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MYC and Human Telomerase Gene (*TERC*) Copy Number Gain in Early-stage Non–small Cell Lung Cancer

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Abstract

Objectives—We investigated the frequency of *MYC* and *TERC* increased gene copy number (GCN) in early-stage non–small cell lung cancer (NSCLC) and evaluated the correlation of these genomic imbalances with clinicopathologic parameters and outcome.

Materials and Methods—Tumor tissues were obtained from 113 resected NSCLCs. *MYC* and *TERC* GCNs were tested by fluorescence in situ hybridization (FISH) according to the University of Colorado Cancer Center (UCCC) criteria and based on the receiver operating characteristic (ROC) classification.

Results—When UCCC criteria were applied, 41 (36%) cases for *MYC* and 41 (36%) cases for *TERC* were considered FISH-positive. *MYC* and *TERC* concurrent FISH-positive was observed in 12 cases (11%): 2 (17%) cases with gene amplification and 10 (83%) with high polysomy. By using the ROC analysis, high *MYC* (mean 2.83 copies/cell) and *TERC* (mean 2.65 copies/cell) GCNs were observed in 60 (53.1%) cases and 58 (51.3%) cases, respectively. High *TERC* GCN was associated with squamous cell carcinoma (SCC) histology ($P = 0.001$). In univariate analysis, increased *MYC* GCN was associated with shorter overall survival ($P = 0.032$ [UCCC criteria] or $P = 0.02$ [ROC classification]), whereas high *TERC* GCN showed no association. In multivariate analysis including stage and age, high *MYC* GCN remained significantly associated with worse overall survival using both the UCCC criteria ($P = 0.02$) and the ROC classification ($P = 0.008$).

Conclusions—Our results confirm *MYC* as frequently amplified in early-stage NSCLC and increased *MYC* GCN as a strong predictor of worse survival. Increased *TERC* GCN does not have prognostic impact but has strong association with squamous histology.

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Keywords

MYC; *TERC*; FISH; non–small cell lung cancer; prognosis

Amplification of genes of the *MYC* family (*MYC*, *MYCN*, and *MYCL1*) has been described in a variety of cancer cell lines and tumor specimens including lung cancer.[1–4] The *MYC* gene localized at 8q24.1 is a well-characterized oncogene involved in cell growth, differentiation, metabolism, and apoptosis.[5]

The prognostic role of *MYC* amplification has been explored both in small cell lung cancer (SCLC) and non–small cell lung cancer (NSCLC). Lockwood et al[6] have investigated 104 cancer cell lines from different tumor tissue origins by array comparative genomic hybridization to identify amplified chromosomal segments. Among 53 lung cell lines, of which 36 were originated from NSCLC, 16 from SCLC, and 1 from Mesothelioma, *MYC* was the most frequently amplified gene.

Some studies on resected NSCLC reported that *MYC* was associated with tumor progression,[7,8] a worse prognosis, and its overexpression was related to metastasis of lung cancer.[9] Kubokura et al[10] showed that *MYC* amplification correlated with lymph node metastasis, suggesting a possible negative effect on survival. Iwakawa et al[11] showed that *MYC* amplification was associated with poor prognosis in patients both with small-sized (2 cm in greatest dimension) and early-stage I lung adenocarcinoma (ADC). Furthermore, *MYC* was expressed in large numbers of NSCLCs[12] and was amplified or overexpressed in SCC and ADC of the lung.[13–15] The *MYC* increased gene copy number (GCN) leads to overexpression of the MYC protein through Max heterodimer transcription factors that alter gene expression in large part by recruiting histone-modifying enzymes.[15]

The gain of sequences on the long arm of chromosome 3 (3q) is also a frequent event in many human malignant diseases, including lung cancer.[2] Human telomerase gene (*TERC*), localized on the chromosome 3q26, encodes the RNA component of human telomerase, a ribonucleoprotein enzyme, which acts as a template for the addition of telomeric repeat sequences 5′-TTAGGG-3′[16] required for the stability and complete replication of chromosome ends, and it has been associated with cell immortality and the development of cancers.

