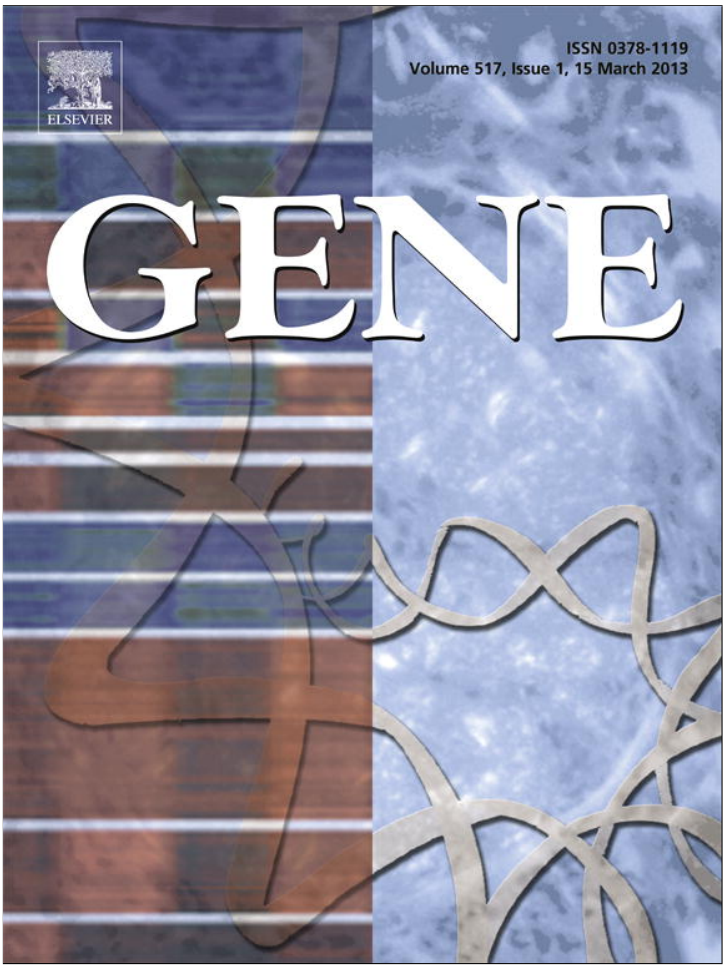

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Role of *EGFR* SNPs in survival of advanced lung adenocarcinoma patients treated with Gefitinib☆

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ABSTRACT

Aim: As a novel molecularly targeting agent for non-small-cell lung cancer (NSCLC), Gefitinib can block its tyrosine kinase activity of the epidermal growth factor receptor (EGFR). Genetic variations in *EGFR* may affect its protein function or expression and lead to diverse outcomes in NSCLC patients after Gefitinib therapy. Therefore, this prospective study examined whether *EGFR* single nucleotide polymorphisms (SNPs) are associated with different survival time in advanced lung adenocarcinoma patients treated with Gefitinib.
Methods: One hundred and twenty-eight patients with stage IIIB or IV lung adenocarcinoma receiving Gefitinib target therapy between 2008 and 2010 were recruited in this study. Six *EGFR* haplotype-tagging SNPs were genotyped by the Sequenom MassArray system. Survival by different genotypes was compared using Kaplan–Meier methods. Cox proportional hazards models were applied to estimate the effect of prognostic factors on overall survival (OS) and progression-free survival (PFS).
Results: After the median 16.6 months of follow-up, the unfavorable *EGFR* rs2293347AA or GA genotype was significantly correlated with shorter OS (AA vs. GG: 2.0 vs. 21.0 months; hazard ratio (HR) = 2.44, 95% confidence interval (CI) = 1.06–5.56; $P = 0.036$; GA vs. GG: 15.0 vs. 21.0 months; HR = 1.75, 95%CI = 1.08–2.86, $P = 0.025$) compared with the favorable rs2293347GG genotype. The prognostic significance of *EGFR* rs4947492 polymorphism on OS also existed (GG carriers vs. AA carriers: median OS = 24.6 vs. 14.9 months, HR = 0.29, 95%CI = 0.10–0.83, $P = 0.021$). No significant associations were found among other *EGFR* SNPs and survival.
Conclusion: EGFR rs2293347 and rs4947492 SNPs might be potential predictive markers of OS in advanced lung adenocarcinoma patients treated with Gefitinib.

