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Clinicopathologic and prognostic significance of *c-MYC* copy number gain in lung adenocarcinomas

A N Seo[1,2], J M Yang[1], H Kim[1,3], S Jheon[4,5], K Kim[4,5], C T Lee[6,7], Y Jin[1,3], S Yun[1,3], J-H Chung*[,1,3] and J H Paik*[,1,3]

[1]Department of Pathology, Seoul National University Bundang Hospital, Seoul National University College of Medicine, 300 Gumi-dong, Bundang-gu, Seongnam, Gyeonggi 463-707, Korea; [2]Department of Pathology, Kyungpook National University College of Medicine, 680 Gukchaebosang-ro, Jung-gu, Daegu 700-842, Korea; [3]Department of Pathology, Seoul National University College of Medicine, 28 Yeongon-dong, Jongno-gu, Seoul 110-799, Korea; [4]Department of Thoracic Surgery, Seoul National University Bundang Hospital, Seoul National University College of Medicine, 300 Gumi-dong, Bundang-gu, Seongnam, Gyeonggi 463-707, Korea; [5]Department of Thoracic Surgery, Seoul National University College of Medicine, 28 Yeongon-dong, Jongno-gu, Seoul 110-799, Korea; [6]Department of Internal medicine, Seoul National University Bundang Hospital, Seoul National University College of Medicine, 300 Gumi-dong, Bundang-gu, Seongnam, Gyeonggi 463-707, Korea and [7]Department of Internal medicine, Seoul National University College of Medicine, 28 Yeongon-dong, Jongno-gu, Seoul 110-799, Korea

Background: *c-MYC* copy number gain (*c-MYC* gain) has been associated with aggressive behaviour in several cancers. However, the role of *c-MYC* gain has not yet been determined in lung adenocarcinomas classified by genetic alterations in epidermal growth factor receptor (*EGFR*), *KRAS*, and anaplastic lymphoma kinase (*ALK*) genes. We investigated the clinicopathologic and prognostic significance of *c-MYC* gain for disease-free survival (DFS) and overall survival (OS) according to *EGFR*, *KRAS*, and *ALK* gene status and stages in lung adenocarcinomas.

Methods: In 255 adenocarcinomas resected in Seoul National University Bundang Hospital from 2003 to 2009, fluorescence *in situ* hybridisation (FISH) with *c-MYC* probe and centromeric enumeration probe 8 (CEP8) was analysed using tissue microarray containing single representative core per each case. *EGFR* (codon 18 to 21) and *KRAS* (codon 12, 13, and 61) mutations were analysed by polymerase chain reaction and direct sequencing method from formalin-fixed, paraffin-embedded tissue sections. *ALK* rearrangement was determined by FISH method. *c-MYC* gain was defined as >2 copies per nucleus, chromosome 8 gain as $\geqslant 3$ copies per nucleus, and gain of *c-MYC*:CEP8 ratio (hereafter, *c-MYC* amplification) as $\geqslant 2$.

Results: We observed *c-MYC* gain in 20% (51 out of 255), chromosome 8 gain in 5.5% (14 out of 255), *c-MYC* amplification in 2.4% (6 out of 255), *EGFR* mutation in 49.4% (118 out of 239), *KRAS* mutation in 5.7% (7 out of 123), and *ALK* rearrangement in 4.9% (10 out of 205) of lung adenocarcinomas. *c-MYC* gain was observed in 19% (22 out of 118) of patients with lung adenocarcinomas with an *EGFR* mutation, but not in any patients with a *KRAS* mutation, or an *ALK* rearrangement. *c-MYC* gain (but not chromosome 8 gain or *c-MYC* amplification) was an independent poor-prognostic factor in the full cohort of lung adenocarcinoma ($P=0.022$, hazard ratio (HR) $=1.71$, 95% confidence interval (CI), 1.08–2.69 for DFS; $P=0.032$, HR $=2.04$, 95% CI, 1.06–3.91 for OS), as well as in stage I subgroup ($P=0.023$, HR $=4.70$, 95% CI, 1.24–17.78 for DFS; $P=0.031$, HR $=4.65$, 95% CI, 1.15–18.81 for OS), and in *EGFR*-mutant subgroup ($P=0.022$; HR $=2.14$; 95% CI, 1.11–4.10 for DFS).

Conclusions: *c-MYC* gain (but not chromosome 8 gain or *c-MYC* amplification) was an independent poor-prognostic factor for DFS and OS in lung adenocarcinomas, both in full cohort and stage I cancer, and possibly for DFS in *EGFR*-mutant adenocarcinomas. Additional studies are required to determine if patients with lung adenocarcinoma with *c-MYC* gain are candidates for additional first-line treatment to mitigate their increased risk for disease progression and death.

*Correspondence: Professor JH Paik; E-mail: paikjh@snu.ac.kr or Professor J-H Chung; E-mail: chungjh@snu.ac.kr

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Lung cancer remains the leading cause of cancer-related death worldwide (Jemal *et al*, 2010; Pao and Girard 2011; Kim *et al*, 2013b), despite therapeutic advances (Pao and Girard, 2011). About 85% of lung cancer is non-small cell lung cancer (NSCLC), and about 50% is adenocarcinoma (Kim *et al*, 2013b). Recently, new treatment strategy targeting 'driver mutations' including epidermal growth factor receptor (*EGFR*) or anaplastic lymphoma kinase (*ALK*) has opened an era of personalised medicine in lung adenocarcinomas (Mok, 2011; Pao and Girard, 2011; Kim *et al,* 2013b). However, the current standard strategy for the management of lung adenocarcinoma is still early detection and curative surgical resection (Iwakawa *et al*, 2011). Even with curative surgical resection in early stage, a considerable number of patients eventually recur, and their survival remains unsatisfactory (Sawabata *et al*, 2010; Shimizu *et al*, 2013). Therefore, it has been an important issue to identify and validate the molecular prognostic factors affecting recurrence and survival in lung adenocarcinoma patients, especially in those with early stage disease. In this context, various clinicopathologic factors have been investigated (Kudo *et al*, 2012; Arrieta Rodriguez *et al*, 2013; Brueckl *et al*, 2013; Chen *et al*, 2013; Kawase *et al*, 2013; Kwon *et al*, 2013; Nentwich *et al*, 2013; Sun *et al*, 2013; Kim *et al*, 2013a). Although the key genetic alterations have been largely revealed in lung adenocarcinoma (Imielinski *et al,* 2012), the useful molecular prognostic factors contributing to or accelerating the carcinogenic process, disease progression, or recurrence have not yet been fully understood, especially in stage I disease (Woo *et al*, 2012; Shimizu *et al,* 2013).

c-MYC gene is an important member of *MYC* proto-oncogene containing *N-MYC*, *c-MYC*, and *L-MYC* (Zhang *et al*, 2010). The *c-MYC* gene is located at chromosome 8q24, and c-MYC protein functions as a transcription factor regulating cell growth, proliferation, differentiation, and apoptosis (Zhang *et al,* 2010; Perez *et al*, 2011; Li *et al*, 2012). The overexpression of c-MYC protein promotes tumorigenesis by enhancing DNA double-strand breaks, genetic instability, and cell migration, as well as preventing escape from cell cycle (as referenced by Hermeking, 2003; Darcy *et al*, 2009; Li *et al,* 2012; Lin *et al*, 2012). c-MYC protein forms a heterodimer with MAX. The MYC/MAX heterodimer binds to E-box sequences near the promoter region of genes, and enhances the transcription of a wide range of genes (Hermeking, 2003; Lin *et al,* 2012).