Recently, Fan et al[17] reported significantly higher percentages of cells with amplification of *TERC* in NSCLC than in SCLC, and the phenomenon occurred more frequently in SCC than in ADC.[18] Using FISH methodology, Pelosi et al[19] studied the 3q26 amplification in preneoplastic/preinvasive squamous cell lesions of the bronchial mucosa and in 2 subsets of lung SCC, the early hilar (EHSCC), and the parenchyma-infiltrating SCC (PISCC). The authors concluded that 3q26 amplification was likely a late event in the development of SCC of the lung and it is more prevalent in EHSCC than in PISCC, suggesting different pathogenesis for these tumor subtypes.

Foster et al[20] showed that the 3q26 amplification was a common feature of pulmonary SCC, confirming its key role in the transition from high-grade preinvasive neoplasia to invasive

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carcinoma, as also documented in uterine cervix[21] and head-and-neck SCC.[22] Yan et al[23] reported that 3q and 8q amplifications were significantly higher in smoker than those in nonsmoker SCC patients and was associated with tumorigenesis and/or progression of the disease.

In summary, only a few studies are available on *MYC* and *TERC* GCN in NSCLC, and no consensus criteria exist on how to assess the status of these genes as prognostic value. This study aimed to evaluate the *MYC* and *TERC* gene copy status in NSCLC by FISH using 2 criteria for interpretation, namely, the University of Colorado Cancer Center (UCCC) scoring system proposed for EGFR in lung cancer[24] and the receiver operating characteristics (ROC) scoring system. We also evaluated the correlation of these genomic imbalances with clinicopathologic parameters and outcome in resected NSCLC patients.

MATERIAL AND METHODS

Patient Selection

This retrospective study was conducted in a cohort of 113 NSCLC patients who received a radical resection for primary NSCLC at the Thoracic Surgery Unit of the Perugia University at S. Maria della Misericordia Hospital, Italy, between 2002 and 2006. Histologic subtypes and grade of differentiation were determined according to the World Health Organization classification.[25] The only criteria used for patient selection was availability of tumor tissue from primary lung cancer and survival data. Neither chemotherapy nor radiotherapy was administered before surgery. A follow-up, including a chest x-ray at 3-month intervals alternated with a total body computed tomography (CT) scan every 6 months, was scheduled for all patients for the first 2 years. Subsequently, the patients underwent a CT scan/y.

Recurrences were detected by imaging techniques and when necessary, confirmed by histologic sampling. The study was reviewed and approved by the local institution's Ethics Committee, and written informed consent was obtained from each patient for surgical specimen analyses.

FISH Assay

FISH assays were carried out on 4 μm (± 1 μm) thick sections from formalin-fixed, paraffin-embedded tissue blocks from surgically resected tumor specimens of NSCLC patients. The 2-color (TERC-MYC) FISH probe was prepared combining LSI TERC Spectrum Gold and LSI c-MYC Spectrum Aqua, both reagents from Abbott Molecular. Slides were incubated at 56 ± 2°C overnight and incubated in CitriSolv 2 times for 10 minutes each and air dried. Thereafter, specimens were dehydrated in 100% ethanol twice, for 5 minutes each, air dried, and subsequently incubated in 2 × saline sodium citrate (SSC) at 75°C for 9 to 25 minutes, 0.25 mg/mL proteinase K at 45°C for 10 to 25 minutes, and 2 – SSC at room temperature for 5 minutes, and dehydrated in 70%, 85%, and 100% ethanol series for 2 minutes each, and air dried. The probe mixture was applied to the target areas of each slide, which were covered with glass coverslips and sealed with rubber cement. Slides were incubated at 85 ± 1°C in a dry oven for 15 minutes to codenature probe and target DNAs and incubated in moisture chamber at 37 ± 1°C for 16 hours. Coverslips were then removed and the slides

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were immersed twice in 2 × SSC/0.3% NP-40 at 73 ± 1°C for 2 minute each, then washed in 2 × SSC at room temperature, dehydrated in 70%, 85% and 100% ethanol, and air dried. Vectashield DAPI in mounting medium (14 mL of 0.3 ug/mL) was applied and the areas were covered with 22 × 50 mm coverslips.

Analysis was performed using fluorescence microscope (Zeiss AxioImager). For documentation, images were captured using a charge-coupled device camera (CoolSnap, Photometrics) and merged using dedicated software (CytoVision, Leica Micro- systems). The scoring was carried out in 100 nonoverlapping tumor cell nuclei per patient from 4 representative tumor areas. According to the Colorado criteria for *EGFR*,[24] the GCN for each gene was classified as increased (FISH-positive) when displaying gene amplification (> 10% of tumor cells with >15 copies of the signals or gene clusters [> 4 gene copies per cluster] or innumerable tight gene clusters) and high polysomy (40% of cells displaying 4 copies of the specific gene signal).

