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Authors: Wang-Yu Zhu, Xiao-Fei Hu, Ke-Xin Fang, Qiong-Qiong Kong, Ri Cui, Hai-Feng Li, Jian-Ying He, Yong-kui Zhang and Han-Bo Le

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Prognostic value of mutant p53, Ki-67, and TTF-1 and their correlation with EGFR mutation in patients with non-small cell lung cancer

Wang-Yu Zhu\textsuperscript{1,2,*}, Xiao-Fei Hu\textsuperscript{2}, Ke-Xin Fang\textsuperscript{1}, Qiong-Qiong Kong\textsuperscript{4}, Ri Cui\textsuperscript{5}, Hai-Feng Li\textsuperscript{2,3}, Jian-Ying He\textsuperscript{1}, Yong-kui Zhang\textsuperscript{2,3}, and Han-Bo Le\textsuperscript{2,3,*}


Wang-Yu Zhu: zhuwangyu24@sina.cn
Xiao-Fei Hu: 1097824003@qq.com
Ke-Xin Fang: 283423935@qq.com
Qiong-Qiong Kong: 240169293@qq.com
Ri Cui: wzmucuiri@163.com
Hai-Feng Li: 1770182534@qq.com
Jian-Ying He: hjyncmcstudent@163.com
Yong-Kui Zhang: zyk801801@126.com
Han-Bo Le: zslehanbo@163.com

\textsuperscript{1} Cell and Molecular Biology Laboratory, Zhoushan Hospital of Wenzhou Medical University, Zhoushan, Zhejiang, China
\textsuperscript{2} Lung Cancer Research Centre, Zhoushan Hospital of Wenzhou Medical University, Zhoushan, Zhejiang, China
\textsuperscript{3} Department of Cardio-Thoracic Surgery, Zhoushan Hospital of Wenzhou Medical University, Zhoushan, Zhejiang, China
\textsuperscript{4} Department of Pathology, Zhoushan Hospital of Wenzhou Medical University, Zhoushan, Zhejiang, China
School of Pharmaceutical Sciences, Wenzhou Medical University, Wenzhou, Zhejiang, China

*Corresponding authors. Wang-yu Zhu, Cellular and Molecular Biology Laboratory, Lung Cancer Research Centre, Zhoushan Hospital of Wenzhou Medical University, Zhoushan, Zhejiang 316021, China. Fax: +86-580-2292659; E-mail: zhuwangyu24@sina.cn.

And Han-bo Le, Lung Cancer Research Centre, Department of Cardio-Thoracic Surgery, Zhoushan Hospital of Wenzhou Medical University, Zhoushan, Zhejiang, China. Fax: +86-580-2292659; E-mail: zslehanbo@163.com

Abstract

**Introduction**: The clinical characteristics of non-small cell lung cancer (NSCLC) with epidermal growth factor receptor (EGFR) mutation have been well studied. However, the correlation of EGFR mutation with mutant p53, Ki-67, and thyroid transcription factor 1 (TTF-1) and their prognostic value remain indistinct.

**Material and Methods**: Clinical and pathological characteristics and overall survival were analysed retrospectively in 523 surgically resected NSCLC patients. The expression levels of p53, Ki-67, and TTF-1 protein were detected by immunohistochemistry, and an amplification refractory mutation system was used to access the status of EGFR mutations.

**Results**: Of 523 patients with surgically resected NSCLC, 210 patients (38.4%) harboured EGFR mutations. Compared to the EGFR wild-type lung cancer, mutated EGFR harboured significantly increased mutant p53-positive or TTF-1-positive tumors ($P < 0.001$ and $< 0.001$, respectively). Former or current smokers, pathological stage and mutant p53-or TTF-1-positive status were independent predictors of EGFR mutation ($P = 0.001$, 0.014, 0.014 and $< 0.001$, respectively). Patients with p53 under expression had significantly better overall survival in the whole cohort and wild-type EGFR cohort ($P = 0.0010$ and 0.0020, respectively) as well as in Ki-67-negative and TTF-1-positive patients ($P < 0.0001$ and 0.0009, and $P < 0.0001$ and 0.0004, respectively). Interestingly, in patients harbouring EGFR mutations, p53-under expression and Ki-67-negative cases still had better survival than positive cases, whereas there was no obvious difference between TTF-1-negative and TTF-1-positive cases ($P = 0.0198$, 0.0068 and 0.3684, respectively). Finally, in NSCLC patients with wild-type EGFR, positive Ki-67 expression was the independent predictor for the worst survival ($P = 0.022$).
**Conclusion:** The expression levels of mutant p53, Ki-67, and TTF-1 were correlated with EGFR mutation. High expression of mutant p53 and Ki-67 correlated with poor survival in the entire cohort, *EGFR* mutation or wild-type cohort. In addition, Ki-67 might have an impact on the prognosis for patients with NSCLC with wild-type *EGFR*.