c-MYC protein is elevated in tumours via several ways including translocation and amplification. In Burkitt lymphoma, the tumorigenesis is mainly mediated by t(8;14) translocation involving *c-MYC* and *IGH@* genes, leading to the extremely enhanced proliferating capacity with very short-doubling time in B-lymphoid cells (Swerdllow *et al*, 2008). Gene amplification or copy number gain of *c-MYC* have also been documented in non-lymphoid solid tumours including cancers from breast, ovary, prostate, bone, and brain (Liao and Dickson, 2000; Ghadimi *et al*, 2003; Morrison *et al,* 2005; Darcy *et al,* 2009; Perez *et al,* 2011; Zitterbart *et al*, 2011; Fromont *et al*, 2013). In these solid tumours, *c-MYC* amplification was associated with lymph node metastasis, recurrence, and disease progression to a variable degree (Ghadimi *et al,* 2003; Darcy *et al,* 2009; Perez *et al,* 2011; Fromont *et al,* 2013). In lung cancer, some early studies revealed frequent *c-MYC* amplification in small cell lung cancer cell lines (Little *et al*, 1983; Johnson *et al*, 1987), and several subsequent studies showed *c-MYC* amplification or *c-MYC* copy number gain (*c-MYC* gain) in NSCLC in animal model or human tumour tissues by using various methods (Kubokura *et al*, 2001; Rapp *et al*, 2009; Job *et al*, 2010; Iwakawa *et al,* 2011). *c-MYC* amplification was associated with lymph node metastasis with indefinite meaning for patient survival (Kubokura *et al,* 2001; Rapp *et al,* 2009), whereas chromosome 8 gain might be a potential prognostic factor (Kubokura *et al,* 2001).

Recently, *c-MYC* amplification was observed as a significant poor-prognostic factor by using whole genome copy number analysis and real-time genomic polymerase chain reaction (RT-G-PCR) in small-sized or early stage lung adenocarcinoma (Iwakawa *et al,* 2011). From this result, we hypothesised that (1) *c-MYC* gain might be a useful molecular marker predicting poor prognosis in early stage adenocarcinoma by using fluorescence *in situ* hybridisation (FISH) method, which is a practical diagnostic tool in the hospital pathology laboratory, and (2) *c-MYC* gain might have selective utility in lung adenocarcinomas with an activating alteration in *EGFR*, *KRAS*, and/or *ALK*.

In the present study, we intended to verify and validate the clinical applicability of the copy number gain of *c-MYC* by using FISH in a relatively large-scale cohort. We investigated the relationship between clinicopathologic parameters and *c-MYC* gain, chromosome 8 gain, and *c-MYC* amplification, and analysed its clinical significance according to *EGFR*, *KRAS*, and *ALK* alteration status in lung adenocarcinomas, especially in stage I adenocarcinomas.

MATERIALS AND METHODS

Patients and samples. A total of 255 patients with primary lung adenocarcinomas who underwent surgical resection in Seoul National University Bundang Hospital from May 2003 to November 2009 were enroled in this retrospective study. None of them received preoperative chemotherapy or radiation therapy. Clinical and pathologic data were retrieved from patients' medical records including pathologic reports. Two pathologists (ANS and JHC) reviewed the hematoxylin and eosin-stained slides. The histological subtypes were determined on the basis of the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) classification (Yoshizawa *et al*, 2011; Warth *et al*, 2012). Disease-free survival (DFS) was estimated from the date of surgical resection to the date of the initial tumour relapse, and overall survival (OS) was measured from the date of surgery to the time of death. Follow-up period for OS ranged from 1 to 84 months (median OS, 40.0 months), and follow-up period for DFS ranged from 1 to 84 months (median DFS, 29.0 months). The Institutional Review Board (IRB) of the Seoul National University Bundang Hospital approved this study as a study with less than minimal risk, and approved a waiver for consent for this study.

Tissue microarrays (TMA). The TMA blocks were manufactured from the most representative areas of individual paraffin blocks, as previously described (Superbiochips Laboratories, Seoul, Korea) (Yoo *et al*, 2010). Briefly, the representative tumour area was selected by two experienced pulmonary pathologists (HK and SBY), and the single core of 2 mm in diameter for each case was taken to TMA block. The cores containing more than 15% of tumour cells by area were considered as valid cores.

Fluorescence *in situ* hybridisation. To evaluate the copy number of *c-MYC* and centromeric enumeration probe 8 (CEP8), FISH assay was performed on the TMA sections of 3 μm thickness by using *c-MYC* probe (Abbott Molecular, Abbott Park, IL, USA) that hybridises to 8q24.12-q24.13 (*c-MYC*) with Spectrum Orange (red) signal, CEP8 probe (Abbott Molecular) that hybridises the centromeric (alpha satellite) region of chromosome 8 (8p11.1-q11.1) with Spectrum Green signal, and Hybrite (Abbott Molecular), according to manufacturer's instruction as previously described (Paik *et al*, 2011).

The FISH slide was interpreted by two experienced pathologists (ANS and JHP) without information about the clinicopathologic characteristics. Tumour tissue was scanned to detect hot spots for *c-MYC* copy numbers by using $\times$600 magnification. If the *c-MYC*

signals were homogeneously distributed, then random areas were selected to count the signals. Twenty non-overlapping tumour nuclei from three hot spots or random areas, that is, a total of 60 nuclei, per case were evaluated, and the numbers of *c-MYC* and CEP8 signals were counted at × 1000 magnification. The 60 nuclei criteria was determined beyond the level of the previous *c-MYC* gain studies in other solid tumours by Darcy *et al* (2009) (50 nuclei per case in ovary cancer), and Perez *et al* (2011) (30 nuclei per case in breast cancer). Small or large clusters of signals were considered as 6 and 12 signals, respectively, according to the interpretive guide for Ventana INFORM HER2 DNA probe staining of breast carcinoma (Ventana Medical Systems, Tucson, AZ, USA). Average copy number of *c-MYC* and CEP8 per nucleus and their ratio (*c-MYC*:CEP8) were calculated to determine *c-MYC* gain, chromosome 8 gain, and *c-MYC* amplification.

Analysis of *EGFR, KRAS* mutations, and *ALK* rearrangement. Epidermal growth factor receptor mutations at exons 18 to 21 and *KRAS* mutations at codons 12, 13, and 61 were analysed by using PCR and a direct DNA sequencing method with formalin-fixed paraffin-embedded (FFPE) tissue samples, as described previously by Paik *et al* (2011) and Lee *et al* (2013). *ALK* rearrangement was evaluated by using FISH method with *ALK* probe (Vysis LSI ALK dual-colour, break apart rearrangement probe; Abbott Molecular) with the cutoff value of 15%, as previously described by Paik *et al* (2011).

Statistical analysis. Statistical analysis was performed by SPSS 18.0 (SPSS Inc, Chicago, IL, USA). To analyse the correlation between clinicopathologic parameters, χ^2-test, Fisher's exact test and Mann–Whitney *U*-test, and/or Pearson correlation test were used. Receiver-operating characteristic (ROC) curve analysis against 5-year survival was performed to determine the clinically relevant cutoff points of *c-MYC* and CEP8 copy numbers. For survival analysis, Kaplan–Meier method with log-rank test and multivariate Cox proportional hazards regression analysis were performed. *P*-values <0.05 were considered as statistically significant (two-tailed). Although all values in this study are from the result of all inclusive enter method where the variables were introduced in one step, the main result was re-tested using other approaches and confirmed that the findings are valid. The influence of *EGFR*-mutational status on DFS and OS was excluded by testing the effect of *EGFR* mutation in any multivariate modelling. Post-operative adjuvant therapy had a significant influence on multivariate modelling. Patients directed to receive adjuvant therapy tended to have more advanced stage and an increased risk of recurrence, and current therapies are not very effective in these high-risk patients. As such, post-operative adjuvant therapy was excluded from analyses of DFS and OS.