Statistical Analysis

The primary end point was to assess whether *MYC* and *TERC* GCN affected survival of surgically resected NSCLC. We used the ROC classification system as a continuous variable to determine a cutoff point for *MYC* and *TERC* GCN.[26] Sensitivity and specificity were expressed in terms of percentage, and the highest value has been chosen as the best cutoff point in discriminating patients who survived compared with those who died. Overall survival (OS) was defined as the time from surgery to the date of death from any cause; patients who were not reported as having died at the time of the analysis were censored at the date they were last known to be alive. Disease-free survival (DFS) was defined as the time from surgery to first local, regional, or distant recurrence; second primary malignancy; or death from any cause, whichever came first. Patients who were alive and did not experience recurrence at the time of the analysis were censored at the last disease assessment date. OS, DFS, and the 95% confidence intervals (CIs) were evaluated by the Kaplan-Meier method comparing the different groups by log-rank test. The Cox proportional hazards model was used to evaluate the prognostic role of each single studied parameter on OS and DFS, in univariate and multivariate analyses. The χ^2 test was used to assess the association between clinical features and the *MYC* and *TERC* GCN. Unless otherwise specified, all tests are with 1 *df*. A probability value of <0.05 was considered as statistically significant. Statistical analysis was carried out using Matlab software (the MathWorks version 7.2.0.232).

RESULTS

Patient Characteristics

The clinical characteristics of all 113 patients are summarized in Table 1. The vast majority of patients were men (84%) and in early pathologic stages (78%; stage I to II). Median age was 66.4 years (range, 40 to 84 years). Most of the patients were diagnosed with either SCC (n = 58, 51.3%) or ADC/bronchioloalveolar carcinomas (n = 38, 33.6%). Patients with ADC were younger than those with SCC ($P = 0.05$). Former or current smokers represented 92% of all patients and were more common in SCC than in ADC ($P = 0.001$). Well- differentiated

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grading was found more frequently in ADC than in SCC patients ($P < 0.0001$). Median OS was 49.5 months (range, 0.5 to 99.7 mo), and median DFS was 41.6 months (range, 0.49 to 99.7 mo). As expected, mean OS and mean DFS were much longer for patients in stage I to II than in stage III (mean ± SD, 53.9 ± 3.0 mo vs. 34.2 ± 5.9 mo; $P = 0.0016$ and 47.2 ± 3.4 vs. 22.1 ± 5.6 mo; $P = 0.0003$, respectively).

Following surgery, 5 patients (4.4%) were treated with chemotherapy, 10 (8.9%) with radiotherapy, 1 (0.9%) with chemoradiotherapy, and the remaining 97 (85.8%) received no adjuvant treatment.

MYC and *TERC* FISH

Increase in the *MYC* GCN was measured using FISH and analyzed according to the Colorado scoring system and the ROC classification. When Colorado criteria were applied, 41 (36.2%) cases in total were considered *MYC* FISH-positive. Specifically, 32 (78%) patients showed high polysomy and 9 (22%) showed gene amplification (GA) (Fig. 2B). *MYC* high polysomy patients included 18 (56.2%) SCC, 9 (28.1%) ADC, 4 (12.5%) large cell carcinoma, and 1 (3.2%) mixed histology; gene amplification patients included 5 (55.6%) SCC, 2 (22.2%) ADC, and 2 (22.2%) mixed histology. *MYC* FISH status was not associated with sex, smoking history, histologic types, grading, or pathologic stage (Table 2). Using the ROC classification, a cutoff of *MYC* copy number per cell at 2.83 identified an area under the curve of 0.62 with sensitivity of 0.61 and specificity of 0.60 (Fig. 1A). Using this cutoff, high *MYC* GCN (2.83 copies per cell) was observed in 60 (53.1%) cases, and no significant differences were observed in sex, smoking status, histology, grading, or pathologic stage between patients with and without high *MYC* GCN (Table 2).

