Prevalence, molecular markers, and outcome of bronchial squamous carcinoma *in situ* in high-risk subjects

STEINN JONSSON,[1,2,*] WILBUR A. FRANKLIN,[3] MARILEILA VARELLA-GARCIA,[1] TIMOTHY C. KENNEDY,[4] DANIEL MERRICK,[3] KATHRYN D. MATNEY,[3] GUDRUN N. OSKARSDOTTIR,[2] ARNI SAEMUNDSSON,[2] ROBERT L. KEITH,[1,5] PAUL A. BUNN[1] and YORK E. MILLER[1,5]

[1]Department of Medicine, University of Colorado Health Sciences Center, Denver, CO, USA; [2]Department of Medicine, Faculty of Medicine, University of Iceland, Reykjavik, Iceland; [3]Department of Pathology, University of Colorado Health Sciences Center; [4]Department of Medicine, Presbyterian/St Luke's Health One Medical Center, Denver; and [5]Pulmonary Division, Department of Medicine, Rocky Mountain Regional Veterans Affairs Medical Center, Aurora, CO, USA

Jonsson S, Franklin WA, Varella-Garcia M, Kennedy TC, Merrick D, Matney KD, Oskarsdottir GN, Saemundsson A, Keith RL, Bunn PA, Miller YE. Prevalence, molecular markers, and outcome of bronchial squamous carcinoma *in situ* in high-risk subjects. APMIS. 2023; 131: 513–527.

Bronchial squamous carcinoma *in situ* (CIS) is a preinvasive lesion that is thought to precede invasive carcinoma. We conducted prospective autofluorescence and white light bronchoscopy trials between 1992 and 2016 to assess the prevalence, molecular markers, and outcome of individuals with CIS and other preneoplastic bronchial lesions. Biopsies were evaluated at multiple levels and selected biopsies were tested for aneuploidy and DNA sequenced for TP53 mutation. Thirty-one individuals with CIS were identified. Twenty-two cases of CIS occurred in association with concurrent invasive carcinomas. Seven of the invasive tumors were radiographically occult. In two cases, CIS spread from the focus of invasive carcinoma into contralateral lung lobes, forming secondary invasive tumors. In nine cases, CIS occurred as isolated lesions and one progressed to invasive squamous carcinoma at the same site 40 months after discovery. In a second case, CIS was a precursor of carcinoma at a separate site in a different lobe. In seven cases CIS regressed to a lower grade or disappeared. High level chromosomal aneusomy was often associated with *TP53* mutation and with invasive carcinoma. CIS most often occurs in association with invasive squamous carcinoma and may extend along the airways into distant lobes. In rare cases, CIS may be observed to directly transform into invasive carcinoma. CIS may be indicative of invasive tumor at a separate distant site. Isolated CIS may regress. Molecular changes parallel histological changes in CIS and may be used to map clonal expansion in the airways.

Key words: Bronchial; carcinoma; *in situ* carcinoma; molecular markers; outcome.

Steinn Jonsson, Department of Medicine, Landspitali-University of Iceland, Fossvogi (E7), 108 Reykjavik, Iceland.
e-mail: steinnj@landspitali.is

Despite recent reductions in mortality, lung cancer (LC) remains the leading cause of cancer deaths in the United States [1] and worldwide [2]. Survival is dependent on the stage at diagnosis but due to the low rate of early diagnosis only 26% of patients survive 5 years with the best treatment available [3]. To improve outcomes for LC, improved early detection and prevention will be required [4]. Screening with low dose helical computed tomography (LDCT) is effective in detecting small peripheral lung tumors that may be cured by surgery [5]. In a large prospective screening trial conducted in the United States, a 20% reduction in mortality was observed in high-risk individuals screened by LDCT compared to controls screened by chest radiograph [6]. This study has recently been confirmed in a large European trial using LDCT technology [7].

Squamous cell carcinoma (SCC) of the lung comprises 25% of all LC in females and 44% in males

according to data obtained from cancer registries worldwide [8]. SCCs are frequently central airway tumors and are thought to arise from a multistep process that affects the whole bronchial mucosa but is rarely observed in individual patients. The WHO classification of lung premalignancy [9] describes graded changes in bronchial lining cells ranging from normal epithelium, through increasing grades of dysplasia to CIS and invasive carcinoma. CIS may arise separately and independently or may be an *in-situ* extension of a contiguous invasive lung cancer [10]. CIS may progress to invasive carcinoma or, occasionally, may regress [10, 11]. The fate of any given CIS lesion cannot be predicted by histology alone and may therefore present formidable diagnostic and therapeutic problems for the clinician.

The identification of biomarkers that can improve the diagnostic accuracy of histology is an important area of research that could guide the selection of individuals that may benefit from early intervention [12]. We have previously shown that aneusomy detected by FISH is a promising adjunct to histology in the evaluation of premalignant lesions [13, 14]. We found that the CIS lesions which resolved were either normal or showed low levels of aneusomy.

In the present study we report data on 31 individuals diagnosed with CIS on bronchial biopsy over a period of 24 years in a prospective multi-institutional bronchoscopy trial conducted in Colorado. We compared histological grade, chromosomal aneusomy by FISH analysis and mutational analysis for the *TP53* gene as biomarkers to assess CIS lesions and correlate the findings with clinical outcomes.

MATERIALS AND METHODS

Subjects

This study (Fig. 1) was performed using patient records and histological sections that have been collected prospectively since 1992 at the University of Colorado Cancer Center (UCCC) through several IRB approved trials.

The subjects included current, former or never smokers who were screened by study coordinators for any of the following: suspicion for lung cancer including radiographic evidence of lung cancer, a history of at least 20 pack-years of cigarette smoking, spirometric evidence of airflow obstruction documented by an FEV1/FVC ratio of <75% and FEV1 of <70% of predicted, or sputum atypia. Former smokers were defined as having quit at least 1 year before the time of enrollment. Duration of smoking was defined as the number of years from initiation of smoking until smoking cessation. A pack-year was defined as the average number of packs smoked per day multiplied by the numbers of years smoked.

Informed consent and procedures

Patients gave informed consent to undergo white light and autofluorescence bronchoscopy with the intent to detect premalignant lesions or cancer. Subjects filled out a standard questionnaire, spirometry was performed, and results recorded. Flexible fiberoptic bronchoscopy was performed by white light with or without autofluorescence examination of the airways using either a Xillix LIFE II or Onco-LIFE system (Xillix Technologies Corp., Richmond, B.C. Canada). Six standard sites were routinely biopsied in all individuals: RUL carina, RML carina, RB6 carina, LUL carina, carina between lingula and upper division bronchus and LB6 carina. In addition, any other sites that were abnormal under white light or auto-fluorescence bronchoscopy were biopsied. Biopsy sites could be omitted at the discretion of the bronchoscopist.

All subjects with CIS at one or more sites were included in this study. Biopsies, surgical specimens, and autopsies from these patients were examined. In most of these cases, multiple biopsies obtained at different times from the same site were assessed. Treatment of individuals with CIS was only initiated when there was evidence of invasive carcinoma. Determination of whether subjects had invasive lung cancer at the time of bronchoscopy was based on biopsy results and review of the clinical history. Subjects were classified as having prevalent LC if an invasive cancer was diagnosed within 6 months of the initial bronchoscopy. Invasive tumors identified after this time were classified as incident cancers. Lung tumors were treated by the most appropriate means for each individual to provide optimal clinical outcome. In cases without invasive tumor, patients were followed with active surveillance by clinical observation and repeat bronchoscopy.

There was consensus among the investigating clinicians that treatment should be offered only if invasive carcinoma was identified. Treatment decisions were then made based on standard of care in each individual case.

Statement of ethics

Protocols for tissue acquisition by bronchoscopy were approved by Colorado Multiple Institutional Review Board (COMIRB), the Research and Development Committee of the Rocky Mountain Regional Veterans Affairs Medical Center and the Health One Institutional Review Board (IRB# 00-1108). Some subjects were enrolled as part of two NCI sponsored chemoprevention trials (N01-CN85188, U01CA96109). This research was conducted in compliance with internationally accepted guidelines for human research and reporting.

Informatics

Data created in this study were recorded and tracked using an SQL relational database.