RESULTS

Clinicopathologic characteristics of patients with total lung adenocarcinomas. The clinicopathologic characteristics of a total of 255 cases of lung adenocarcinoma were summarised in Table 1. Briefly, median age was 64 years with similar frequencies between men (50.6%, 129 out of 255) and women (49.4%, 126 out of 255). Histologically, acinar-predominant type (59.6%, 152 out of 255) was most common. Stage I cases accounted for 59.6% (152 out of 255), while stage II and stage III cases were 14.5 (37 out of 255) and 25.9% (66 out of 255), respectively. Post-operative adjuvant chemotherapy was performed in 39.6% (101 out of 255) and adjuvant radiation therapy in 11.8% (30 out of 255). The clinical profiles of the 'adjuvant therapy group' and 'no adjuvant therapy group' were not different except for stage (Table 1).

Determining the clinically relevant cutoff value in *c-MYC* and chromosome 8 gain. The median of *c-MYC* and CEP8 copy number per nucleus was 1.57 (range, 1.0–22.68) and 1.98 (range: 1.0–5.88). The median ratio (*c-MYC*:CEP8) were 0.78 (range, 0.32–6.84). Representative FISH patterns were shown in Figure 1. Owing to the lack of established criteria for *c-MYC* gain, we tested the previously published criteria (Morrison *et al,* 2005; Darcy *et al,* 2009; Perez *et al,* 2011), but clinical significance was limited.

By ROC curve analysis for predicting 5-year survival (Figure 2), several candidate cutoff values were tested, and the cutoff value representing maximum χ^2 (minimum *P*-value) was selected. '*c-MYC* >2.0 copies per nucleus' and 'CEP8 $\geqslant 3.0$ copies per nucleus' were observed as the most predictive cutoff criteria. In the present study, we defined the '*c-MYC* gain' as '*c-MYC* >2.0 copies per nucleus', 'chromosome 8 gain' as 'CEP8 $\geqslant 3.0$ copies per nucleus', and '*c-MYC* amplification' as '*c-MYC*:CEP8 ratio $\geqslant 2$', respectively. Additionally, to descriptively analyse the characteristics of the *c-MYC* gain cancer, we arbitrarily subdivided the *c-MYC* gain (>2.0) into high-level gain (>5.0) and low-level gain (*c-MYC* copies >2 and $\leqslant 5$). By the present criteria, '*c-MYC* gain' includes specific gain of *c-MYC* region and chromosome 8 gain.

In the full cohort, *c-MYC* gain was observed in 20.0% (51 out of 255). Low-level gain was observed in 18.4% (47 out of 255) of cancers, and high-level gain was seen in 1.6% (4 out of 255) of cases. Chromosome 8 gain accounted for 5.5% (14 out of 255), and *c-MYC* amplification was observed only in 2.4% (6 out of 255).

***Mutations in EGFR, KRAS, ALK* rearrangement, *c-MYC* gain, chromosome 8 gain, and *c-MYC* amplification.** In full cohort, *EGFR* and *KRAS* mutation data were obtained in 94% (239 out of 255) and 48% (123 out of 255) of full cohort. *ALK* rearrangement data were available in 80% (205 out of 255). As shown in Table 1, *EGFR* mutation was observed in 49.4% (118 out of 239), *KRAS* mutation in 5.7% (7 out of 123) and *ALK* rearrangement in 4.9% (10 out of 205). In two cases, mutations for both *EGFR* and *KRAS* were observed. However, *ALK* rearrangement was not observed in *EGFR*- or *KRAS*-mutant cases.

c-MYC gain was observed in 20% (51 out of 255), chromosome 8 gain in 5.5% (14 out of 255), and *c-MYC* amplification in 2.4% (6 out of 255) (Table 2).

Associations with clinical covariates. Table 2 and Supplementary Table S1-2 show the correlation between *c-MYC* gain, chromosome 8 gain, *c-MYC* amplification status and clinical covariates in full cohort (Table 2), stage I (Supplementary Table S1), *EGFR* wild-type, and *EGFR*-mutant subsets (Supplementary Table S2). Supplementary Table S3 summarises the significant correlations with clinical covariates. Briefly, *c-MYC* gain was correlated with lymphatic invasion and recurrence in full cohort and *EGFR*-mutant subset. Chromosome 8 gain tended to be associated with ever smoker, male sex, and/or lymphatic invasion. But the significance of correlation might be limited owing to relatively small number of cases with chromosome 8 gain ($n=14$ in full cohort) or *c-MYC* amplification ($n=6$ in full cohort).

c-MYC gain was detected in *EGFR*-mutant cases and *EGFR/KRAS/ALK* non-mutated cases. Specifically, *c-MYC* gain was observed in 19% (22 out of 118) of *EGFR*-mutant cases and 20% (24 out of 121) of *EGFR* wild-type cases. However, *c-MYC* gain was not observed in patients with *KRAS* mutation or *ALK* rearrangement (Table 2).

Associations with DFS in full cohort. As shown in Table 3, in univariate analysis in full cohort, the conventional clinicopathologic variables, that is pleural invasion, venous invasion, lymphatic invasion, perineural invasion, and high stage (III), were observed as significant poor-prognostic factors for DFS. Both of the *c-MYC* gain and *c-MYC* amplification were also poor-prognostic factors for DFS (Figure 3A), while chromosome 8 gain had only marginal

Table 1. Clinicopathologic characteristics of the patients in full cohort of lung adenocarinomas ($N = 255$)

	Total	No post-operative adjuvant therapy	Post-operative adjuvant therapy
Clinicopathologic characteristics	***N* (%)**	***N* (%)**	***N* (%)**
Age, years			
Median	64	64	64
Range	33–84	33–84	33–82
Sex			
Male	129 (50.6)	67 (46.9)	62 (55.4)
Female	126 (49.4)	76 (53.1)	50 (44.6)
Smoking history			
Ever	141 (55.3)	84 (58.7)	57 (50.9)
Never	114 (44.7)	59 (41.3)	55 (49.1)
IASLC subtype			
Acinar predominant	152 (59.6)	92 (64.3)	60 (53.6)
Papillary predominant	37 (14.5)	17 (11.9)	20 (17.9)
Solid predominant	34 (13.3)	10 (7.0)	24 (21.4)
Lepic predominant	30 (11.8)	24 (16.8)	6 (5.4)
Mucinous predominant	1 (0.4)	0 (0)	1 (0.9)
Micropapillary predominant	1 (0.4)	0 (0)	1 (0.9)
Pathologic stage			
IA	105 (41.2)	92 (64.3)	13 (11.6)
IB	47 (18.4)	35 (24.5)	12 (10.7)
IIA	28 (11.0)	10 (7.0)	18 (16.1)
IIB	9 (3.5)	2 (1.4)	7 (6.3)
IIIA	58 (22.7)	3 (2.1)	55 (49.1)
IIIB	8 (3.1)	1 (0.7)	7 (6.3)
Post-operative adjuvant chemotherapy			
No	154 (60.4)	143 (100.0)	11 (9.8)
Yes	101 (39.6)	0 (0)	101 (90.2)
Post-operative adjuvant radiotherapy			
No	225 (88.2)	143 (100.0)	82 (73.2)
Yes	30 (11.8)	0 (0)	30 (26.8)
***EGFR* mutation**[a]			
Negative	121 (50.6)	69 (53.1)	52 (47.7)
Positive	118 (49.4)	61 (46.9)	57 (52.3)
***KRAS* mutation**[a]			
Negative	116 (94.3)	56 (96.6)	60 (92.3)
Positive	7 (5.7)	2 (3.4)	5 (7.7)
***ALK* rearrangement**[a]			
Negative	195 (95.1)	95 (94.1)	100 (96.2)
Positive	10 (4.9)	6 (5.9)	4 (3.8)