TERC GCN was similarly evaluated according to both the Colorado scoring system and ROC classification. When Colorado scoring was applied, 41 (36.2%) cases were considered *TERC* FISH-positive, of which 26 (23.0%) showed high polysomy and 15 (13.3%) showed gene amplification (Fig. 2C). *TERC* high polysomy patients included 20 (76.9%) SCC, 2 (7.7%) ADC, 1 (3.9%) large cell carcinoma, and 3 (11.5%) mixed histology; gene amplification patients included 11 (73.4%) SCC, 3 (20%) ADC, and 1 (6.6%) large cell carcinoma. *TERC* FISH-positive was associated with SCC histology (75.6% vs. 24.4% in non-SCC; $P = 0.0001$) but no significant differences were observed with sex, smoking history, grading, or pathologic stage (Table 2). The ROC curve classification identified a cutoff of TERC copy number per cell at 2.65 for an area under the curve of 0.57, with sensitivity of 0.58 and specificity of 0.52 (Fig. 1B). Using this cutoff, high *TERC* GCN was observed in 58 (51.3%) cases (mean per cell 2.65 copies). *TERC* FISH-positive was associated with SCC histology (72.4% vs. 29.1% in non-SCC; $P < 0.0001$) but was not associated with other clinicopathologic features (Table 2).

Concurrent *MYC* and *TERC* FISH-positive patterns were observed in 12 cases (11%): 2 (17%) cases with GA and 10 (83%) cases with high polysomy for both genes.

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Survival Analysis

At a median follow-up time of 53.9 months, 55 patients (48.6%) had died: 41 (74.5%) deaths were due to disease recurrence and 14 (25.5%) to unrelated causes. Eleven (18.9%) of the 58 patients still on follow-up experienced recurrence: local recurrence was observed in 7 patients (63.6%) and recurrence in lung and other sites in 4 patients (36.4%).

We first analyzed patient survival according to *MYC* and *TERC* FISH status based on the UCCC criteria. In the univariate analysis summarized in Table 3, *MYC* FISH-positive showed a shorter DFS and OS than *MYC* FISH-negative NSCLC patients (median DFS, not reached vs. 26.5 mo; $P = 0.032$, Fig. 3A; median OS, not reached vs. 45.5 mo; $P = 0.032$, Fig. 3B). No difference in DFS and OS was found between *TERC* FISH-positive and negative patients. Similar results were observed for each of the genes adopting the ROC classification. Patients with high *MYC* GCN (2.83 per cell) showed a tendency for shorter DFS than those with low *MYC* GCN (median DFS, not reached vs. 33.3 mo; $P = 0.06$, Fig. 3C). Patients with high *MYC* GCN (2.83 per cell) had shorter OS than those with low *MYC* GCN (median OS, not reached vs. 42.9 mo; $P = 0.02$, Fig. 3D). No difference in DFS and OS was detected according to high *TERC* GCN (2.65per cell) or low *TERC* GCN (< 2.65 per cell).

Two multivariate Cox regression models for DFS and OS were built according to UCCC criteria (model 1) and ROC classification (model 2). Both the multivariate Cox regression models included the variables (stage and age) that were found significant at the univariate analysis. Increased *MYC* FISH-positive according to the UCCC criteria or ROC classification remained significantly associated with worse DFS (HR, 1.92; 95% CI, 1.09-3.38; $P = 0.02$ and HR, 1.97; 95% CI, 0.97-3.54; $P = 0.02$, respectively) and OS (HR, 1.01; 95% CI, 1.07-2.97; $P = 0.02$ and HR, 2.05; 95% CI, 1.20-3.50; $P = 0.008$, respectively). The stage (II to III vs. I) was confirmed to be an independent poor prognostic factor for DFS (model 1: HR, 3.68; 95% CI, 2.01-6.73; $P < 0.0001$; model 2: HR, 3.52; 95% CI, 1.93-6.43; $P < 0.0001$) and OS (model 1: HR, 2.68; 95% CI, 1.56-4.61; $P < 0.0001$; model 2: HR, 2.88; 95% CI, 1.66-4.98; $P < 0.0001$) (Table 4).