**Keywords:** non-small cell lung cancer; epidermal growth factor receptor; mutant p53; Ki-67; TTF-1; prognosis
Introduction

Lung cancer remains the cause of highest morbidity and the main cause of cancer-related mortality worldwide and in China (Travis et al., 2013; Hung et al., 2014; Torre et al., 2016). Non-small cell lung cancer (NSCLC) accounts for 85% of lung cancers and presents three different histological subtypes including adenocarcinoma (ADC), squamous cell carcinoma (SCC) and large cell carcinoma (LCC). Recently, the diagnosis of lung cancer in early stages was become possible due to the advances in imaging techniques, such as computed tomography (CT) and positron emission tomography CT (PET-CT) (Rami-Porta et al., 2015). Different subtypes of lung cancer have distinct immunohistochemical (IHC) markers that could be used to distinguish ADC and SCC, resulting in the definition of appropriate therapeutic strategy and less severe side effects for the patients (Montezuma et al., 2013). However, the roles of IHC markers in the prediction of prognosis for lung cancer patients are not fully clarified, and their correlation with epidermal growth factor receptor (EGFR) mutations remains unclear.

Targeted therapy has recently emerged as a new therapeutic method for patients with advanced NSCLC, especially in patients with EGFR mutations who could benefit from the molecular targeted drugs such as tyrosine kinase inhibitors (TKIs) (Chiu et al., 2014). Notably, lung adenocarcinoma was reported to harbour more EGFR mutations and showed better progression-free survival than squamous cell carcinoma treated with TKIs (Chiu et al., 2014; Wu et al., 2014). However, thus far, only a few studies have reported the predictive value of EGFR mutations in patients with resected lung cancer without TKI treatment (Liu et al., 2014; Jiang et al., 2016).

IHC markers such as thyroid transcription factor 1 (TTF-1) have been advocated to distinguish ADC and SCC, whereas Ki-67 and mutant p53 were used to define the prognosis of lung cancer patients (Anagnostou et al., 2009; Lei et al., 2013; Wei et al., 2016). TTF-1 is a transcriptional factor involved in lung cancer differentiation and lung development (Snyder et al., 2013). Previous studies have also shown that low expression of TTF-1 predicted poor
prognosis and increased metastasis tendency in vivo (Lei et al., 2013). Wei et al. reported that positive TTF-1 expression was closely related with EGFR mutation in patients with NSCLC in China (Wei et al., 2016). Furthermore, Ki-67-positive tumors were reported to occur more frequently in males, smokers and non-ADC patients, which adversely correlate with EGFR mutation status (Ahn et al., 2014). p53 is a tumor suppressor that plays a central role in tumor development, and mutant p53 expression detected by IHC correlated with poor survival in patients with non-small cell lung cancer (Lei et al., 2013). However, the association between mutant p53, Ki-67 or TTF-1 proteins and EGFR mutation is not clear. Additionally, the Zhoushan archipelago, which is located in the eastern area of China, is the only archipelago city in China, and the people living on the islands have a particular life style eating seafood or salted seafood every day with less vegetables and meat. Hence, the purpose of the present study is to investigate correlations of EGFR mutations with the expressions of mutant p53, Ki-67 or TTF-1 protein and to assess the prognostic value of such mutations combined with the three protein expressions in patients with NSCLC in eastern Chinese islands.