Abbreviations: ALK = anaplastic lymphoma kinase; EGFR = epidermal growth factor receptor; IASLC = International Association for the Study of Lung Cancer.
[a]These numbers exclude missing values.

significance. In multivariate analysis with *c-MYC* gain and conventional significant variables, *c-MYC* gain was a significant prognostic factor for DFS ($P = 0.022$; HR = 1.71), while *c-MYC* amplification was not significant ($P = 0.589$) when *c-MYC* amplification was included instead of *c-MYC* gain in the modelling. Although adjuvant therapy was another influential factor for DFS and OS ($P < 0.001$ for both), we did not include it in the multivariate model of Table 3, because 'adjuvant therapy group' showed significantly high rate of recurrence and poor prognosis (13.3% (19 out of 143) of recurrences in no adjuvant therapy subgroup; 73.2% (82 out of 112) of recurrence in adjuvant therapy subgroup; $P < 0.001$). This might suggest that the adjuvant therapy had the role of clinical surrogate marker of high probability of recurrence overriding its innate therapeutic effects. Consistent with this, by including adjuvant therapy in the multivariate modelling, the significance of *c-MYC* gain became

limited ($P = 0.080$), while the inclusion of *EGFR* mutation did not significantly influence on DFS using univariate and multivariate models (data not shown).

Associations with OS in full cohort. In univariate analysis for OS, the *c-MYC* gain and chromosome 8 gain were significant prognostic factors, along with conventional clinicopathologic variables (Table 3 and Figure 3B). In multivariate analysis for OS, *c-MYC* gain, high stage (III), pleural, and venous invasions were independent poor-prognostic factors. High stage (III) was the most powerful prognostic factor ($P < 0.001$, HR = 4.88), while *c-MYC* gain was a less powerful significant prognostic factor ($P = 0.032$, HR = 2.04). The multivariate modelling was not influenced by inclusion of adjuvant therapy or *EGFR* mutation (data not shown).

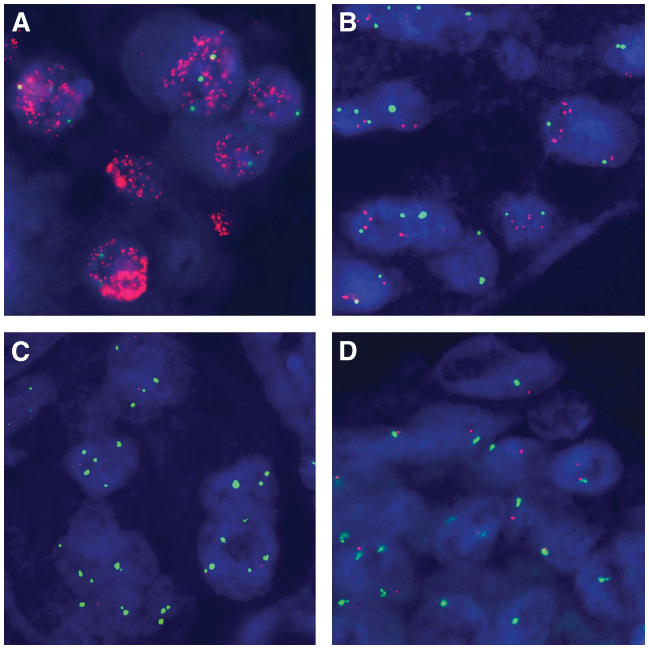

Figure 1. Representative patterns of FISH of *c-MYC* gene (red colour) and chromosome 8 (CEP8) (green colour) copy number status. (**A**) High-level gain of c-*MYC* (*c-MYC*:CEP8 ratio = 6.84), (**B**) low-level gain of *c-MYC* (*c-MYC*:CEP8 ratio = 1.82), (**C**) chromosome 8 gain (*c-MYC*:CEP8 ratio = 0.30), and (**D**) non-gain of *c-MYC* or chromosome 8 (*c-MYC*:CEP8 ratio = 0.90).

Subset analysis of DFS. To investigate the clinical and prognostic significance of *c-MYC* gain in several clinicopathologic subgroups, we analysed the survival effect of *MYC* gain in stage I subgroup, *EGFR*-mutant and wild-type subgroups.

As shown by Iwakawa *et al* (2011), we performed survival analysis in the stage I adenocarcinoma subgroup containing 152 patients. In this subgroup, *c-MYC* gain was observed in 15.8% (24 out of 152), chromosome 8 gain in 4.6% (7 out of 152), and *c-MYC* amplification in 1.3% (2 out of 152). In univariate survival analysis of stage I adenocarcinoma subgroup, *c-MYC* gain tended to show poor prognosis but the association with DFS did not achieve statistical significance ($P = 0.065$, Table 4 and Figure 3C). In multivariate analysis with *c-MYC* gain and conventional variables, *c-MYC* gain was the independent prognostic factor for DFS in stage I subgroup ($P = 0.023$, HR = 4.70; Table 4; Figure 4A).

Next, we investigated the clinical meaning of *c-MYC* gain according to *EGFR*-mutational status, since *c-MYC* gain was frequently observed in lung adenocarcinoma with *EGFR*-mutation (Table 2). The frequency of *c-MYC* gain was similar between the two subgroups (19% (22 out of 118) of *EGFR*-mutant subgroup and 20% (24 out of 121) of *EGFR* wild-type subgroup) (Table 2 and Supplementary Table S2).

In patients with a mutation in *EGFR*, *c-MYC* gain was associated with DFS using univariate analysis ($P = 0.008$; Figure 3D and E) and exhibited independent prognostic relevance in multivariate analysis ($P = 0.022$) after adjusting for the conventional clinical covariates (Table 5 and Figure 4B). In patients without *EGFR* mutation, *c-MYC* gain, chromosome 8 gain, and *c-MYC* amplification were not associated with DFS (Table 5 and Figure 4C).