DISCUSSION

To our knowledge, this is the first study, investigating in the same time the prognostic role of both *MYC* and *TERC* GCN in early NSCLC patients. Our results showed that high *MYC* GCN, an event occurring in approximately half of the patients, was an independent prognostic factor in resected NSCLC. In our series, high *MYC* GCN (either by UCCC criteria or ROC classification) was significantly associated with worse OS. These results are essentially consistent suggesting that NSCLC tumors with increased *MYC* GCN harbor biologically aggressive phenotypes as previously reported by other authors.[10,11] The study of Kubokura et al[10] conducted on a small number (31 patients) of resected NSCLC patients showed that *MYC* amplification correlated with lymph node metastasis, suggesting a possible negative effect on survival. Recently, Iwakawa et al[11] found in 65 cases of small-sized ADCs by GeneChip Human Mapping 10-K SNP array and in 162 stage I lung ADCs based on real-time genomic PCR that MYC amplification was a prognostic marker for patients with both small-sized and early-stage I lung ADCs. Two studies by Volm et al[9,27]

reported that tumors with MYC overexpression protein showed a significant increased of metastasis in early NSCLC patients.

In several studies, the frequency of *MYC* amplification in NSCLC has been variably reported to be from 5% to 88% in patients having NSCLC who were not treated with EGFR-TKIs.[1,6,10] These studies have used FISH method but with different interpretation criteria, which may account for the wide range of rates detected. To address this question, we analyzed *MYC* gene amplification in NSCLC using FISH and 2 interpretation criteria: the UCCC criteria and the ROC classification. FISH is generally accepted as more accurate than RT- PCR for evaluating GCN because it allows in situ examination of tumor cells, with little or no contamination from non-neo- plastic cells. Nevertheless, the interpretation criteria of *MYC* GCN using FISH in NSCLC have not been well established. The UCCC criteria used in our manuscript at the state of the art is the standard scoring method applied in clinical studies regarding not only EGFR but also other target genes.[24] In a recent study in advanced NSCLC, Cappuzzo et al,[28] using ROC analysis, defined high *MYC* GCN as a mean of 2.8 copies per cell for the evaluation of *MYC* amplification to discriminate responsive from nonresponsive patients to EGFR- TKIs, and demonstrated that high *MYC* GCN was detected in 53.7% of patients without significant association with clinicopathologic features. In agreement with this study, we also found high *MYC* GCN in 53.1% (by ROC classification) of our population, without significant correlation between increased *MYC* GCN (either by UCCC criteria or ROC classification) and sex, smoking history, histology, grading, and stage. Kubokura et al[10] reported a much higher incidence of *MYC* amplification in NSCLC (88%), probably because of the less stringent criteria used for defining gene amplification (*MYC* GA defined as *MYC* > centromere 8).

Beyond its prognostic value, *MYC* has been considered as a possible specific target of biological treatment as demonstrated in a preclinical mouse model of Ras-induced lung ADC.[29] Interestingly, Cappuzzo et al[28] showed that the contemporary amplification of *MYC* and eukaryotic translation initiation factor 3 subunit H (EIF3H) was associated with better outcome after gefitinib therapy; among EGFR-positive patients (FISH-positive and/or mutated), only individuals with increased *MYC/EIF3H* GCN had a significant tumor shrinkage. The results of this study showed for the first time that MYC and EIF3H were coamplified in NSCLC, and this biologic event could positively affect the response and survival of patients treated with EGFR-TKIs. However, these data originated from a retrospective analysis and must be validated with a prospective study. The mechanism responsible for the highest sensitivity to anti-EGFR agents in presence of *MYC* GCN gain is not clear; however, similar results have been detected in breast carcinomas.[30]

TERC has been suggested to be a novel putative target in 3q26[31] for molecular therapy of NSCLC, as some strategies have been evaluated to target the proteins associated with telomerase such as small molecules, antisense RNA, and ribozymes[32] but without a clear impact on the clinical practice up to now.

We found *TERC* FISH positivity in 36.2% of cases by the UCCC criteria and high *TERC* GCN in 51.3% of cases according to ROC classification, and no statistically significant difference in DFS and OS was observed between patients with positive and negative *TERC*

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GCN using both criteria. This observation confirmed that high *TERC* GCN is not a prognostic factor for NSCLC, but it showed phenotypic properties strongly associated with SCC histology using both criteria (UCCC, $P = 0.0001$; ROC, $P < 0.0001$). These data agree with earlier studies of NSCLC in which DNA copy number gains at 3q26 occurred more frequently in SCC than in ADC.[18]

Contrary to the observations of Yan et al[23] that the amplification of the chromosomal arm 3q (in particular 3q26.2-q29) was significantly higher in smoker than in non-smoker SCC patients, we found no association between high *TERC* GCN and smoking history. However, this could be because of the low percentage of nonsmoker patients (7.9% cases) in our population.