Materials and Methods

Patients

Between July 2011 and March 2016, consecutive patients who underwent surgical resection with curative intent for lung cancer (N = 1140) were retrospectively enrolled at Zhoushan Hospital in Zhejiang, China. Patients’ preoperative work-flow (including enhanced thoracic CT, brain magnetic resonance imaging, bone scan and abdominal ultrasonography) was examined to exclude those with secondary lung cancer and those with systemic disease. Lymphadenectomy was routinely performed according to the National Comprehensive Cancer Network (NCCN) for all the patients (Rami-Porta et al., 2014). Histodiagnosis of the haematoxylin and eosin-stained slides of the specimens was performed and verified by two board-certified pathologists with crosschecked diagnosis. The criteria used for pathology diagnosis were in accordance with the standards of the World Health Organisation
classification (WHO) Classification of Lung Tumors and the International Association for the Study of Lung Cancer, American Thoracic Society and European Respiratory Society (IASLC/ATS/ERS) (Warth et al., 2012). The maximum diameter of the resected tumor was also decided by the pathologists. Otherwise, pathological tumor-node-metastasis (TNM) staging was conducted according to the proposed 8th edition of lung cancer classification (Asamura et al., 2015; Eberhardt et al., 2015; Rami-Porta et al., 2015). The following patients were excluded: patients who died within 30 days after surgery and those with incomplete records and follow-up data. Finally, 523 patients with primary NSCLC with completed records and EGFR mutation, mutant p53, Ki-67 and TTF-1 protein expression detection were included in the study. Overall survival was recorded between the time from surgical resection to the time of death or the final follow-up of surviving patients.

This study was approved by the Ethics Committee of Zhoushan Hospital, Wenzhou Medical University (Zhoushan, Zhejiang, China) with written informed consent before study enrolment by the participants, or their next of kin.

Genomic DNA extraction and EGFR mutation detection

A 10% formalin solution was used to fix resected lung tumors, which were then embedded in paraffin and sectioned at 10 µm thickness. The QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from five tissue sections of which tumor lesions were selected. DNA concentration and purity were assessed using a Quawell 3000 spectrophotometer (Quawell Technology, Sunnyvale, CA, USA). Human EGFR genotypes were assessed in all patients in the laboratory of Zhoushan Hospital on a 7500 Real-/Time PCR (polymerase chain reaction) system (ABI, Foster City, CA, USA) by an amplification refractory mutation system (Yuanqi Diagnostics, Shanghai, China), and all subsequent assays followed the manufacturer’s instructions. PCR amplification condition was 42°C 5min, 94°C 3min; 94°C 15sec, 60°C 60sec, 40 cycles, and collected the fluorescence signal in the second step of 60°C through FAM-TAMRA channel. The detected mutations are

Mutant p53, Ki-67 and TTF-1 protein expression analysis by immunohistochemistry

Five-micrometre-thick formalin-fixed, paraffin-embedded sections were used for IHC detection of mutant p53, Ki-67 and TTF-1 protein expression. The sections were de-paraffinized in xylene, hydrated and immersed in peroxidase blocking solution. They were then boiled in a heat-mediated antigen retrieval solution and cooled at room temperature. The sections were blocked with 3% bovine serum albumin (BSA) tris-buffered saline and then incubated in a 1:300 dilution of Ki-67 (ZM-0166, Zhongshan Golden Bridge Biotechnology, China), 1:200 dilution of p53 (ZM-0408, Zhongshan Golden Bridge Biotechnology), and 1:100 dilution of TTF-1 (MAB-0599, MXB Biotechnology, China) overnight at 4°C. The secondary anti-mouse or rabbit immunoglobulin G (IgG, 253-4290, Ventana Medical System, Roche, USA) and peroxidase-antiperoxidase conjugate were applied to detect the three proteins. Finally, the sections were stained in dianminobenzidine (DAB) and counterstained with haematoxylin. Phosphate-buffered saline (PBS) was used as a blank control, whereas preimmune serum was used as the negative control and the known positive sections were used as the positive control. The stained slides were observed under the Leica pathological image analysis instrument (DM25000B) at 400 times (×400) magnification by two experienced pathologists. The three proteins were located in the nucleus, and brown or yellow staining was identified as positive expression. Scores were calculated according to the percentage of cells showing positive immunoreactivity (score: 0 = ≤10%; 1 = 10%–39%; 2 = 40%–69%; and 3 =
≥70% of cells) and the staining intensity (score: 0 = negative; 1 = weak; 2 = moderate; and 3 = strong). The total score of 0 points for the specimen was defined as negative (-), 1-2 points as positive (+), and > 2 points as strongly positive. Discordant results were resolved by consensus reviews.