1. Introduction

Non-small cell lung cancer (NSCLC) accounts for >80% of primary lung cancers and about one third of NSCLC patients are diagnosed at a locally advanced stage (Gandara et al., 2005). Although most advanced NSCLC patients could benefit from platinum-based chemotherapy, their median survival time was only 8–11 months and the 1- and 2-year survival rates were 35–40% and 10–20%, respectively (Fossella et al., 2003; Schiller et al., 2002). As novel molecularly targeting agents for the treatment of patients with advanced NSCLC refractory to chemotherapy, Gefitinib and Erlotinib [epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, TKIs] can reversibly bind the ATP pocket in the EGFR intracellular tyrosine kinase domain and block its tyrosine kinase activity (Fukuoka et al., 2003). In clinics, EGFR TKIs showed great benefits to these advanced NSCLC patients, especially female patients, never-smokers, and ones with adenocarcinoma histology (Giaccone, 2005).

Although the above-mentioned clinical characteristics are helpful during identification of suitable candidate patients for TKI treatment, patients' somatic and germline genetic backgrounds might also determine their response to TKIs. Several somatic mutations in exons 18–21 of the *EGFR* gene have been identified to account for the increased sensitivity to Gefitinib (Janne et al., 2005). It has also been reported that *EGFR* copy number and EGFR expression are useful in predicting which patients are more likely to respond to TKIs. However, since patients with advanced lung cancer can not undertake surgery to remove their tumors, tissue samples are usually unavailable for detection of somatic mutation, copy number change, and/or amplification of *EGFR*. Therefore, if some non-invasive (or minimally

Abbreviations: NSCLC, non-small-cell lung cancer; EGFR, epidermal growth factor receptor; SNPs, single nucleotide polymorphisms; OS, overall survival; PFS, progression-free survival; HR, hazard ratio; CI, confidence interval; TKI, tyrosine kinase inhibitor.

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invasive) biomarkers could be identified, it will be helpful in clinics. Interestingly, several studies showed that *EGFR* genetic polymorphisms could also be potential biomarkers for efficacy prediction of Gefitinib treatment. In these reports, the most studied polymorphisms include an intron 1 CA simple sequence repeat (CA-SSR) (Amador et al., 2004; Liu et al., 2008; Ma et al., 2009; Tiseo et al., 2008, 2010), −216G>T (Gregorc et al., 2008; Ichihara et al., 2007; Liu et al., 2008), −191C>A (Gregorc et al., 2008; Ichihara et al., 2007; Liu et al., 2008), and Arg497Lys (Gregorc et al., 2008; Ichihara et al., 2007; Liu et al., 2008). Using a haplotype-tagging single nucleotide polymorphism (htSNP) approach, Ma and colleagues systematically evaluate the association between SNPs in the *EGFR* gene locus and clinical outcomes in 84 advanced NSCLC patients from a northern Chinese population (Ma et al., 2009). Interestingly, it was found that *EGFR* rs2293347 (D994D) and CA-SSR polymorphisms are associated with the outcome of Gefitinib therapy. There might be significant differences in the allelic and genotype frequencies of some SNPs among Chinese in different geographical areas (Ma et al., 2009), which may impact relationship between SNPs and clinical outcomes of Gefitinib treatment. Therefore, we conducted this study to evaluate the role of *EGFR* htSNPs in a southern Chinese population.