Recently, Eid et al[33] found that *TERC* amplification and grading were significantly correlated with cervix cancer. Furthermore, there was significant correlation between *MYC* amplification and grading. *TERC* and *MYC* genes amplification were correlated and showed an inverse correlation with patients' age. The study highlighted the importance of using *TERC* and *MYC* copy number gain by FISH in cervix cancer or premalignant lesions as a sensitive technique for early diagnosis and poor prognostic assessment.

In conclusion, both criteria (UCCC criteria and ROC classification) used to evaluate *MYC* genomic status showed similar results regarding survival, even though ROC classification seems to be more appropriate in terms of predicting the prognosis of NSCLC patients. Moreover, our data suggest that NSCLC with high *MYC* GCN is able to develop a more aggressive behavior, possibly representing as a biomarker, as tumors with high *MYC* GCN showed a significantly worse survival. Furthermore, high *TERC* GCN showed to be as a typical feature of lung SCC. Finally, the limited number of patients and the short follow-up in our study require that these results be interpreted cautiously and be confirmed in large prospective trials.

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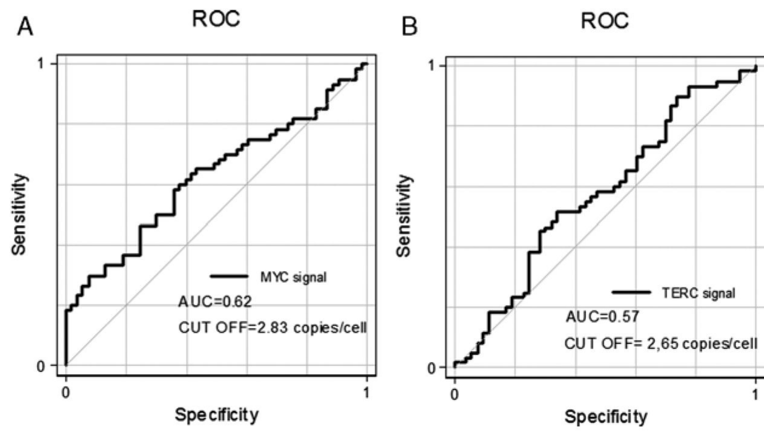

FIGURE 1.
Receiver operating characteristic (ROC) curves for *MYC* (A) and *TERC* (B).

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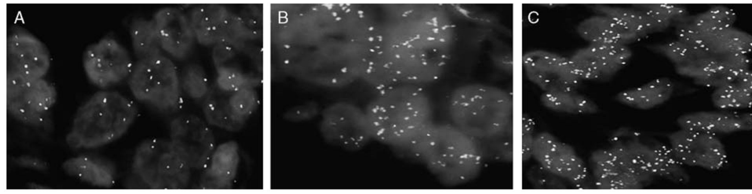

FIGURE 2.

Non–small cell lung cancer sections hybridized with the MYC (Spectrum Aqua)/TERC (Spectrum Gold) probe set showing low copy numbers (A), high copy numbers for *MYC* (B), and high copy numbers for *TERC* (C).

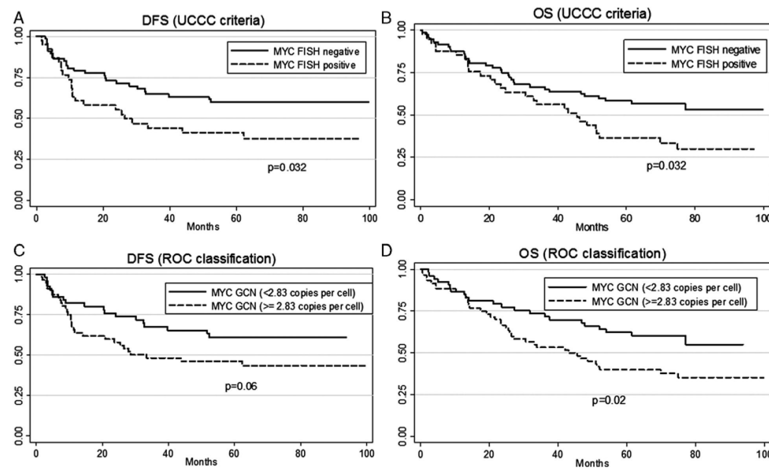

FIGURE 3.