**Statistical analysis**

Pearson’s chi-square test or Fisher’s exact test was used to investigate differences between categorical groups. Comparison of continuous variables was performed by independent Student’s t-test or Mann-Whitney U test. Logistic regression was used to perform multivariate analysis of the risk factors for EGFR mutation. Overall survival was evaluated by Kaplan-Meier curves, and log-rank tests were used to compare the two categories in univariate analysis. Multivariate analysis was conducted using Cox’s proportional hazards regression model of all prognostic factors with univariate P value less than 0.05 to identify independent prognostic factors. All statistical tests were two-sided, with P < 0.05 considered as significant.

**Results**

**EGFR mutations and clinicopathologic characteristics with mutant p53, TTF-1, and Ki-67 expression**

A total of 523 patients with NSCLC in eastern Chinese islands who underwent surgical resection were analysed. The clinical and pathological characteristics associated with EGFR mutation status are shown in Table 1. The histological staining of mutant p53, TTF-1 and Ki-67 protein expression is shown in Fig. 1. Of 523 patients, 201 harboured EGFR mutations (38.4%) as follows: G719X in exon 18 (n = 5, 1.0%), exon 19 deletion (n = 58, 11.1%), exon 20 insertion (n = 1, 0.2%), L858R and/or L861Q in exon 21 (n = 136, 26.0%) and co-existing mutations in exons 19 and 21 (n = 1, 0.2%). There was no significant difference in EGFR mutations between genders (P = 0.098); however, those who never smoked harboured more
mutations than former and current smokers ($P < 0.001$). Mutations were also correlated with larger tumor size ($P < 0.001$), pleural invasion ($P = 0.023$), ADC histology ($P < 0.001$) and pathological stage I and stage II / IIIA ($P < 0.001$). In this cohort, $EGFR$ mutations were harboured in patients with lung ADC, whereas no $EGFR$ mutation was detected in patients with SCC and LCC. In the subtypes of lung adenocarcinoma, patients with lepidic, acinar or papillary-dominant subtypes more commonly had $EGFR$ mutations ($P = 0.038$, $< 0.001$ and $< 0.001$, respectively). Moreover, compared to $EGFR$ wild-type tumors, mutated $EGFR$ was significantly harboured in mutant p53-high expression or TTF-1-positive tumors ($P < 0.001$ and $< 0.001$, respectively). Ki-67-positive tumors harboured a higher frequency of $EGFR$ mutations ($P < 0.001$). The details are shown in Table 1. The relationships between clinical features and IHC markers were further analysed by multivariate logistic regression analysis, as summarised in Table 2. Non-smokers, pathological stage and mutant p53-high expression or TTF-1-positive status were independent predictors for $EGFR$ mutation ($P = 0.001$, 0.014, 0.014 and $< 0.001$, respectively).

The correlation of mutant p53, Ki-67 and TTF-1 protein expression with exon 19 deletion or exon 21 point mutation is shown in Table 2. Exon 19 deletions were more frequent in Ki-67-positive tumors, whereas exon 21 mutations were more common in Ki-67-negative tumors ($P = 0.039$). However, the frequency of mutations was comparable between under and high expression p53 and TTF-1 tumors (Table 3).

**Overall survival for patients with NSCLC in eastern Chinese islands**

The last follow-up time was Dec, 2017. Overall survival outcomes were compared between mutant p53, Ki-67, and TTF-1-negative and TTF-1-positive patients; the mean and median follow-up time for these patients was 34.1 months and 32 months, respectively, and the range was 3 to 76 months. Patients with p53-under expression had significantly better overall survival than high expression cases ($P = 0.0010$, Fig. 2a), and patients with Ki-67-negative and TTF-1-positive had better overall survival ($P < 0.0001$, = 0.0009, Fig.
Additionally, in patients with wild-type EGFR, p53-under expression cases also presented better survival than high expression cases, and the same results were observed in Ki-67-negative and TTF-1-positive cases ($P = 0.0020, < 0.0001$ and $0.0004$, respectively, Fig. 2beh). However, in patients harbouring $EGFR$ mutations, p53-under expression and Ki-67-negative cases still had better survival than positive cases, whereas there was no obvious difference in survival between TTF-1-negative and TTF-1-positive cases ($P = 0.0198$, $0.0068$ and $0.3684$, respectively, Fig. 2cfi).