To relate histological diagnoses, digital images of stained slides, bronchoscopic mucosal images, location of biopsy sites, demographic data and chronology, where used to create a bronchial map tool (Fig. 5) utilizing metadata stored in the database. The tool allowed side by side comparison of bronchial epithelial samples from different
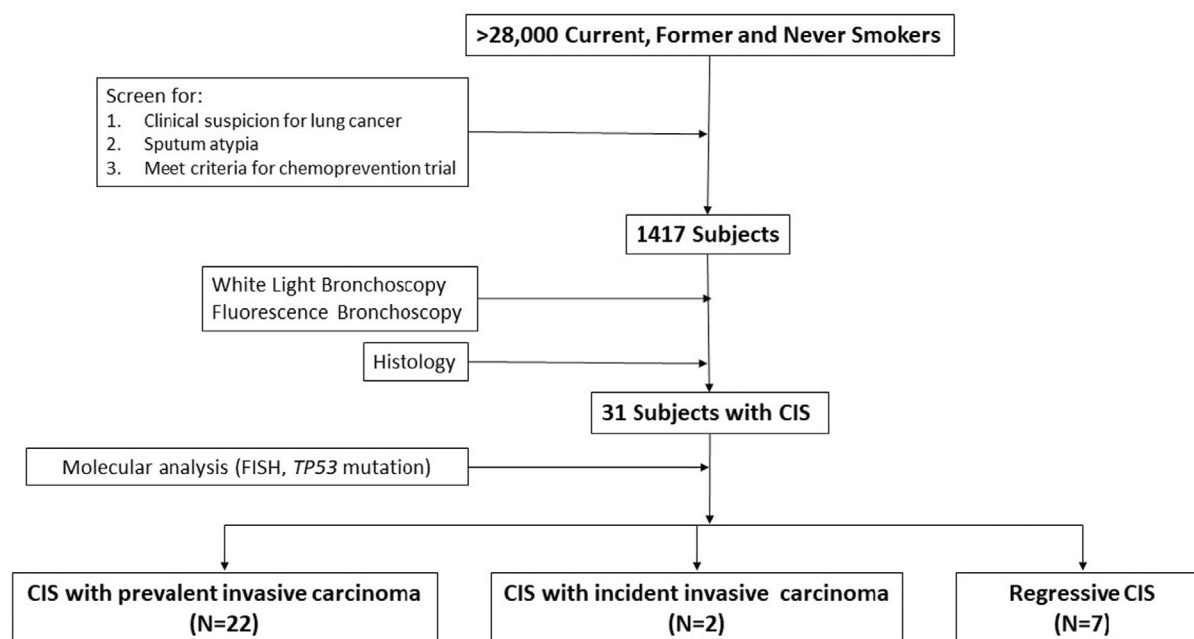
Fig. 1. Consort diagram illustrating study plan of this long-term observational trial. Patients seen at Colorado SPORE clinics for multiple clinical trials with one or more of the listed attributes were offered participation in a bronchoscopy trial with biopsy that were ultimately grouped by outcome as shown.

sites and dates. Color coded icons are applied on a graphical representation of the tracheobronchial tree to easily follow dysplasia progression at specific bronchial sites. Changes in histology scores could be quantified by subtracting later scores from earlier scores at the same site.

Histological analysis

Biopsies were processed according to a standard protocol that was consistently applied to all biopsy material obtained by bronchoscopy. Because it is possible to miss a small focus of CIS if tissue is inadequately sampled, our sectioning protocol required examination of multiple levels of the biopsy so that no focus of abnormal cells was missed. Four to eight 4-μm sections of formalin-fixed paraffin embedded biopsy tissue were mounted onto 12 slides. Slides 4 and 9 were stained with H&E. Epithelia in each biopsy were graded according to the World Health Organization criteria [17] in which bronchial dysplasia is assigned the following numerical scores (1) normal, (2) reserve cell hyperplasia, (3) squamous metaplasia, (4) mild dysplasia, (5) moderate dysplasia, (6) severe dysplasia, (7) CIS, and (8) invasive carcinoma. All slides were read by two investigators (WAF and DM) and any disagreements resolved by joint slide review.

FISH analysis

The LAVysion probe set (Vysis/Abbott Molecular, Abbott Park, IL, USA) was used for FISH analysis. This set includes four DNA targets: 6p11.1-q11 (CEP 6, labeled

with Spectrum Aqua); 5p15.2 (D523, D5S721 labeled in Spectrum Green—encompasses the SEMA5A gene), 7p12 (labeled in Spectrum Red encompasses the *EGFR* gene), and 8q24.12-q24.13 (labeled in Spectrum Gold, encompasses the *MYC* gene).

At the time of the histological evaluation, the most appropriate area for FISH analysis was selected and a digital image created and printed to guide selection of cells for FISH signal enumeration and for microdissection and sequencing analysis. Initially unstained paraffin sections encompassing the selected area were incubated for 2 h at 56°C, deparaffinized in Citri-Solv (Fisher Scientific, Hampton, New Hampshire, USA) and washed in 100% ethanol for 5 min. The slides were then incubated in 2xSSC at 75 °C for 15 min, digested in 0.25 mg/mL Proteinase K/ 2xSSC at 45 °C for 16 min and washed in 2xSSC for 5 min before being dehydrated in an ethanol series. The probe set was applied according to the manufacturer's instructions to the selected hybridization areas, which were covered with 12 mm glass coverslips and sealed with rubber cement. Co-denaturation and hybridization were performed in the Hybrite platform (Vysis), with temperature set for 1 min at 85 °C and 24 h at 37 °C. Post hybridization washes were performed with 2xSSC/0.3% NP40 at 72 °C for 2 min followed by wash in 2xSSC for 2 min at room temperature and dehydration in ethanol series. Chromatin was counterstained with DAPI (0.3 μg/mL in Vectashield Mounting Medium; Vector Laboratories, Newark, CA, USA).

Analysis was performed on epifluorescence microscopes equipped with single band pass interference filter sets for blue (DAPI), aqua (Spectrum Aqua), green (FITC), yellow (Spectrum Gold), and red (Texas red). For

documentation, images were acquired with a cooled CCD camera (Photometrics, Tucson, Arizona, USA) in monochromatic layers and merged and processed using the CytoVision software (Applied Imaging Inc., Grand Rapids, MI, USA). The areas selected by the pathologist in the H&E-stained sections were identified in the hybridized slide and 30–50 nuclei were scored per area. As a control, we analyzed a total of 30 areas with normal histology, scoring 30–50 nuclei per area. A nucleus was considered abnormal (aneusomic) when showing three or more signals for at least one of the DNA targets. The highest frequency of abnormal nuclei in the normal epithelium was 6.7%, and for only a single DNA target. Therefore, we chose 100% specificity for the test (>6.7% of abnormal nuclei) as the cutoff for determining the classification of premalignant areas as exhibiting or not chromosomal aneusomy and the level of aneusomy displayed. Based on the level of abnormalities detected, specimens were classified as follows [1]: no aneusomy, if abnormalities were not present [2]; low aneusomy, if copy number gains occurred in one or two markers, and [3] high aneusomy, if gains were displayed for more than two markers (Fig. 2).