Kaplan-Meier curves of disease-free survival (DFS) and overall survival (OS) according to *MYC* FISH-positive and MYC FISH- negative based on the University of Colorado Cancer Center (UCCC) criteria (A and B) and according to *MYC* gene copy number (GCN) 2.83 per cell and *MYC* GCN < 2.83 per cell based on the receiver operating characteristic (ROC) classification (C and D) in non–small cell lung cancer (NSCLC) patients. FISH indicates fluorescence in situ hybridization.

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TABLE 1

Clinicopathologic Characteristics of NSCLC Patients According to Histologic Types

	SCC (n = 58)	ADC (n = 38)*	Others (n = 17)†	*P*
Age (y)				
Median	69	65	68	0.05
Mean ± SD	67.4 ± 7.5	64.5 ± 9.6	67 ± 7.3	
Sex, n (%)				
Male	53 (91.4)	28 (73.7)	14 (82.4)	0.05
Female	5 (8.6)	10 (26.3)	3 (17.6)	
Smoking status, n (%)				
Former/current	58 (100.0)	31 (81.6)	15 (88.2)	0.001
Never	0 (0.0)	7 (18.4)	2 (11.8)	
pTNM stage, n (%)				
I	34 (58.6)	28 (73.7)	8 (47.1)	0.11
II	13 (22.4)	2 (5.3)	3 (17.7)	
III	11 (19.0)	8 (21.0)	6 (35.3)	
Grading, n (%)				
Well differentiated	4 (6.9)	8 (21.0)	0 (0.0)	< 0.0001
Moderately differentiated	31 (53.5)	20 (52.6)	0 (0.0)	
Poorly differentiated	23 (39.6)	10 (26.4)	17 (100.0)	

ADC indicates adenocarcinoma; SCC, squamous cell carcinoma.

*ADC category also includes bronchioloalveolar carcinoma.

†Other histology include large cell carcinoma (n = 9) and mixed histology (n = 8).

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TABLE 2

Clinicopathologic Variables of Patients According to *MYC* and *TERC* Gene Copy Status Based on the UCCC Criteria and ROC Classification

	UCCC Criteria		ROC Classification		UCCC Criteria		ROC Classification	
	***MYC* FISH + (n = 41, 36.2%)**	***MYC* FISH − (n = 72, 63.8%)**	***MYC* 2.83 (n = 60, 53.1%)**	***MYC*<2.83 (n = 53, 46.9%)**	***TERC* FISH + (n = 41, 36.2%)**	***TERC* FISH − (n = 72, 63.8%)**	***TERC* 2.65 (n = 58, 51.3%)**	***TERC*<2.65 (n = 55, 48.7%)**
Sex, n (%)								
Female	6 (14.6)	12 (16.7)	9 (50.0)	9 (50.0)	4 (9.8)	14 (19.4)	7 (38.9)	11 (66.1)
Male	35 (85.4)	60 (83.3)	51 (53.6)	44 (46.3)	37 (90.2)	58 (80.6)	51 (53.7)	44 (46.3)
P	1.0		0.77		0.28		0.25	
Smoking, n (%)								
Former/ current	40 (97.6)	64 (88.9)	56 (53.8)	48 (46.3)	40 (97.6)	64 (88.9)	56 (53.8)	48 (46.2)
Never	1 (2.4)	8 (11.1)	4 (44.4)	5 (55.6)	1 (2.4)	8 (11.1)	2 (22.2)	7 (77.8)
P	0.15		0.58		0.15		0.06	
pTNM stage, n (%)								
I	25 (61.0)	45 (62.5)	39 (55.7)	31 (44.2)	24 (58.5)	46 (63.9)	36 (51.4)	34 (48.6)
II	9 (22.0)	9 (12.5)	10 (55.6)	8 (44.8)	7 (17.1)	11 (15.3)	10 (55.6)	8 (44.4)
III	7 (17.0)	18 (25.0)	11 (44.0)	14 (56.0)	10 (24.4)	15 (20.8)	12 (48.0)	13 (52.0)
P	0.32		0.58		0.81		0.88	
Histology, n (%)								
Squamous	23 (56.1)	35 (48.1)	32 (55.2)	26 (44.8)	31 (75.6)	27 (37.5)	42 (72.4)	16 (27.6)
Non Squamous	18 (43.9)	37 (51.4)	28 (50.9)	27 (40.1)	10 (24.4)	45 (62.5)	16 (29.1)	39 (20.9)
P	0.56		0.65		0.0001		< 0.0001	
Grading, n (%)								
Well	2 (4.9)	10 (13.9)	6 (50.0)	6 (50.0)	3 (7.3)	9 (12.5)	6 (50.0)	6 (50.0)
Moderately	19 (46.3)	32 (44.4)	27 (53.0)	24 (47.0)	20 (48.8)	31 (43.0)	24 (47.1)	27 (52.9)
Poorly	20 (48.8)	30 (41.7)	27 (54.0)	23 (46.0)	18 (43.9)	32 (44.5)	25 (50.0)	25 (50.0)
P	0.34		0.56		0.72		0.95	