**Univariate and multivariate analysis for patients with NSCLC in eastern Chinese islands**

Univariate and multivariate Cox regression analysis results of the survival of patients with NSCLC in eastern Chinese islands are summarised in Table 4. In the multivariate analysis, unfavourable overall survival was associated with tumor size, which was an independent predictor of reduced overall survival ($P < 0.001$). In NSCLC patients with $EGFR$ wild-type, larger tumor size and positive Ki-67 expression were the independent predictors for worse survival ($P = 0.017$ and $0.022$, Table 5). However, in the cohort of NSCLC patients with mutated $EGFR$, these factors were not associated with reduced overall survival ($P > 0.05$, Table 6).

**Discussion**

In the present study, we showed the $EGFR$ mutation status in patients with NSCLC from eastern Chinese islands and its correlation with mutant p53, Ki-67 and TTF-1 protein expression. Mutant p53 and Ki-67 expressions were positively related to worse overall survival in the presence of $EGFR$ mutation, whereas TTF-1 was negatively related to overall survival in patients with NSCLC or with wild-type $EGFR$, but not with mutated $EGFR$.

People in eastern Asia have a higher frequency of $EGFR$ mutation than western populations. In this study, we reported that the frequency of $EGFR$ mutation of the studied patients with NSCLC from eastern Chinese islands was 38.4% (201/523); this finding was in
EGFR mutations were mainly detected in patients who were never smokers. The mechanism by which smoking status contributes to EGFR mutation, however, remains unknown. With regard to the different histological subtypes, none of the patients with SCC and LCC in this cohort harboured EGFR mutation; Rekhtman et al reported that only one patient with LCC as variant of ADC harboured EGFR mutation, but none of patient with LCC as variant of SCC harboured EGFR mutation (Rekhtman et al., 2013); Wang et al. revealed that the mutation rate of EGFR was 3.9% in 310 patients with lung SCC (Wang et al., 2015). We speculate that these differences can be attributed to the limited number of cases and different genetic backgrounds in our study. In line with the other studies, all EGFR mutations in patients with lung ADC showed high frequency of lepidic, papillary and acinar dominant subtypes in our cohort (Lu et al., 2016; Nakamura et al., 2015). Moreover, EGFR mutations more commonly occurred in p-stage I, II, and IIIA; this finding was similar to the results obtained from population in southern China (Liu et al., 2014; Wang et al., 2017; Wei et al., 2016). Moreover, it has been reported that larger tumor diameter is correlated with EGFR mutation. A northern Chinese group showed that EGFR mutations were more likely to occur in large tumors, whereas in tumors with diameters of 0 to 10 mm, the frequency of EGFR mutation was low (Ning et al., 2017).

Mutant p53, Ki-67 and TTF-1 are the common IHC markers for lung cancer, although the association between the expression levels of these three proteins and EGFR mutation remains unclear. In our cohort, patients with EGFR mutation were negatively correlated with TTF-1 expression. TTF-1 is a homeodomain transcription factor that is used as a biomarker for the respiratory unit cells. Previous studies have revealed that TTF-1 expression was negatively correlated with EGFR mutation; this finding is in line with our data (Wei et al., 2016; Zhang et al., 2015). TP53 is a well-known tumor suppressor in various cancers, and mutated TP53 or the loss of TP53 accelerates the progression of tumors (Chen et al., 2012).
VanderLaan and colleagues revealed that lung cancer patients with \( EGFR \) mutation also harboured 50% \( TP53 \) mutation (VanderLaan et al., 2017). This finding was similar to the observation in our cohort where the mutant \( p53 \) protein was correlated with \( EGFR \) mutation. Ki-67 is activated in all phases of the cell cycle except the resting stage (G0) and is recognised as a marker to evaluate cell proliferation in NSCLC (Wen et al., 2015). However, there are few reports on the correlation between Ki-67 and \( EGFR \) mutation. In our cohort, Ki-67-positive cases harboured more \( EGFR \) mutations. This might suggest that \( EGFR \) mutation accelerates the progression of tumors. Moreover, \( EGFR \) exon 19 deletions were more frequently observed in Ki-67-positive tumors, whereas exon 21 mutations were more commonly found in Ki-67-negative tumors. Further large-scale cohort studies should be conducted to confirm the relationship between Ki-67 and \( EGFR \) mutation.