DNA sequencing for p53 and other genes

Diagnostic cells were identified in deparaffinized, hematoxylin-stained 10-micron tissue sections under a dissecting microscope and microdissected under molecular grade glycerol using a fine bore glass pipette connected to a foot controlled pneumatic cell collection device. Glycerol was removed from microdissected cells by washing with PBS and 70% ethanol and resuspended in ATL tissue lysis buffer containing proteinase K and incubated overnight at 56 °C followed by 1 h at 90 °C. The resulting DNA was purified using a QIA cube (QIAGEN, Valencia, CA, USA) automated purification device. After nucleic acid extraction, *TP53* exons 5a, 5b, 6, 7, and 8 were amplified in a 45-cycle polymerase chain reaction (PCR) using a KAPA Robust 2G Hotstart Enzyme Kit (cat#KK5525) in an ABI 9700 thermocycler. Primers and unamplified DNA were removed from amplification products using Agencourt AMPure XP beads (Beckman Coulter Inc., Brea, CA,USA) Sequencing of amplicons was accomplished using the Big Dye terminator Cycle Sequencing Kit in a 30 cycle PCR reaction. The dye-tagged products were then directly sequenced by capillary electrophoresis with an
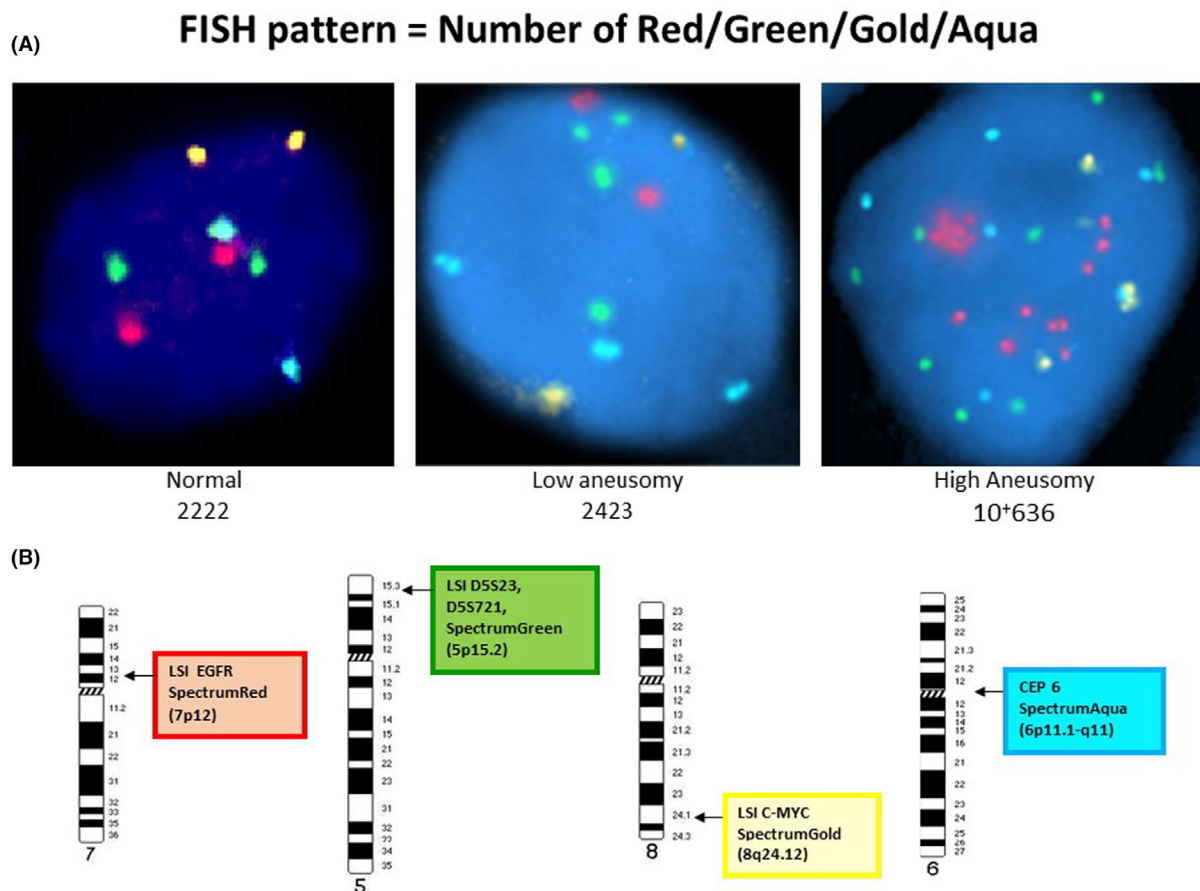
Fig. 2. (A) FISH analysis used four probes as illustrated above. The number of signals for each probe were combined into an aneuploidy score in which two signals for each probe were classified as normal, >2 signals for one or two probes were classified as low aneusomy and >2 signals for >2 probes were classified as high aneusomy. In the figure, the number of signals for each probe is recorded below each photomicrograph. (B) The chromosomal mapping of each probe is shown.
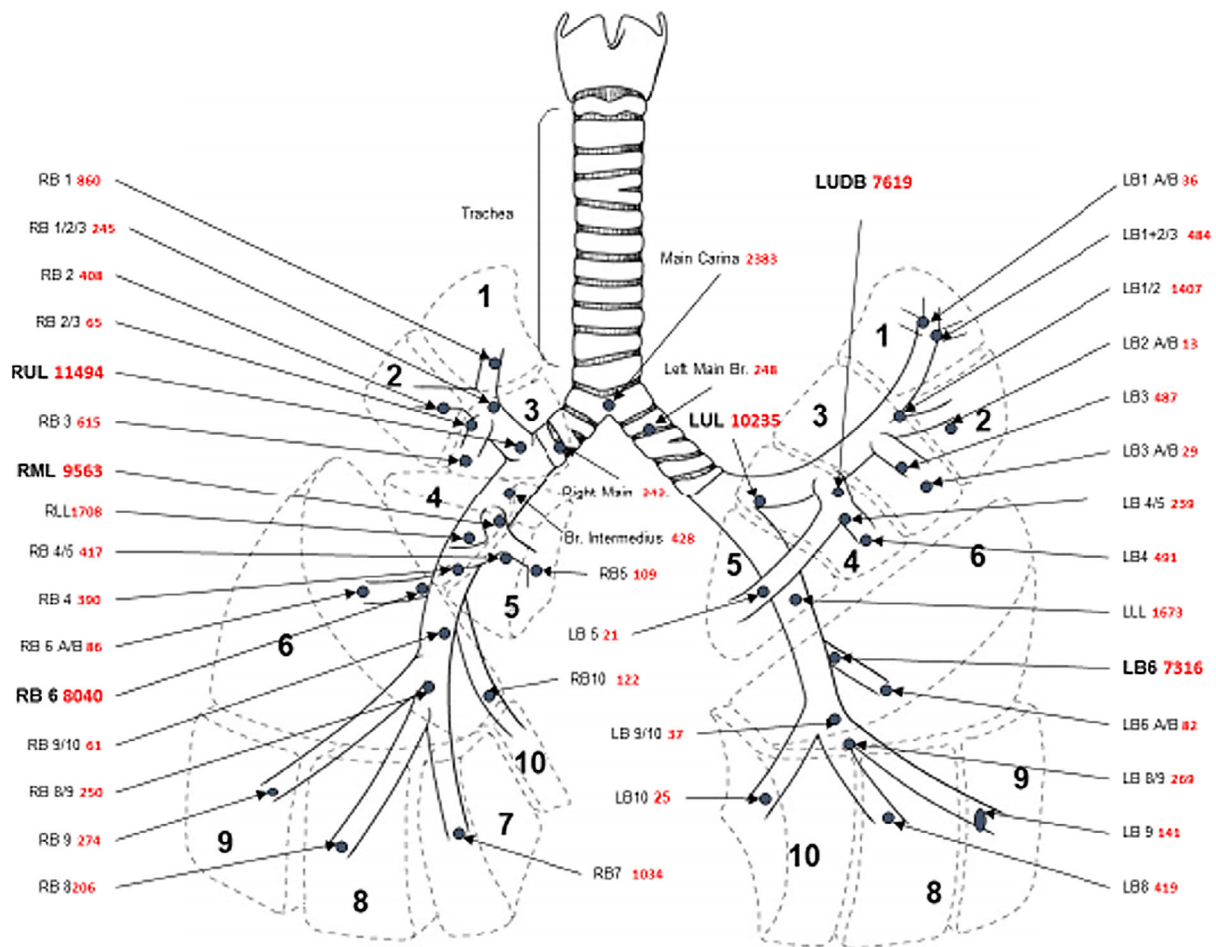
Fig. 3. Map of bronchial sites biopsied during bronchoscopy. Number in red indicates number of biopsies taken at each site. Figure does not include LB7 at which 1034 biopsies were taken. Predetermined biopsy sites were RUL, RML, RB6, LUL, LUDB, and LB6.

ABI 3500xL DNA Analyzer. Mutations were identified by evaluation of sequencing electrophoretograms using Mutation Surveyor software (v4.0.9).

In addition to single gene analysis, multiplex PCR was used in a single case [3] in which same site progression to invasive carcinoma was observed. Eighty exon sequences from 26 cancer-related genes in the Illumina TruSight panel were amplified and analyzed using the Illumina Miseq system which identifies and quantifies variants and (mutant) alleles.

