmed in up to 30% of squamous carcinomas.

p63 immunostaining data shows progressive increase throughout the depth of the epithelium from metaplasia to severe dysplasia and relates to the pathology. These findings, obtained in 43 preinvasive lesions, confirm data previously reported by Pelosi et al. (31) and Sniezek et al. (32) in a small series of head and neck and lung tumors. In particular, it is apparent that p63 expression increases progressively from preinvasive to invasive lesions during the transformation of squamous epithelia. Moreover, p63 immunoreactivity has been shown to be inversely correlated with both squamous cell maturation and nonsquamous differentiation in cervical intraepithelial neoplasia (24). This progressive increase of p63 expression according to pathological grade makes p63 less attractive to early detection efforts and does not offer a major advantage to pathological distinction. In contrast, amplification of p63 is characteristic of high-grade (severe dysplasia and CIS) preinvasive and invasive squamous lung cancers but not earlier. In our set of 43 preinvasive lesions, 13 were found adjacent to invasive squamous carcinomas. For every high-grade preinvasive lesion where p63 was amplified, the corresponding invasive tumor was also amplified. Low-grade preinvasive lesions found in the vicinity of invasive tumors did not show p63 CN gain regardless of the degree of amplification of the invasive tumor. This suggests that p63 amplification occurs during tumorigenesis when the preinvasive lesions commit to invasion and may therefore be a good biomarker for early detection of patients with or at risk for lung cancer. Given the quantifiable nature of p63 CN by FISH and to address obvious limitations of a retrospective study, we are planning a prospective evaluation of 3q amplification as a predictive biomarker for lung cancer development in biopsy specimens and in sputum from patients at risk for or with lung cancer. Because not all high-grade lesions develop into invasive tumors (33), the management of preinvasive lesions is still a subject of controversy. Our study suggests that special

![Fig. 6. H&E, p63, and Ki-67 immunostaining for a severe dysplastic preinvasive bronchial lesion on adjacent sections. This is a representative sample of preinvasive lesions illustrating the differential pattern of expression of the two markers.](image)

![Fig. 7. Effect of p63 CN and immunostaining on survival in NSCLCs. A, Kaplan-Meier survival curve shows significant prolonged survival associated with patients with tumors exhibiting genomic amplification of p63 (p63 \( \geq 3 \), n = 73; p63 CN <3 (n = 6)) in squamous carcinoma of the lung. P < 0.05. B, effect of p63 immunostaining intensity on odds of surviving from squamous carcinoma of the lung. Scores of 1–4 were obtained based on staining intensity. The stronger the staining intensity, the lower the risk of dying. P < 0.014, n = 94.](image)

![Fig. 8. Frequency distribution of immunostaining intensity for p53 and p63. Staining intensity was graded 0–4. p53 and p63 staining intensities were considered weak when the score was \( \leq 2 \) and strong when \( \geq 3 \). No significant correlation was found between the two markers.](image)
consideration should be given to preinvasive lesions with p63 amplification. Furthermore, identification of these lesions may assist in monitoring the response to new chemopreventive agents.

The mechanism by which p63 amplification and p63 overexpression participate in tumor progression remains unclear. We confirmed that p63 is expressed at the basal layer of the airway epithelium, a layer that has regenerative potential (3, 6, 31). Because specific antibodies to the ΔNp63α splice variant are not currently available, we could not confirm the predominant expression of this splice variant in preinvasive and invasive lung tumors by immunohistochemistry. Our data on localization of p63 and Ki-67 demonstrates a different pattern of expression suggesting that ΔNp63α may not play a direct role in cell cycle regulation. At some concentration, ΔNp63α may be toxic and cause cells to die (4). Once the cells have committed to proliferation, p63 may become less important functionally and may potentially be toxic. This may account for the reduced expression seen in more differentiated cancer cells with persistent genomic amplification.

We found that p63 amplification and overexpression are extremely prevalent in squamous carcinomas and that they are also associated with better survival. In a recent study, Pelosi et al. (31) examined the percentage of p63-positive cells by IHC, yet did not find an association between p63 expression and survival. Nevertheless, we found that the majority of cancer cells (>80%) stained for p63 in squamous carcinomas, and therefore, we tested whether the staining intensity of the cells rather than the percentage might correlate with outcome. Our data demonstrate a prolonged survival in patients with p63 amplification and a lower risk of dying for patients with tumors that have strong p63 immunostaining. We speculate that the reason for a survival advantage could involve the following: p63 functions as a survival factor that promotes transformation in the appropriate unstable genetic background. Once transformed, cells develop and keep p63 genomic amplification. As the cells further differentiate, p63 expression is decreased and may then be an important marker of cell differentiation and confer a survival advantage.

Because of sequence homology with p53, it has been suggested that p63 may play a similar pattern of expression in tumors. Our observation that p53 and p63 overexpression occurs simultaneously in only 30% of squamous carcinomas of the lung and that there is no relationship between the two markers among the three histological subtypes studied. We also could find no correlation between p53 and p63 immunostaining for any stage or grade of differentiation. These results suggest independent roles for p63 and p53 during tumorigenesis. The functional interpretation of p53 and of p63 assessed by immunostaining remains a limitation of this study. There is no data available correlating p63 immunostaining and activity in human tumors. Similarly for p53, although mutated p53 generally shows strong immunoreactivity (17), mutation of the gene can lead to overexpression of the protein or complete loss of expression (truncated protein; Ref. 34), making the immunostaining an imperfect predictor of p53 mutation status.

This genomic amplification of p63 in preinvasive lesions is also likely to be part of the same amplicon found in invasive squamous carcinomas. This genomic region of amplification on chromosome 3q26–3q28 also contains a number of other important genes, some of which have been called potential candidate oncogenes or are thought to be involved in tumor progression (PIK3CA, somatostatin gene, telomerase RNA component gene, and neutral endopeptidase gene). The specific role of these other candidate biomarkers in tumor progression and their functional interactions remain to be determined.

Finally, p63 amplification allows us to identify two different classes of NSCLCs. Upon transformation, the majority of tumors developing from the bronchial epithelium acquire 3q amplification. This pattern suggests a different mechanism of tumor development from those of other cell types. Although all NSCLCs are currently treated identically, genes in the chromosome 3q amplicon may open a window for identification of potential targets for molecular intervention in preinvasive and invasive lung cancer. Taken together, our data suggest that p63 amplification and overexpression of ΔNp63α are critical steps in the early development of NSCLC and that p63 amplification in particular may prove to be an excellent biomarker of squamous carcinoma progression.

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