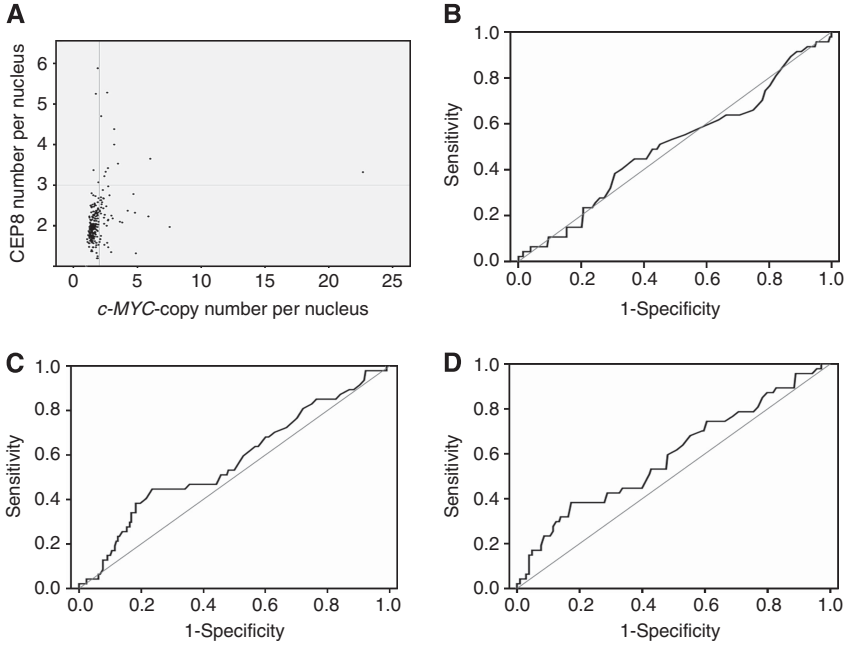

Figure 2. Scatter plot and ROC curves for *c-MYC* gain, chromosome 8 gain, and *c-MYC* amplification (gain of *c-MYC*:CEP8 ratio) in lung adenocarcinomas. (**A**) Scatter plot with *c-MYC* (X-axis) status and chromosome 8 (Y-axis) status. (**B**) ROC curves for *c-MYC*:CEP8 ratio, (**C**) *c-MYC* gain, and (**D**) chromosome 8 gain for predicting 5-year survival.

Table 2. The association between clinicopathologic parameters and c-MYC,chromosome 8 status in full cohort of lung adenocarcinomas

		c-MYC status			Chromosome 8 status			c-MYC:CEP8 ratio		
	Total	Non-gain	Gain		Non-gain	Gain		Negative	Amplification	
Characteristics	*N* (%)	*c-MYC* ⩽2	2< *c-MYC*	*P*	CEP 8 <3	CEP 8 ⩾3	*P*	Ratio <2.0	Ratio ⩾2.0	*P*
Age										
Median Range	64 33–84	64 33–84	63 33–80	0.708[a]	64 33–84	70 48–78	0.053[a]	64 33–84	59 57–70	0.444[a]
Sex										
Male Female	128 (50.2) 127 (49.8)	101 (49.5) 103 (50.5)	27 (52.9) 24 (47.1)	0.755	115 (47.7) 126 (52.3)	13 (92.9) 1 (7.1)	0.001[b,c]	127 (51.0) 122 (49.0)	1 (16.7) 5 (83.3)	0.120[c]
Smoking history										
Never Ever	141 (55.3) 114 (44.7)	118 (57.8) 86 (42.2)	23 (45.1) 28 (54.9)	0.102	139 (57.7) 102 (42.3)	2 (14.3) 12 (85.7)	0.002[b,c]	137 (55.0) 112 (45.0)	4 (66.7) 2 (33.3)	0.694[c]
Tumour size (cm)										
⩽3 3<size⩽7 7<size	164 (64.3) 84 (32.9) 7 (2.8)	138 (67.6) 60 (29.4) 6 (2.9)	26 (60.0) 24 (47.1) 1 (2.0)	0.059[c]	157 (65.1) 79 (32.8) 5 (2.1)	7 (50.0) 5 (35.7) 2 (14.3)	0.061[c]	162 (65.1) 80 (32.1) 7 (2.8)	2 (33.3) 4 (66.7) 0 (0)	0.311[c]
Pleural invasion										
Absent Visceral invasion Parietal invasion	155 (60.8) 89 (34.9) 11 (4.3)	126 (61.8) 69 (33.8) 9 (4.4)	29 (57.9) 20 (39.2) 2 (3.9)	0.821[c]	146 (60.6) 85 (35.3) 10 (4.1)	9 (64.3) 4 (28.6) 1 (7.1)	0.597[c]	152 (61.0) 86 (34.5) 11 (4.4)	3 (50.0) 3 (50.0) 0 (0)	0.748[c]
Lymphatic invasion										
Absent Present	154 (60.4) 101 (39.6)	133 (65.2) 71 (34.8)	21 (41.2) 30 (58.8)	0.002[b]	151 (62.7) 90 (37.3)	3 (21.4) 11 (78.6)	0.003[b,c]	152 (61.0) 97 (39.0)	2 (33.3) 4 (66.7)	0.218[c]
Venous invasion										
Absent Present	228 (89.4) 27 (10.6)	182 (89.2) 22 (10.8)	46 (90.2) 5 (9.8)	0.839	216 (89.6) 25 (10.4)	12 (85.7) 2 (14.3)	0.649[c]	223 (89.6) 26 (10.4)	5 (83.3) 1 (16.7)	0.493[c]
Perineural invasion										
Absent Present	242 (94.9) 13 (5.1)	196 (96.1) 8 (3.9)	46 (90.2) 5 (9.8)	0.144[c]	231 (95.5) 10 (4.1)	11 (78.6) 3 (21.4)	0.027[b,c]	236 (94.8) 13 (5.2)	6 (100.0) 0 (0)	1.000[c]
Recurrence										
Absent Present	154 (60.4) 101 (39.6)	131 (64.2) 73 (35.8)	23 (45.1) 28 (54.9)	0.013[b]	149 (61.8) 92 (38.2)	5 (35.7) 9 (64.3)	0.088	152 (61.0) 97 (39.0)	2 (33.3) 4 (66.7)	0.218[c]
pTNM stage										
I II III	152 (59.6) 37 (14.5) 66 (25.9)	128 (62.7) 26 (12.7) 50 (24.5)	24 (47.1) 11 (21.6) 16 (31.4)	0.099	145 (60.2) 35 (14.5) 61 (25.3)	7 (50.0) 2 (14.3) 5 (35.7)	0.631[c]	150 (60.2) 35 (14.1) 64 (25.7)	2 (33.3) 2 (33.3) 2 (33.3)	0.223[c]
***EGFR* mutation[d]**										
Negative Positive	121 (50.6) 118 (49.4)	97 (50.3) 96 (49.7)	24 (52.2) 22 (47.8)	0.815	109 (48.4) 116 (51.6)	12 (85.7) 2 (14.3)	0.011[b,c]	120 (51.5) 113 (48.5)	1 (16.7) 5 (83.3)	0.117[c]
***KRAS* mutation[d]**										
Negative Positive	116 (94.3) 7 (5.7)	89 (92.7) 7 (7.3)	27 (100.0) 0 (0)	0.346[c]	106 (93.8) 7 (6.2)	10 (100.0) 0 (0)	1.000[c]	113 (94.2) 7 (5.8)	3 (100.0) 0 (0)	1.000[c]
***ALK* rearrangement[d]**										
Negative Positive	195 (95.1) 10 (4.9)	151 (93.8) 10 (6.2)	44 (100.0) 0 (0)	0.123[c]	183 (94.8) 10 (5.2)	12 (100.0) 0 (0)	1.000[c]	190 (95.0) 10 (5.0)	5 (100.0) 0 (0)	1.000[c]
Total	255 (100.0)	204 (100.0)	51 (100.0)		241 (100.0)	14 (100.0)		249 (100.0)	6 (100.0)	

Abbreviations: ALK = anaplastic lymphoma kinase; CEP = centromeric enumeration probe; EGFR = epidermal growth factor receptor. *P*-values are calculated by using χ^2-test.

[a]Mann–Whitney *U* test.

[b]Indicates that *P*-values<0.05.

[c]Fisher's exact test

[d]These numbers exclude missing values.