Statistical analysis

Categorical data were summarized using frequencies and percents. Distribution differences between groups were examined using the chi-squared test. Relationships among histological score, chromosomal aneusomy and *TP53* mutation status were also investigated by chi-squared test (Microscoft Excel). Kaplan–Meier survival curves were created for separate staging categories and differences

compared by log rank test using the SPSS software package.

RESULTS

Distribution of CIS lesions in the airways

In this prospective study of current, former, and never smokers at risk for lung cancer (Fig. 1), over 28 000 patients were screened. Based on clinical suspicion of cancer, sputum atypia or fulfillment of criteria for chemoprevention trials, 1471 subjects were examined by white light bronchoscopy with or without autofluorescence bronchoscopy under IRB approved protocols between January1992 and July 2016. Over 44,000 bronchoscopic biopsies were obtained at abnormal or predetermined bronchial sites as depicted in Fig. 3.
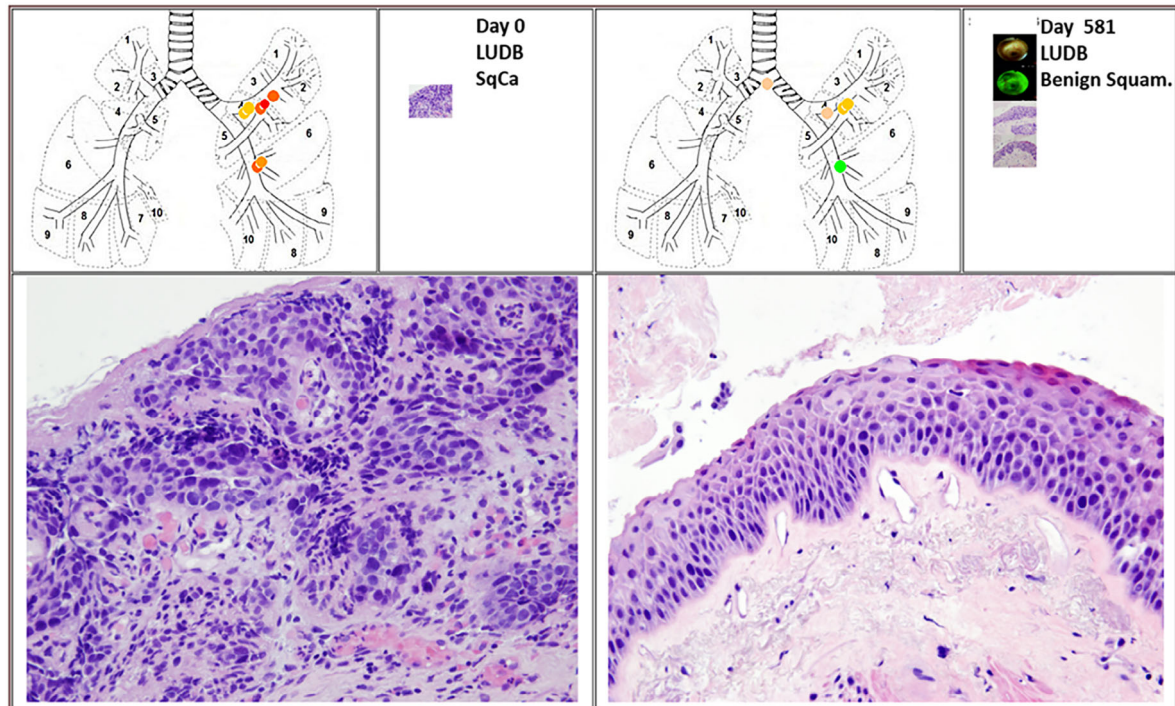
Fig. 4. Site in left upper lobe contained CIS which transformed into small focus of invasive carcinoma shown on left. Patient received SBRT to the lesion but died 4 years later of COPD. At autopsy, only low-grade dysplasia remains (right) following irradiation of invasive carcinoma site.

Multiple same site biopsies were obtained from 290 individuals. A histological diagnosis of CIS was made in 31 individuals at 66 bronchial sites. Nine individuals had more than one CIS lesson. Twenty-six of these subjects were males and 5 were female. Mean age was 63 years. The mean duration of smoking was 43 years, and most of the subjects (58.1%) were former smokers.

Individual lesions could be separated into categories according to whether they occurred simultaneously with invasive tumor (concurrent), preceded invasive tumor (progressive) or regressed (regressive).

**Concurrent: CIS With simultaneous invasive carcinoma**

CIS discovered within 6 months of a diagnosis of invasive carcinoma were classified as concurrent CIS. Twenty-two cases of concurrent carcinoma were included among the 31 cases of CIS. Concurrent CIS occurred in several configurations including:

1. *CIS spread from a focus of invasive carcinoma a short distance along the bronchial surfaces.* Treatment of these tumors was surgical

resection in nine, radiotherapy in three, and chemotherapy in one. Four patients who were felt to be unsuitable for curative surgery or radiotherapy received endobronchial treatment only and one declined treatment. Median survival in this group was 16 months. One of these cases stands out as an exception to this dire outcome. In this case, invasive tumor was identified at an early stage as depicted in Fig. 4.

CIS with a small focus of invasive carcinoma was found at the upper division bronchus of the left lung. This site was treated with stereotactic bronchial radiation therapy (SBRT) and was tumor-free 4 years after treatment.

1. *CIS spreading widely as a continuous, broad front from a focus of invasive carcinoma.* Two cases of CIS with concurrent invasive carcinoma were remarkable for the extended spread of CIS with both patients developing bilateral CIS. In one patient (Fig. 5) bilateral invasive squamous tumors formed with the same *TP53* mutation. Invasive tumors in this group pursued an aggressive course and the two patients in this group died 15 and 44 months after initial surgery.
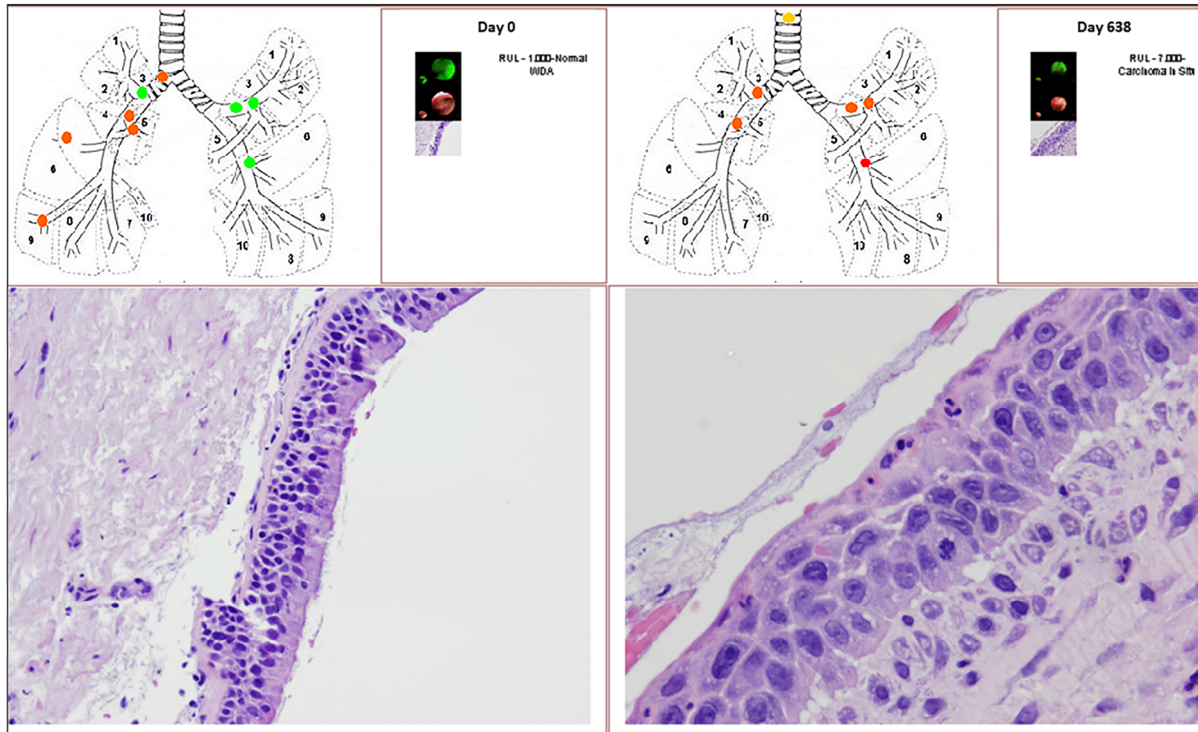
Fig. 5. Bronchial map showing biopsies obtained before resection of invasive squamous tumor in right lower lobe in 2006. Biopsy on left in 2006 where free of CIS. Two years later CIS had spread to left lung.