FISH indicates fluorescence in situ hybridization; ROC, receiver operating characteristic classification; UCCC, The University of Colorado Cancer Center criteria.

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TABLE 3

Univariate Analysis of Disease-free Survival and Overall Survival for Variables Considered

Variables	Disease-free Survival			Overall Survival		
	HR	**95% CI**	***P***	**HR**	**95% CI**	***P***
Sex (male/female)	0.82	0.39-1.73	0.60	0.78	0.31-1.57	0.48
Age (y) continuous	1.01	0.98-1.05	0.25	1.03	1.00-1.06	0.042
Smoking (never/ever)	0.86	0.31-2.38	0.77	0.70	0.30-1.64	0.42
Histology (squamous/other)	1.27	0.76-2.13	0.35	1.05	0.65-1.68	0.85
Grading (G3/G1-G2)	1.53	0.92-2.55	0.10	1.5	0.93-2.40	0.095
TNM stage (II-III/I)	2.86	1.64-4.97	< 0.0001	2.47	1.48-4.12	0.001
MYC FISH (positive/negative)[*]	1.84	1.05-3.24	0.032	1.74	1.05-2.90	0.032
MYC GCN (2.83/<2.83 copies)[†]	1.71	0.96-3.04	0.06	1.80	1.06-3.06	0.02
TERC FISH (positive/negative)[*]	1.49	0.84-2.63	0.16	1.53	0.91-256	0.10
TERC GCN (2.65/<2.65 copies)[†]	1.34	0.76-2.37	0.30	1.37	0.82-2.29	0.22

CI indicates confidence interval; FISH, fluorescence in situ hybridization; GCN, gene copy number; HR, hazard ratio.

[*]Using the University of Colorado Cancer Center (UCCC) criteria the tumors were considered FISH-positive when 4 gene copies displayed in 40% of tumor cells.

[†]Receiver operating characteristic (ROC) classification.

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TABLE 4

Multivariate Analyses of Disease-free Survival and Overall Survival for Variables Considered

	Multivariate Analysis					
	Disease-free Survival			Overall Survival		
Variables	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Model 1						
MYC FISH (positive/negative)*	1.92	1.09-3.38	0.02	1.01	1.07-2.97	0.02
TNM stage (II-III/I)	3.68	2.01-6.73	< 0.0001	2.68	1.56-4.61	< 0.0001
Age (y) continuous	1.0	0.97-1.04	0.64	1.01	0.98-1.05	0.26
Model 2						
MYC GCN (2.83/<2.83 copies)†	1.97	0.97-3.54	0.02	2.05	1.20-3.50	0.008
TNM stage (II-III/I)	3.52	1.93-6.43	< 0.0001	2.88	1.66-4.98	< 0.0001
Age (y) continuous	1.01	0.97-1.04	0.49	1.02	0.98-1.05	0.18

CI indicates confidence interval; FISH, fluorescence in situ hybridization; GCN, gene copy number; HR, hazard ratio.

*Using the University of Colorado Cancer Center (UCCC) criteria the tumors were considered FISH-positive when 4 gene copies displayed in 40% of tumor cells

†Receiver operating characteristic (ROC) classification.