Table 3. Survival analysis for disease-free survival and overall survival in full cohort of lung adenocarcinomas ($N = 255$)

		Disease-free survival			Overall survival		
		Univariate analysis	Multivariate analysis		Univariate analysis	Multivariate analysis	
Clinicopathologic variables	Category	*P*	*P*	HR (95% CI)	*P*	*P*	HR (95% CI)
Age	⩾64 *vs* <64	0.235	–	–	0.007[a]	0.059	1.77 (0.98–3.21)
Pleural invasion	Present *vs* absent	<0.001[a]	0.003[a]	1.86 (1.23–2.83)	<0.001[a]	0.045[a]	1.82 (1.01–3.26)
Venous invasion	Present *vs* absent	<0.001[a]	0.004[a]	2.17 (1.29–3.65)	<0.001[a]	0.037[a]	1.99 (1.04–3.80)
Lymphatic invasion	Present *vs* absent	<0.001[a]	0.015[a]	1.74 (1.11–2.72)	<0.001[a]	0.935	1.03 (0.55–1.93)
Perineural invasion	Present *vs* absent	0.013[a]	0.900	0.96 (0.46–1.97)	0.002[a]	0.562	1.31 (0.52–3.30)
Stage	III *vs* I, II	<0.001[a]	<0.001[a]	2.65 (1.66–4.23)	<0.001[a]	<0.001[a]	4.88 (2.52–9.43)
c-MYC status	Gain *vs* non-gain	0.006[a]	0.022[a]	1.71 (1.08–2.69)	0.013[a]	0.032[a]	2.04 (1.06–3.91)
Chromosome 8 status	Gain *vs* non-gain	0.050	–	–	0.015[a]	0.405	1.55 (0.55–4.33)
c-MYC:CEP8 ratio	Amplification *vs* negative	0.016[a]	–	–	0.105	–	–

Abbreviations: ALK = anaplastic lymphoma kinase; CEP = centromeric enumeration probe; EGFR = epidermal growth factor receptor.
[a]Indicates that *P*-values are less than 0.05.

Subset analysis of OS. In stage I subgroup, *c-MYC* gain was observed as a significant poor-prognostic factor for OS ($P = 0.008$; Table 4 and Figure 3F), while chromosome 8 gain and *c-MYC* amplification were not significant (Table 4) in univariate analysis. In multivariate analysis with *c-MYC* gain and significant conventional variables, *c-MYC* gain was the independent poor-prognostic factor for OS ($P = 0.031$, HR = 4.65, Table 4 and Figure 4D).

In patients with a mutation in *EGFR*, *c-MYC* gain, chromosome 8 gain, and *c-MYC* amplification were not associated with OS (Table 5 and Figure 4E). In patients without *EGFR* mutation, high stage (III) (but not *c-MYC* gain or chromosome 8 gain) was a significant prognostic factor for OS in multivariate analysis ($P < 0.001$, Table 5 and Figure 4F).

DISCUSSION

In the present retrospective study with FISH method, we investigated the clinicopathologic significance of *c-MYC* gain, chromosome 8 gain, and *c-MYC* amplification in lung adenocarcinomas according to the genetic alteration status of *EGFR*, *KRAS*, and *ALK,* and validated the PCR-based results by Iwakawa *et al* (2011) in early stage adenocarcinoma. Herein, we observed *c-MYC* gain was an independent poor-prognostic factor for DFS and OS in lung adenocarcinomas, both in full cohort and stage I cancer, and possibly in *EGFR*-mutant adenocarcinomas for DFS.

c-MYC is known to link the stimulation by growth factors and cellular proliferation in normal cells, and pathologic activation through translocation or amplification is thought to constitutively enhance the transcription of a certain group of genes contributing to cell proliferation without the stimulation by growth factors (Lin *et al*, 2012). The extremely high-proliferative activity in Burkitt lymphoma is mediated via t(8;14) translocation where *c-MYC* gene is juxtapositioned with one of the immunoglobulin loci, and regulated by immunoglobulin gene regulatory elements (Boxer and Dang, 2001). The detection of *c-MYC* translocation by using FISH has been established as a hallmark for the diagnosis or prognosis in malignant lymphoma (Swerdllow *et al,* 2008; Slack and Gascoyne, 2011). Similarly, *c-MYC* gain detected by FISH method in some solid tumours tended to be associated with poor-clinical outcome (Sauter *et al*, 1995; Ghadimi *et al,* 2003; Morrison *et al,* 2005; Darcy *et al,* 2009; Perez *et al,* 2011). However, the clinical application of *c-MYC* gain as a prognostic marker in lung cancer has been limited owing to lack of solid evidence (Kubokura *et al,* 2001). Recently, the Iwakawa's study using RT-G-PCR method showed *c-MYC* gain as a significant prognostic marker in early stage lung adenocarcinomas (Iwakawa *et al,* 2011). By using FISH method, we observed the prognostic significance of *c-MYC* gain in lung adenocarcinomas.

In the present study, low-level gain of *c-MYC* (18.4%) was more common than high-level gain (1.6%) in lung adenocarcinoma. Despite the slightly different criteria, similar 'low-level gain'-predominant patterns have been described in other solid tumours (Sauter *et al,* 1995; Perez *et al,* 2011; Zitterbart *et al,* 2011). Therefore, the copy number analysis of 8q24 or *c-MYC* locus might be very delicate, and inevitably raises important issues about how to define the '*c-MYC* gain/amplification' and whether to include the 'low-level gain' in the '*c-MYC* gain' or not. In a recent study with prostate cancer, the relatively 'low-level gain' was included in the '*c-MYC* gain/amplification' group with the criteria of *c-MYC*/CEP8 ratio > 1.5, and a poor prognosis was observed in this group (Fromont *et al,* 2013). Another recent study with lymphoma, the cases with 2.2 copies per nucleus (44 copies/20 cells) were classified as the increased *c-MYC* copy number group, and c-MYC protein expression was correlated with increased *c-MYC* copy number and mRNA expression (Valentino *et al,* 2013). In agreement with these results, '*c-MYC* gain' criteria by Iwakawa *et al* (2011) with lung cancer by using RT-G-PCR was set as low as *c-MYC* copy number > 1.59, while the criteria might not be directly applied to the FISH method. The '*c-MYC* gain' criteria in the present study (*c-MYC* > 2.0 copies/nucleus) appears to largely contain duplication of 8q24 or *c-MYC* locus and/or chromosome 8 polysomy, as shown in Figure 2. In the clinical point of view, the relatively simple criteria in the present study with FFPE tissue-based FISH method might be practically useful in the assessment of patients with lung adenocarcinomas, because it could clearly distinguish the poor-prognostic group, especially in stage I cancer, regardless of *c-MYC*:CEP8 ratio or chromosome 8 status.