2. *CIS occurring remotely and discontinuously from an invasive focus.* That CIS is a marker for field carcinogenesis is indicated by three cases in which invasive carcinoma was found at a site distant from the CIS lesion. One case was peripheral lung adenocarcinoma. None of the patients in this group died of squamous carcinoma. Two patients died of unrelated causes and a third is alive 94 months after resection for an invasive squamous carcinoma.

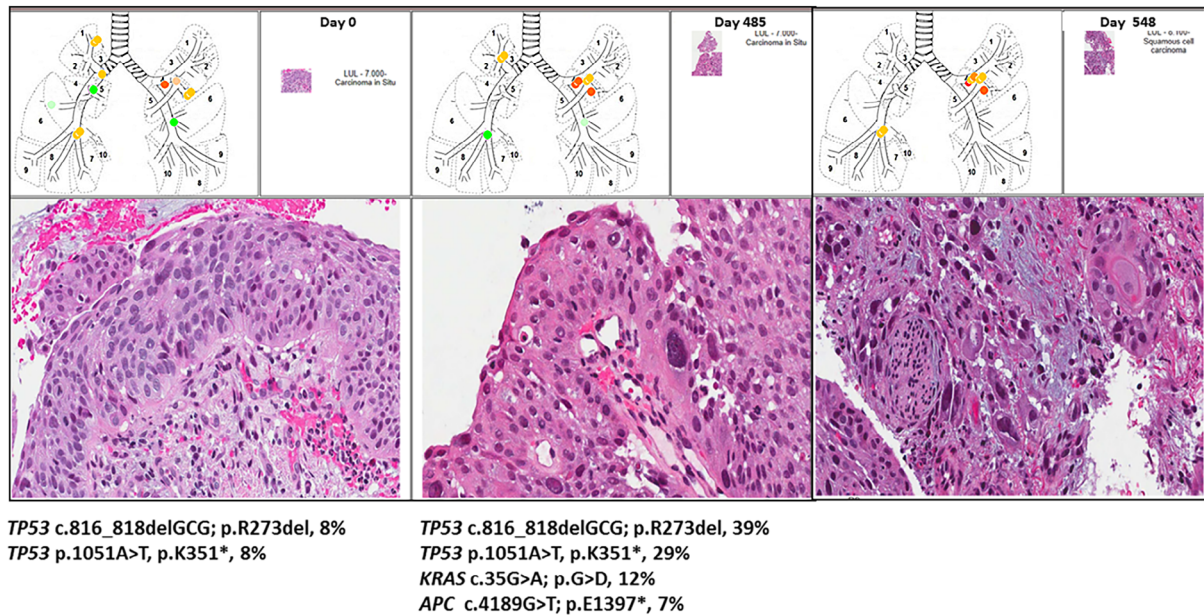**Progressive: CIS with subsequent invasive carcinoma**

Only a single case was observed in whom invasive carcinoma developed at a CIS site that had been under observation for more than 6 months. Invasive tumor was found 40 months after CIS had been observed. This tumor was classified as CIS with incident invasive lung carcinoma. This case is presented in detail since it coincides with current models of multistep carcinogenesis and shows that invasive squamous carcinoma can be successfully treated if detected early. The case also is an illustration of the use of the bronchial map and integration of data from multiple platforms and times to plot the fate of bronchial mucosa at a specific site.

This patient was discovered through a Colorado SPORE screening program to have CIS in biopsies at the junction between the anterior and apical posterior segments of the upper lobe, left lung (Fig. 6 left). CIS persisted for many months (Fig. 6 middle frame). Note the increase in the percentage of *TP53* mutant copies in the CIS DNA prior to the identification of invasive tumor. Other areas not containing CIS did not harbor TP53 mutation, consistent with the somatic nature of the mutation. A year and a half after discovery of the initial CIS, invasive carcinoma was found at the site (Fig. 6 right) and was treated with stereotactic radiotherapy. On revisiting the tumor site no recurrence has been identified. The patient remains alive and well.

A second case of incidental carcinoma shows (Fig. 7) that CIS may be a risk factor not only for the specific site where it occurs, but also for the rest of the aerodigestive tract the process of field carcinogenesis. This patient was also identified through a SPORE screening program.

Regressive CIS with reversion to lower grade or death without evidence of progression to invasive carcinoma (seven cases)

Among the seven patients who did not develop invasive lung cancer during this study, five (16%)

Fig. 6. Case 3 illustration of CIS with same-site progression to invasive carcinoma. This patient was discovered through a Colorado SPORE screening program to have CIS in biopsies at the junction between the anterior and apical posterior segment of the upper lobe, left lung (Day 0). CIS persisted for many months (middle frame) accompanied by increasing mutational burden. Finally, a year and a half after discovery of the initial CIS, invasive carcinoma was found at the site (Day 548). The site was treated with stereotactic radiotherapy. On revisiting the tumor site no recurrence has been identified. The patient remains alive and well.

lesions regressed at the same site to lower grades of dysplasia or normal mucosa (Fig. 8). Two individuals died of unrelated causes: one of pneumonia following lung transplantation for COPD and a second of respiratory failure due to COPD, 2.8 and 5 years after CIS diagnosis, respectively. At the time of death there was no radiographic evidence of invasive carcinoma in these two cases, but no follow up bronchoscopy was available before death. Outcomes for the various categories are plotted in a Kaplan Meyer survival graph which plots survival for each tier in the proposed classification (Fig. 9).

No cases of persistent CIS without regression or progression to invasion were identified.

There is a wide difference in survival between CIS with concurrent carcinoma and regressive CIS. Twenty of the 22 patients with concurrent carcinoma died during the study; 12 died of lung cancer, four of cardiopulmonary failure, four of nonthoracic or unknown causes. In contrast, none of the seven regressive CIS patients died of lung cancer but 2 died of COPD. Survival in CIS with incident carcinoma was intermediate. This pattern of survival is significant at p = 0.034 by log rank statistic.

Molecular changes

Chromosomal aneusomy

A total of 182 specimens from 26 individuals were tested for elevated chromosomal copy number (aneusomy) by FISH. There was a strong correlation between aneusomy and histological grade (Fig. 10).

The rates of aneusomy (high and low) in invasive carcinoma and CIS were 87% and 63%, respectively. However, even in the normal epithelium of this smoking population, there was some low aneusomy (14%). In hyperplastic epithelium, the rate of aneusomy, both high and low was 39% with 3 of 18 cases exhibiting high aneusomy. By chisquared test the association between histology score and aneusomy was significant at p = 2.88393E-05. In three of eight biopsies from four cases of regressive CIS, low aneusomy was found while in the remaining five biopsies there was no evidence of aneusomy. However, the relationship between the CIS staging classification and aneusomy by chisquared analysis was not significant (p = 0.470).