2. Patients and methods

2.1. Patients

This study included 128 patients with advanced lung adenocarcinoma (Table 1). Patients were recruited between January 2008 and December 2010 at Tongji Hospital, Huazhong University of Science and Technology (Wuhan, Hubei Province). Eligible patients had at least one measurable lesion with a minimum size in at least one diameter of ≥10 mm for liver, lung, brain or lymph node metastases, WHO performance status of 0–1, and life expectancy of ≥3 months. Each patient was treated with Gefitinib orally at a daily dose of 250 mg as 2nd or 3rd line monotherapy. The exclusion criteria included previous other EGFR-TKI treatment, pneumonectomy or severe cardio-pulmonary diseases. This study was approved by the Review Board of the Tongji Hospital. Written informed consent was obtained from each patient for the use of their DNA and clinical information.

2.2. Treatment and follow-up

All patients were treated with Gefitinib orally at Tongji Hospital, Huazhong University of Science and Technology between January 2008 and December 2010. Survival information was collected every 2 months from the specific doctor's follow-up records, and the patients were asked to return to the hospital for review. Progression-free survival (PFS) was defined as the time from first dose of Gefitinib to the date of disease progression or death due to any cause. Overall survival (OS) was defined as the time between the first dose of Gefitinib and death or the last follow-up. Dates of death were obtained from (a) inpatient and outpatient records, (b) patient's family, or (c) local Public Security Census Register Office. Patients who were not departed were censored at the last date they were known to be alive based on the date of the last contact.

Table 1
Demographic and base line clinical characteristics of patients.

Variable	No. of patients (n = 128)	%
Sex		
Male	62	48.4
Female	66	51.6
Smoking status		
Smoker	41	32.0
Non smoker	87	68.0
Tumor stage at diagnosis		
IIIB	32	25.0
IV	96	75.0
EGFR mutation status		
Positive	28	21.9
Negative	29	22.6
Unidentified	71	55.5
Median age (year)		55.2 (32.0–80.0)
Median time of follow up		16.6 (1.3–45.7)
Median time of OS		15.3 (1.2–45.7)
Median time of PFS		8.8 (0.6–29.7)

Abbreviations: OS, overall survival; PFS, progression free survival.

2.3. SNP genotyping

EGFR htSNPs (rs4947492, rs11977388, rs2075102, rs7809028, rs2293347, and rs1154848) were selected using the HaploView software with HapMap HCB data in a pairwise mode and all correlated alleles were captured at $r^2 > 0.8$ (Ma et al., 2009). Before genotyping, five-mL blood sample was collected from each patient before therapy. DNA was extracted with standard proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation (Yang et al., 2009). Genotypes of the *EGFR* htSNPs were determined by the MassArray system (Sequenom Inc., San Diego, California, USA). Genotyping was performed without knowledge of clinical outcomes of the patients. A 15% blind, random sample of study subjects was genotyped in duplicates and the reproducibility was 100%.

2.4. Statistical analysis

Student's *t*-test or χ^2-test was used to calculate the difference of patient clinical characteristics. Survival distributions were estimated with the Kaplan–Meier method and compared with the log-rank test. Multivariate Cox proportional hazards models were applied to estimate the effect of prognostic factors on OS and PFS, using proverbial clinical factors, including age, sex, smoking status, EGFR mutation status and clinical stages. Hazard ratios (HRs) with 95% confidence intervals (CIs) were computed using the Cox model. Statistical significance was set at a level of 0.05, and all the analyses were performed using the SPSS software package (version 16.0, SPSS Inc., Chicago, IL).

3. Results

3.1. Patient characteristics and clinical outcomes

The distribution of demographic and clinical characteristics of patients is showed in Table 1. The median follow-up time of the patients was 16.6 months when the final analysis was done (November 2011). The median age of patients was 55.2 years (range, 32.0–80.0 years), and 48.4% were male. Forty one patients (32.0%) were smokers and 87 patients (68.0%) were nonsmokers. Thirty two (25.0%) had stage IIIB disease and 96 (75.0%) had stage IV disease. EGFR mutation status was available for 57 patients. In this cohort, eighty five patients (66.4%) died and the median OS time was 15.3 months (range, 1.2–45.7 months). The median PFS time was 8.8 months (range, 0.6–29.7 months).