It might not be clear how the 'low-level gain' of *c-MYC* gene in the present study could lead to the aggressive biology of the lung cancer, and it is still poorly understood whether the prognostic

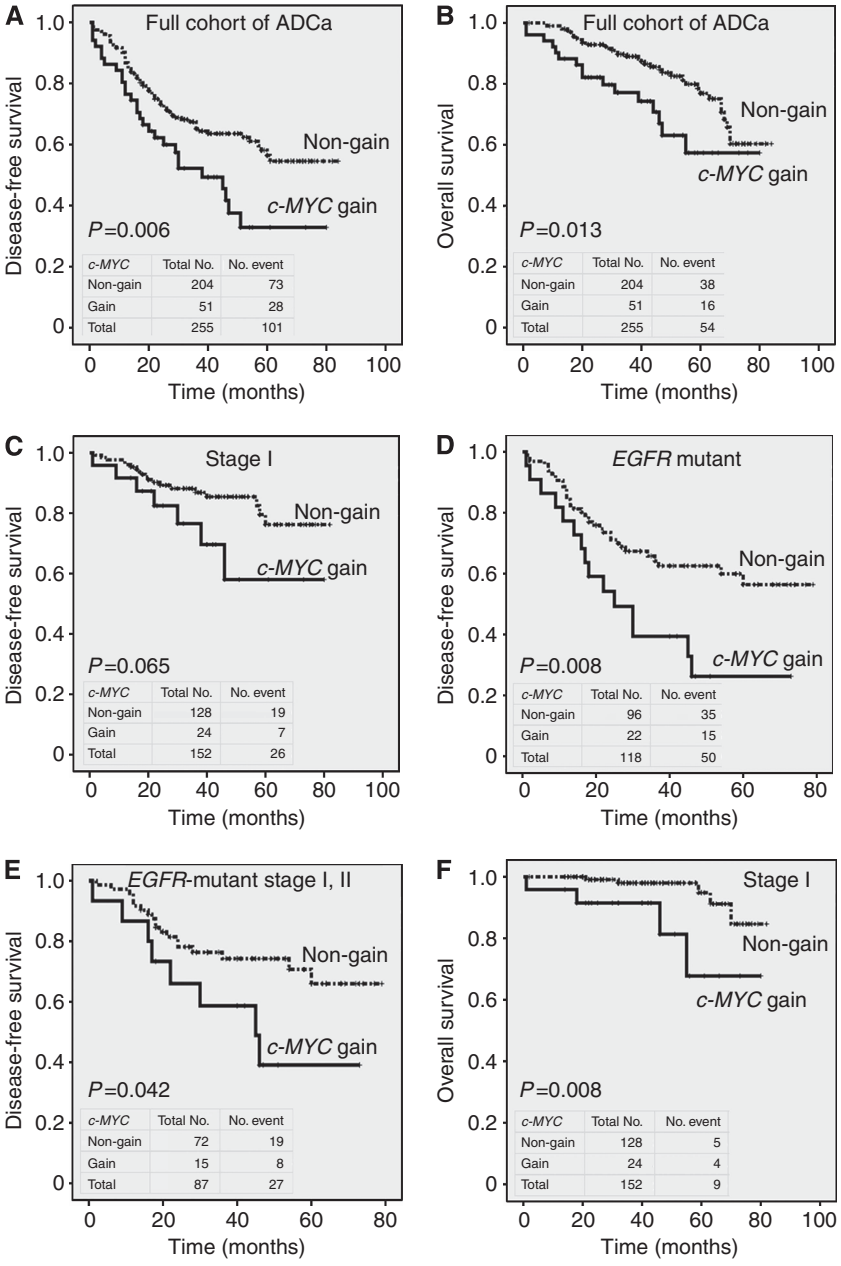

Figure 3. Kaplan–Meier survival curves illustrating prognostic effects of *c-MYC* gain in lung adenocarcinomas. (**A**) *c-MYC* gain for DFS and (**B**) OS in full cohort. (**C**) *c-MYC* gain for DFS in stage I, (**D**) *EGFR*-mutant, and (**E**) *EGFR*-mutant low stage (I, II) subgroups. (**F**) *c-MYC* gain for OS in stage I subgroup.

effect of *c-MYC* gain is achieved solely by the increased expression of c-MYC protein, since the *c-MYC* gene status but not c-MYC protein expression did influence the prognosis in a recent prostate cancer study (Fromont *et al,* 2013). Considering the complex way of regulation of c-MYC protein and multiple interaction with other molecules, it might be possible that the gain of 8q24 or *c-MYC* locus could enhance c-MYC activity at certain level, which might be sufficient to effectively trigger amplification of transcription involving a various set of genes in tumour cells (Lin *et al,* 2012), which remains to be investigated further. Additionally, other factors including non-coding RNAs and some regulatory proteins encoded at 8q24 locus or nearby chromosomal regions might be involved in the '*c-MYC* gain'-associated cellular changes leading to poor-clinical outcome. Especially, several candidate protein-coding and non-coding oncogenes including *ATAD2*, *PVT1*, and *MIR1204*, have been known to be mapped in 8q24 locus, coamplified with *c-MYC*, and transduce or modify the *c-MYC*-induced signals to other regulatory pathways (Carramusa *et al*, 2007; Huppi *et al*, 2008, 2012; Raeder *et al*, 2013), while the level of functional contribution of these molecules in the tumour biology of lung adenocarcinoma needs to be clarified. In this context, to evaluate the '*c-MYC* gain'-associated phenotypic changes at either cellular or clinical levels, it might not simply be sufficient to investigate the immunohistochemical expression of c-MYC protein, which harbours various epitopes, short half-life, complex interaction with other protein factors, and still unclear significance of various subcellular localisation including cytoplasmic cleavage product (Conacci-Sorrell *et al*, 2010; Fromont *et al,* 2013).

The solid tumours with '*c-MYC* gain' have tended to be associated with invasiveness and lymph node metastasis (Ghadimi *et al,* 2003; Heselmeyer-Haddad *et al*, 2012), which are required phenotypes for the localised early cancers to progress and eventually disseminate. Moreover, the *c-MYC* has been known to

Table 4. Survival analysis for overall survival in stage I lung adenocarcinoma ($n = 152$)

		Disease-free survival			Overall survival		
		Univariate analysis	Multivariate analysis		Univariate analysis	Multivariate analysis	
Clinicopathologic variables	Category	*P*	*P*	HR (95% CI)	*P*	*P*	HR (95% CI)
Age	⩾64 *vs* <64	0.318	–		0.017[a]	0.067	7.14 (0.87–58.49)
Pleural invasion	Present *vs* Absent	0.005[a]	0.107	5.08 (0.70–36.67)	0.004[a]	0.272	3.29 (0.39–27.50)
T stage	T2 *vs* T1	0.002[a]	0.348	2.60 (0.35–19.20)	0.034[a]	0.424	2.41 (0.28–20.69)
c-MYC status	Gain *vs* Non-gain	0.065	0.023[a]	4.70 (1.24–17.78)	0.008[a]	0.031[a]	4.65 (1.15–18.81)
Chromosome 8 status	Gain *vs* Non-gain	0.102	–		0.482	–	–
c-MYC:CEP8 ratio	Amplification *vs* Negative	0.589	–		0.804	–	–

Abbreviations: CI = confidence interval; HR = hazard ratio.
[a]Indicates that *P*-values are less than 0.05.