Expansion of aneuploid clones might be expected to yield similar aneusomy test results at multiple
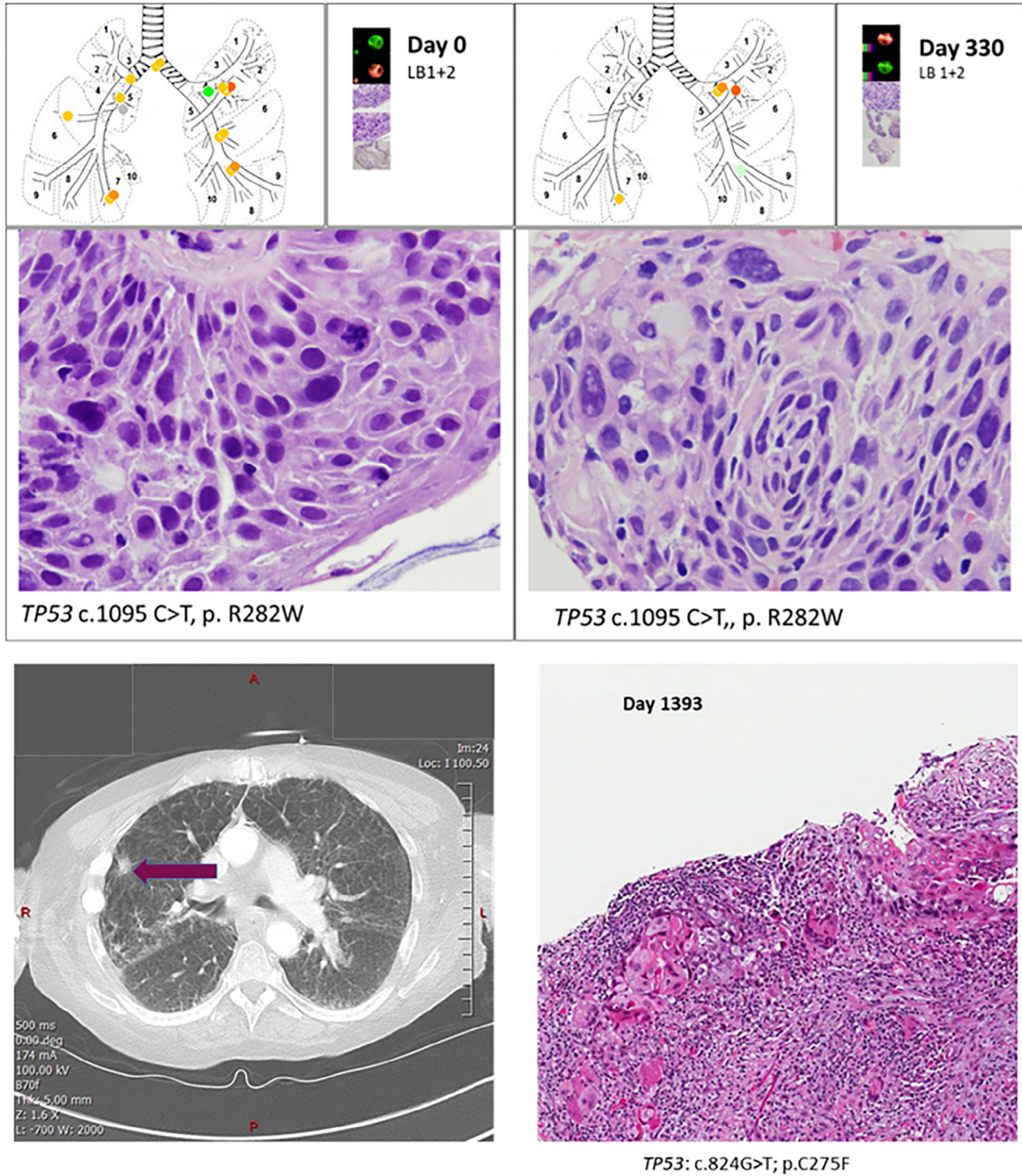
Fig. 7. In this patient, several sites of severe dysplasia were identified on initial bronchoscopy along with one focus of CIS at LB1 + 2 (upper left). Repeat biopsies at that site 11 months later again showed CIS with the same *TP53* mutation as in the first biopsy (Fig. 5 upper right). No evidence of invasive carcinoma was found. Nearly 4 years later the patient presented again, this time with invasive squamous carcinoma in the upper lobe on the right (contralateral) side (lower right). The invasive tumor had a different *TP53* mutation than that previously found in the left lung (lower right). The tumor was surgically removed but the patient died shortly after surgery.
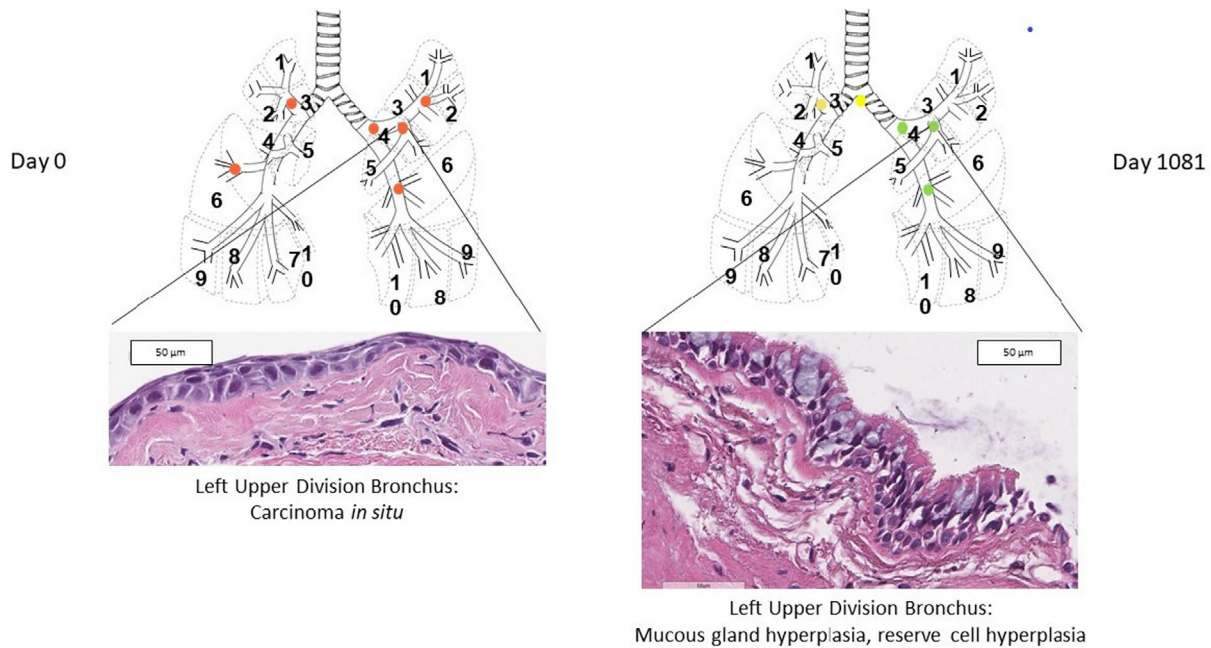
Fig. 8. This case was referred to the Colorado SPORE because of a consensus diagnosis of multifocal CIS. No definite TP53 mutations could be identified in these lesions A lesion in the left lung is illustrated in 5, left. By Day 1081 of observation without treatment, all CIS lesions had regressed.

tracheobronchial sites. High level aneusomy was found in 11 CIS cases. In six of these cases, high level aneusomy was present at multiple sites. In three cases, high level aneusomy was present in both invasive and noninvasive epithelium. In two cases high level aneusomy was found only in CIS.

TP53

One hundred twenty-seven specimens from 29 individuals were tested for *TP53* somatic mutation. Thirty-two mutations were found in 20 individuals. Mutations were correlated with histological score (Fig. 11). Sixty-five percent of the sites with invasive carcinomas proved to have a *TP53* mutation as did 30% of the CIS. Moderate to severe dysplasia also had a *TP53* mutation rate of slightly more than 18% in 38 biopsies from 12 individuals. Finally, no *TP53* mutations were found in 26 biopsies from 17 individuals showing low grade (mild) dysplasia or normal bronchial epithelium. This mutational distribution was significant at the $p = 3.07E-04$ by chi-squared test.

Same site testing was conducted for both aneusomy and *TP53* on 84 specimens. Sixty percent of biopsies with high aneusomy were *TP53* mutation positive while only 13% of those without evidence of aneusomy were mutation positive (Fig. 12). By chi-squared analysis the relationship between *TP53*

mutation and aneusomy was significant at $p = 4.61E-03$.

In six cases, *TP53* mutations were detected at multiple bronchial sites. In four of these cases the same mutation was found at separate sites, suggesting intraepithelial clonal expansion.