The evaluation of mutant \( p53 \), Ki-67 and TTF-1 as prognostic biomarkers for cancer is important. However, the prognostic role of these three IHC markers in different \( EGFR \) mutation statuses is not well known. Similar to other studies, poor overall survival of patients with NSCLC was correlated with positive expression of mutant \( p53 \), Ki-67 or negative expression of TTF-1 in the eastern Chinese islands population (Anagnostou et al., 2009; Lei et al., 2013; Wei et al., 2016). Moreover, TTF-1 was negatively related to worse overall survival in patients with NSCLC (Zhang et al., 2015). We further revealed that in cases with mutated or wild-type \( EGFR \), the positive expression of mutant \( p53 \) and Ki-67 was associated with poor survival. Nevertheless, wild-type \( EGFR \) cases with TTF-1-positive expression had better survival than those with TTF-1-negative expression. TTF-1 was first used as a biomarker for distinguishing lung ADC from SCC, and EGFR mutation commonly occurs in lung ADC along with only a few negative TTF-1 cases. The association between \( EGFR \) mutation and TTF-1 needs further study. Multivariate analysis showed that Ki-67 was an independent prognostic factor for patients with wild-type \( EGFR \) NSCLC rather than patients with mutated EGFR and the whole cohort. This might be because the mutant EGFR accelerates the tumor progression by activating other genes, and the Ki-67 might not be an important factor in
EGFR mutated tumors. A meta-analysis of 32 studies showed that high Ki-67 expression was more likely to be associated with a poorer outcome, and this condition particularly occurred in Asian patients with lung ADC (Wen et al., 2015).

Limitations of the current study should be considered. The study was retrospective in nature and was conducted solely at a single institution; there was also a lack of data on disease-free survival. A future prospective study with data on disease-free survival should be performed to validate the current findings. Additionally, because of the relatively small number of squamous cell lung cancer and large cell lung cancer cases, a future study with a large number of cases should be performed to eliminate bias. In our study, we did not verify mutation status of TP53, p53 protein negative was considered as null mutation and included these patients in under expressed p53 protein group (Oros Klein et al., 2016), a future study should be done to determine the correlation of TP53 mutation and p53 protein in NSCLC patients.

In summary, we first showed the EGFR mutation status in the population of eastern Chinese island and then revealed the correlation of mutant p53, Ki-67 and TTF-1 expression with EGFR mutation. Moreover, high expression of mutant p53 and Ki-67 correlated with poor survival of the entire cohort, regardless of whether the patient had wild-type or mutated EGFR. Additionally, Ki-67 might have an impact on the prognosis for patients with NSCLC with wild-type EGFR.

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Figure Legends

Fig. 1. Immunohistochemical (IHC) staining of mutant p53, Ki-67, TTF-1 in non-small cell lung cancer. a. Minimally invasive lung adenocarcinoma with haematoxylin and eosin (H&E) stained. b. Corresponding faint nuclear mutant p53 staining in minimally invasive lung adenocarcinoma. c. Squamous cell carcinoma (SCC) with H&E staining. d. Corresponding strong mutant p53 staining in SCC. e. SCC with H&E staining. f. Corresponding Ki-67 < 5% negative nuclear expression in SCC. g. SCC with H&E staining. h. Corresponding Ki-67 40% moderate nuclear expression in SCC. i. SCC with H&E staining. j. Corresponding TTF-1 negative nuclear TTF-1 staining in SCC. k. Lung adenocarcinoma in situ with H&E staining. l. Corresponding strong nuclear TTF-1 staining in lung adenocarcinoma in situ. ×400, scale bar 200 um.

Fig. 2. Kaplan-Meier curves for overall survival in 523 lung cancer patients according to EGFR mutation. a. Overall survival in all patients stratified by p53 expression. b. Overall survival in patients with wild-type EGFR stratified by p53 expression. c. Overall survival in patients with mutated EGFR stratified by p53 expression. d. Overall survival in all patients stratified by Ki-67 expression. e. Overall survival in patients with wild-type EGFR stratified by Ki-67 expression. f. Overall survival in patients with wild-type EGFR stratified by Ki-67 expression. g. Overall survival in all patients stratified by TTF-1 expression. h. Overall survival in patients with mutated EGFR stratified by TTF-1 expression. i. Overall survival in patients with mutated EGFR stratified by TTF-1 expression.
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