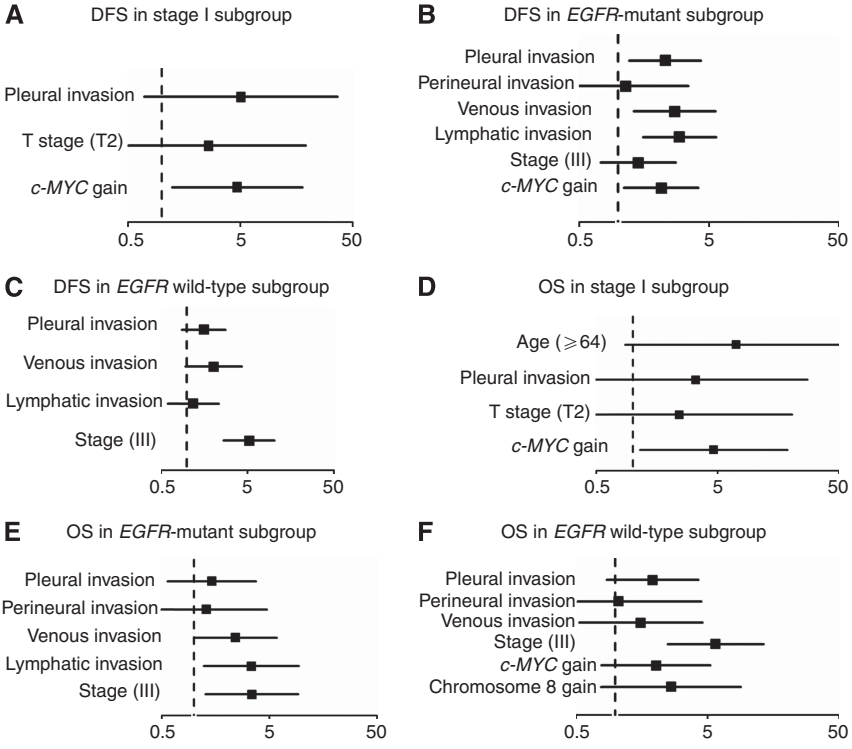

Figure 4. Forest plot showing multivariate survival analysis for DFS and OS in stage I, *EGFR*-mutant, and *EGFR* wild-type subgroups. (**A**) DFS in stage I, (**B**) *EGFR*-mutant, and (**C**) *EGFR* wild-type subgroups. (**D**) OS in stage I, (**E**) *EGFR*-mutant, and (**F**) *EGFR* wild-type subgroups.

be a downstream target of *EGFR/RAS/RAF/MEK/ERK* signalling pathway (Dang, 2012). *c-MYC* played an important role in tumour progression in *RAF*- or *RAS*-driven lung cancer models *in vitro* or *in vivo*, and targeting *c-MYC* effectively suppressed or reversed tumorigenesis (Tran *et al*, 2008; Rapp *et al*, 2009; Fukazawa *et al*, 2010; Soucek *et al*, 2013). These findings suggest that *c-MYC* gain might be involved in the progression of early stage lung adenocarcinoma, especially in conjunction with *EGFR/RAS/RAF* pathway, and be consistent with the observations in the present study that *c-MYC* gain was a significant prognostic factor in stage I adenocarcinoma subgroup, as well as in *EGFR*-mutant subgroup. The meaning of *c-MYC* gain according to *KRAS* mutation status could not be sufficiently analysed owing to low *KRAS* mutation rate, and it remains to be clarified further.

In addition to *c-MYC* gain, chromosome 8 gain also harboured a poor-prognostic effect in lung adenocarcinomas, while the significance was limited. It might be assumed that the '*c-MYC* gain' defined in the present study would technically contain a broad spectrum of genomic gains involving *c-MYC* (8q24) locus, up to some proportion of chromosome 8 gains (Figure 2). Considering that chromosomal regions around 8q24 are unstable (Huppi *et al*, 2008), our observations from clinical samples suggest that whether or not *c-MYC* (8q24) region is included in the process of genomic gain involving some parts of chromosome 8 might be a surrogate marker for genomic instability and critical determinant of poor-clinical outcome in lung adenocarcinomas. Conversely, stable genomic status at *c-MYC* (8q24) locus or nearby regions might indirectly reflect that one of

Table 5. Survival analysis for disease-free survival and overall survival in *EGFR*-mutant and *EGFR* wild-type subgroups of lung adenocarcinoma

		Disease-free survival			Overall survival		
		Univariate analysis	Multivariate analysis		Univariate analysis	Multivariate analysis	
Clinicopathologic variables	Category	*P*	*P*	HR (95% CI)	*P*	*P*	HR (95% CI)
***EGFR* mutant subgroup (*n* = 118)**							
Pleural invasion	Present *vs* absent	<0.001[a]	0.010[a]	2.30 (1.22–4.31)	0.015[a]	0.436	1.46 (0.57–3.74)
Perineural invasion	Present *vs* absent	0.015[a]	0.818	1.14 (0.37–3.45)	0.037[a]	0.693	1.30 (0.35–4.76)
Venous invasion	Present *vs* absent	<0.001[a]	0.007[a]	2.71 (1.32–5.56)	<0.001[a]	0.053	2.40 (0.99–5.85)
Lymphatic invasion	Present *vs* absent	<0.001[a]	0.001[a]	2.96 (1.56–5.62)	<0.001[a]	0.018[a]	3.40 (1.23–9.39)
Stage	III *vs* I & II	<0.001[a]	0.302	1.42 (0.73–2.77)	<0.001[a]	0.014[a]	3.43 (1.28–9.22)
c-MYC status	Gain *vs* non-gain	0.008[a]	0.022[a]	2.14 (1.11–4.10)	0.332	–	–
Chromosome 8 status	Gain *vs* non-gain	0.193	–	–	0.557	–	–
c-MYC:CEP8 ratio	Amplification *vs* negative	0.113	–	–	0.458	–	–
***EGFR* wild-type subgroup (*n* = 121)**							
Pleural invasion	Present *vs* absent	0.012[a]	0.134	1.56 (0.87–2.77)	0.048[a]	0.114	1.91 (0.86–4.27)
Perineural invasion	Present *vs* absent	0.197	–	–	0.027[a]	0.943	1.05 (0.25–4.48)
Venous invasion	Present *vs* absent	0.001[a]	0.65	2.03 (0.96–4.30)	0.009[a]	0.422	1.56 (0.53–4.61)
Lymphatic invasion	Present *vs* absent	0.015[a]	0.63	1.18 (0.60–2.33)	0.252	–	–
Stage	III *vs* I & II	<0.001[a]	<0.001[a]	5.22 (2.66–10.23)	<0.001[a]	<0.001[a]	5.76 (2.50–13.32)
c-MYC status	Gain *vs* Non-gain	0.112	–	–	0.006[a]	0.147	2.04 (0.78–5.26)
Chromosome 8 status	Gain *vs* Non-gain	0.169	–	–	0.012[a]	0.119	2.64 (0.78–8.95)
c-MYC:CEP8 ratio[b]	Amplification *vs* Negative	0.032[a]	–	–	0.001[a]	–	–

Abbreviations: CI = confidence interval; EGFR = epidermal growth factor receptor; HR = hazard ratio.
[a]Indicates that *P*-values are less than 0.05.
[b]Since *c-MYC* amplification was observed in only one case in this subgroup, the significance on multivariate survival model could not be determined.

the vulnerable sites of genomic instability associated with the clinical outcome was spared.

As a tissue biomarker of lung adenocarcinoma, *c-MYC* gain might be a good candidate, because it accounts for 15–20% of full cohort or subsets (Supplementary Table S3), and shows an independent prognostic value in full cohort and stage I cancer. In contrast, the cases with chromosome 8 gain and *c-MYC* amplification were observed in less than 6% and 3% of full cohort and subsets (Supplementary Table S3), respectively, and did not show any significant prognostic value. Therefore, the clinical meaning of *c-MYC* gain needs to be validated for clinical application in prospective and larger scale study.

In conclusion, we observed that *c-MYC* gain was associated with lymphatic invasion, and was an independent poor-prognostic factor for DFS and OS in lung adenocarcinomas, both in full cohort and stage I subgroup, and possibly for DFS in *EGFR*-mutant subgroup. These findings might provide the useful way of detailed risk stratification in patients with lung adenocarcinomas, and an insight into pathogenesis and mechanism of progression in lung adenocarcinomas. Furthermore, patients with lung adenocarcinoma with *c-MYC* gain, even in early stage, appear to have an increased risk of disease progression and death, which merits further prospective evaluation across multiple institutions to validate the clinical utility of *c-MYC* gains in this disease setting and to determine whether these patients might benefit from additional first-line therapy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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