DISCUSSION

This study focuses on the highest grade noninvasive lesion of the central airways, CIS. In this microscopic lesion, the normal mucociliary epithelium is replaced by multilayered, mitotically active epithelium with irregular nuclei, high nuclear to cytoplasmic ratio and no recognizable orientation in relation to the mucosal surface [16]. Few studies specifically address CIS [15, 17] and these are short term and conducted using different protocols in variable patient populations. The present study takes advantage of the large cohort of high-risk subjects in University of Colorado SPORE premalignancy trials and provides information on the extent and distribution of these lesions in relation to invasive squamous carcinoma and the fate of lesions over an extended period of time. Several observations were made that may be of clinical importance.
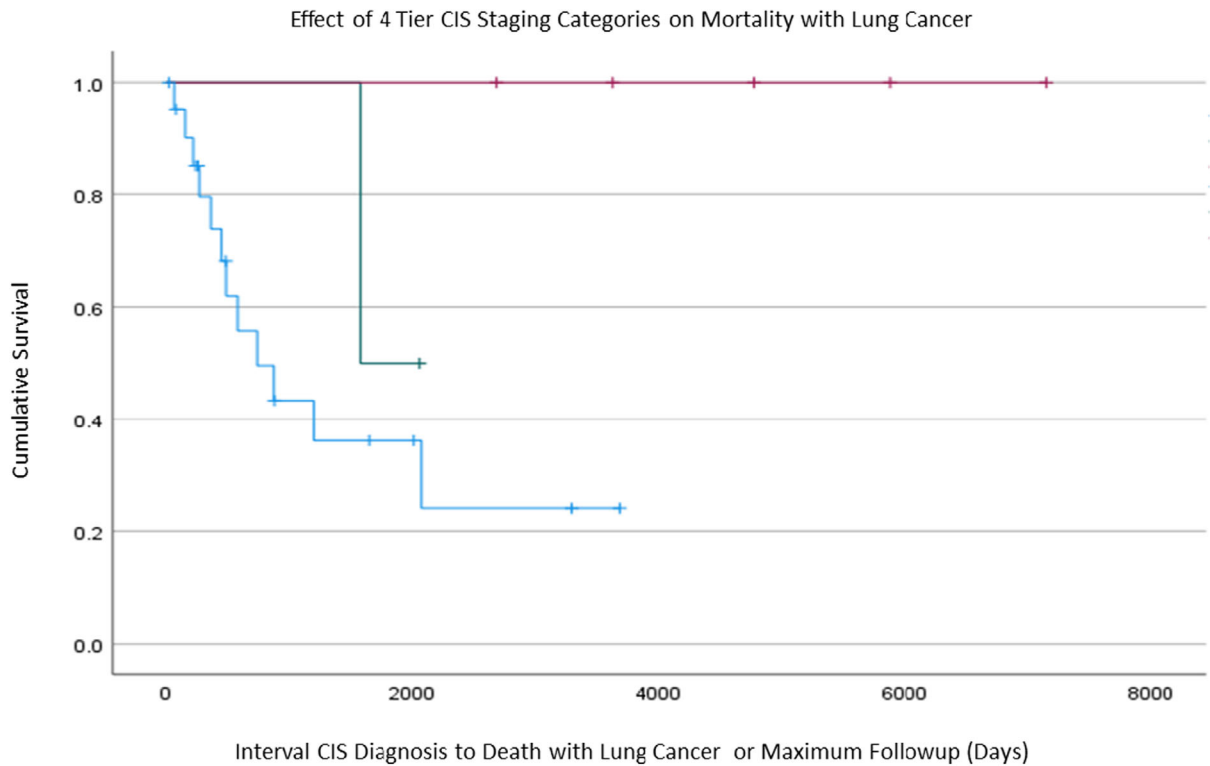
Fig. 9. Kaplan–Meier plot comparing mortality from lung cancer in patients with different CIS staging categories. Blue represents CIS associated with concurrent invasive carcinoma, green represents CIS with incident invasive carcinoma, and red represents regressive CIS. Crosses represent censored cases (maximum follow up time or death from unrelated causes).

We observed that most CIS was associated with concurrent invasive squamous carcinoma. In 22 of 31 cases (71%), invasive carcinoma was discovered at the same bronchoscopy or within 6 months of finding the initial CIS and was considered concurrent. This study also documents a dichotomy of behavior between two types of CIS lesions. In one type, CIS remains circumscribed within a few millimeter of invasive focus and does not extend laterally. The second type of CIS extends aggressively as a solid front into adjacent lobes and contralateral lung.

In a second type CIS recurred in the residual bronchial stump after resection of invasive carcinoma and eventually spread as a solid front across the main carina into the opposite lung. There, CIS formed secondary invasive squamous tumors with the same TP53 mutations as the CIS lesions, confirming the origins of the invasive tumors. This case documents the potential of CIS to spread through the airways and to form invasive tumors at multiple distant sites.

These cases raise the question of whether the CIS coexisting with invasive tumor is a precursor lesion or an extension from invasive tumor. We suggest

that the answer may be both. In some cases, cytological details in the CIS were less atypical than the corresponding invasive tumor while in others there was no discernable difference between the two types of lesions. In 10 invasive tumor/CIS pairs from 9 patients, FISH results were identical in five pairs and discrepant in four pairs. In all cases where there was a difference in FISH patterns, the invasive tumor exhibited a more severe aneusomy, consistent with clonal evolution to more unstable genome. In two cases where *in situ* and invasive carcinomas could both be sequenced, *TP53* mutations were identical in one case but differed in another. Taken together, molecular findings suggest that both progression to invasive carcinoma and lateral spread of already invasive tumor may explain the simultaneous presence of CIS and invasive carcinoma.

In rare cases, it is possible to identify CIS that transforms over time into invasive squamous carcinoma at the same site. CIS progressed to invasive carcinoma at the same site over a period of 18 months. A biopsy at 16 months showed progression of cellular atypia and a more complicated mutational profile. Additionally, the increased

## Relationship of Aneusomy to Histology Score*
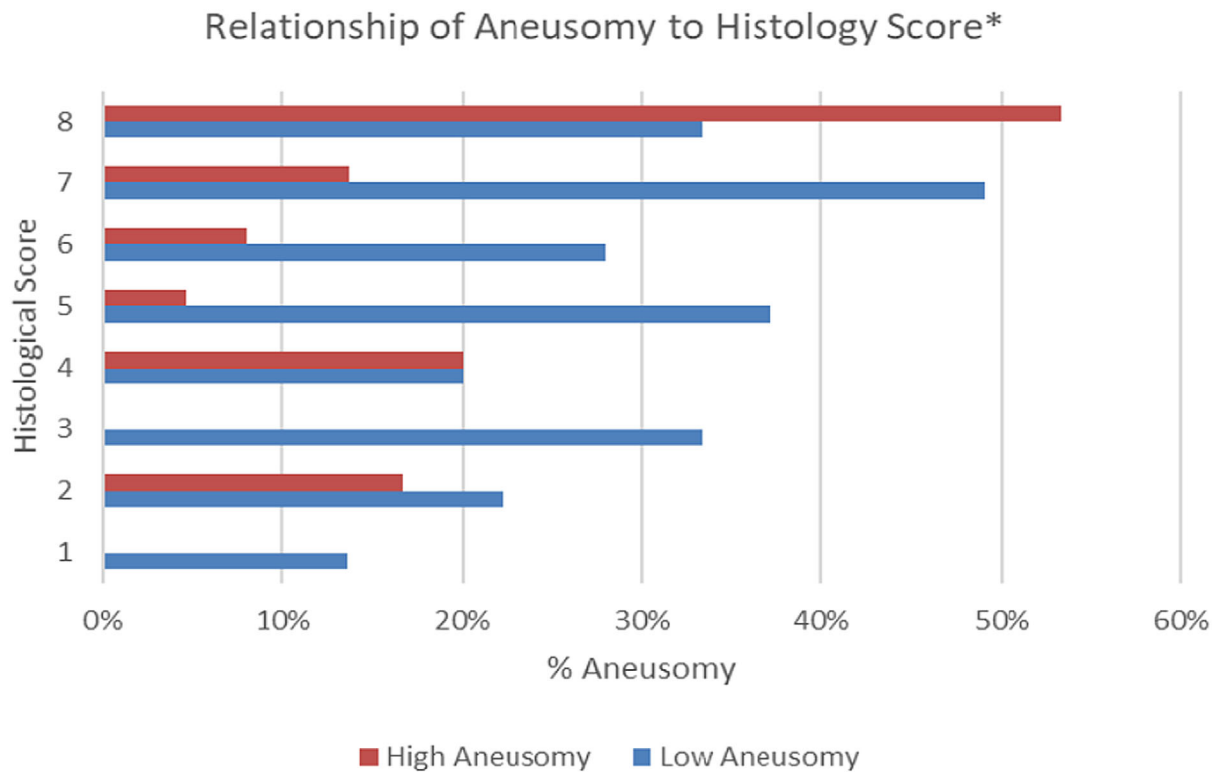
Fig. 10. Relationship between aneusomy and histology score (1. Normal 2. Reserve cell hyperplasia, 3. Squamous metaplasia, 4. Mild dysplasia, 5. Moderate dysplasia, 6. Severe dysplasia, 7. CIS, 8. Invasive carcinoma). Most high aneusomy was found in invasive tumors. This distribution was significant at the p = 2.88E-05 level.

Mutations and Histology Scores
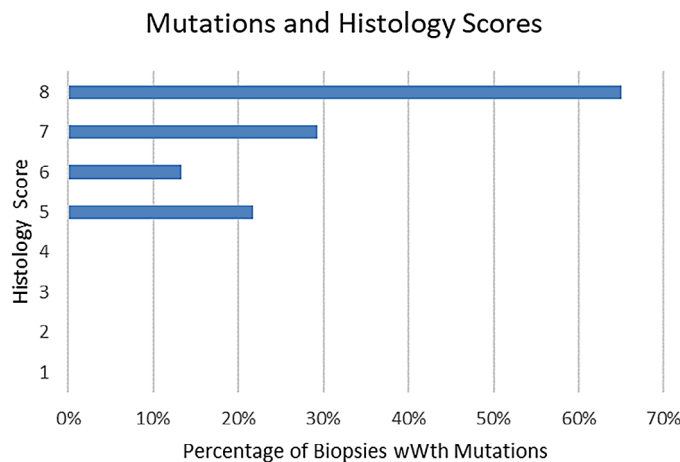
Fig. 11. *TP53* mutation in relation to histology score (WHO classification 1–8). All mutations occurred in biopsies with histology scores >4. This pattern was significant at the p = 3.07E-04 by chi-squared testing.

TP53 variant allele frequency at 16 months vs that seen in the baseline CIS, provides evidence that progressive CIS have a growth advantage that supports clonal expansion on the way to developing invasive features. This lesion was treated with stereotactic radiation and to this date several years later, the patient survives and is without evidence of either invasive or *in situ* carcinoma. This case provided an opportunity to observe transformation of neoplastic cells from *in situ* to invasive carcinoma at an extremely early stage. Microscopically invasive tumor was accompanied by increasing
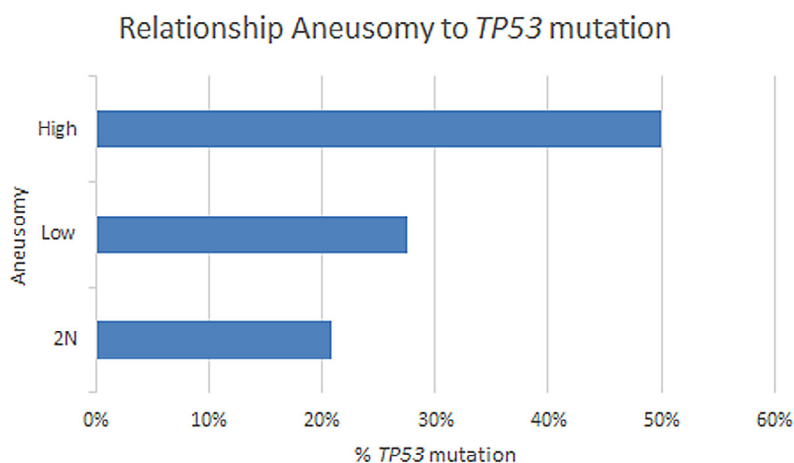
Fig. 12. Relationship between aneusomy and *TP53* mutation. Aneusomy was most frequent and severe in biopsies with *TP53* mutations.

molecular complexity of this lesion as it became invasive and supports current concepts of mutation load and clonal expansion ultimately leading to an invasive tumor [18–20].

A second patient who was discovered to have a small focus of invasive squamous carcinoma detectable only by histological examination. The lesion had a complete response to SBRT. This patient died several years after SBRT of unrelated causes. At autopsy, the entire airway was microscopically examined in serial sections and showed only radiation changes marking the site of the tumor but no evidence of carcinoma anywhere in the airways.

Both cases successfully treated by SBRT highlight the advantage of early detection of invasive carcinoma and provide incentive to continue to develop better methods for surveillance of the bronchial lining. However, these cases were extremely unusual and represent only two cases in over 1700 high-risk smokers enrolled in the SPORE program who underwent thousands of biopsies. These cases nevertheless represent proof of principle that CIS followed by or accompanying invasive cancer can, on occasion, be identified and successfully treated. This finding may be helpful in the design of future surveillance trials aimed at early detection and reduction of mortality from squamous carcinoma of central airway epithelial surfaces, a region of the lung where radiographic detection is less effective.

CIS may be an indication of field change and can precede the development of carcinomas elsewhere in the airways. This was illustrated by a case in which CIS was found in one area of the bronchi and was followed for several years without progression. However, a radiologically visible invasive tumor appeared in the opposite lung which ultimately resulted in the patient's demise. This case illustrates the challenge of dealing with the wide field of exposure that must be taken into account in patients with CIS that is a manifestation of field change.

In seven cases, isolated CIS without an invasive component showed reversion to lower grade or to normal respiratory epithelial histology. The explanation for this lack of progression is not certain at the present time. It could be argued that there could have been reader error in the interpretation of biopsy histology. This seems unlikely as biopsies were all reviewed by at least two expert lung pathologists and the diagnosis was a consensus agreement by all reviewers.

Regression of CIS is not unknown in other organs, notably uterine cervix. While lung and cervix are not highly analogous as cervical lesions have a well-established viral etiology, the finding does indicate that cells with ominous morphological appearances may not be clinically aggressive. In cervix, regression preferentially occurs in CIS lesions associated with subtypes other than HPV16 [21, 22]. At the present time, conservative management of CIS lesions until definitive evidence of stromal invasion occurs is appropriate.

These studies document complex molecular changes that accompany and confirm the histological abnormalities that define CIS. We found that histological score correlated with the presence of both TP53 mutation and aneuploidy, and that the presence of aneuploidy correlated with the presence of TP53 mutation. These findings support previous observations that TP53 is associated with invasive lung cancer and that aneuploidy is frequently present in high-grade airway dysplasia [13, 14]. It seems likely that the ablation of the TP53 checkpoint results in chromosomal mis segregation and aneuploidy since TP53 is known to play a role in

chromosomal segregation and its ablation may result in chromosomal mis-segregation [23, 24].

These studies also suggest that *TP53* mutation can be used as a measure of clonal expansion in the airways. Demonstration of the same mutation at multiple sites documented clonal spread in four cases in this series. This is consistent with the recently described spatial dispersion of clonal progenitors within invasive tumors [20, 25], that probably occurs in the premalignant airways as well [26]. Recent studies of CIS indicate advanced genomic instability with complex genetic, epigenetic, and transcriptomic changes that can be used to predict progression to invasive cancer or regression [12]. This hypothesis could not be directly explored in this series since comprehensive molecular characterization of these lesions was not feasible in this long-term study.

We were not able to find CIS persisting at a specific site for an extended period. The explanation of this finding may be that CIS rarely remained in a static condition and either progressed to invasive carcinoma or regressed. We observed that chromosomally unstable CIS lesions are likely to indicate invasive carcinoma while those lesions without the requisite degree of genetic abnormality spontaneously resolve.

These studies provide guidance for future management of CIS in clinical trials and suggest study design for further clinical investigations on how to detect and treat CIS. Isolated CIS is not a medical emergency and can be carefully monitored over an extended period. A second implication is that the number of CIS cases even in a large cohort of high-risk smokers is going to be small and large numbers of subjects will have to be collected, probably through multicenter trials, to establish criteria for prediction of progression to invasive carcinoma.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data in this study are not available for sharing.

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