3.2. Comparison of survival according to baseline characteristics of patients

To test whether various clinical characteristics contribute to survival, patients were grouped according to age, sex, smoking status, clinical stages, or EGFR mutation status. Both PFS and OS were compared among different groups. Sex had a significant impact on PFS (Univariate analyses: HR = 0.43, 95%CI = 0.29–0.63, $P < 0.001$; multivariate analyses: HR = 0.42, 95%CI = 0.25–0.71, $P = 0.001$). For OS, sex also significantly influenced patient prognosis (Univariate analyses: HR = 0.51, 95%CI = 0.28–0.93, $P = 0.027$; multivariate analyses: HR = 0.54, 95%CI = 0.35–0.83, $P = 0.005$) (Table 2).

Table 2
Univariate and multivariate Cox-regression analyses for PFS and OS.

Variable	PFS				OS			
	Univariate analyses		Multivariate analyses		Univariate analyses		Multivariate analyses[a]	
	HR (95%CI)	*P*	HR (95%CI)	*P*	HR (95%CI)	*P*	HR (95%CI)	*P*
Age	0.98 (0.96–1.00)	0.044	0.98 (0.96–1.00)	0.017	0.98 (0.96–1.00)	0.118	0.99 (0.97–1.01)	0.185
Sex	0.43 (0.29–0.63)	<0.001	0.42 (0.25–0.71)	0.001	0.51 (0.28–0.93)	0.027	0.54 (0.35–0.83)	0.005
Smoking status	1.94 (1.30–2.89)	0.001	0.95 (0.55–1.63)	0.852	0.98 (0.52–1.81)	0.936	1.68 (1.06–2.64)	0.026
Stage	1.00 (0.64–1.54)	0.982	0.92 (0.59–1.45)	0.718	0.77 (0.45–1.29)	0.318	1.11 (0.67–1.83)	0.690
EGFR mutation status	0.40 (0.24–0.68)	0.001	1.16 (0.90–1.50)	0.258	1.23 (0.91–1.66)	0.182	1.14 (0.85–1.53)	0.394

Note: PFS, progression free survival; OS, overall survival.
[a] From separate Cox regression models, each was adjusted for sex, age, smoking status, EGFR mutation and stages, where it was appropriate.

3.3. Effects of SNPs on overall survival and progression-free survival

Associations between *EGFR* htSNPs and OS of advanced lung adenocarcinoma patients treated with Gefitinib are summarized in Table 3 and Fig. 1. Patients carrying different genotypes of *EGFR* rs11977388, rs2075102, rs7809028 and rs1154848 SNPs did not show significant differences of OS in Cox regression models (all $P > 0.05$; Table 3 and Figs. 1B–D and F) with or without adjusting for sex, age, ECOG, smoking status, EGFR mutation and EGFR stages. However, the *EGFR* rs2293347 polymorphism was significantly associated with the OS of patients. As shown in Table 3, the median OS for patients with the rs2293347AA genotype (median OS = 2.0 months, 95%CI = 0.0 to 5.4 months) was significantly shorter than that of patients with the rs2293347GG genotype (median OS = 21.0 months; 95%CI = 14.0 to 27.9 months) (multivariate model: HR = 2.44, 95%CI = 1.06–5.56, $P = 0.036$ for log-rank test) (Table 3 and Fig. 1E). There was also an association between the rs2293347GA genotype and the patients' OS (median OS = 15.0 months, 95%CI = 8.3 to 21.8 months; multivariate model: HR = 1.75, 95%CI = 1.08–2.86, $P = 0.025$ for log-rank test), demonstrating this polymorphism also functions in a dominated genetic model (Table 3 and Fig. 1E). In the multivariate Cox proportional hazards analyses, after adjustment for the clinicopathologic factors including sex, age, smoking status and disease stages, the prognostic significance of *EGFR* rs4947492 polymorphism also existed (GG carriers vs. AA carriers: median OS = 24.6 vs. 14.9 months, HR = 0.29, 95%CI = 0.10–0.83, $P = 0.021$; Table 3). No significant difference in PFS was observed in advanced lung adenocarcinoma patients related to any *EGFR* htSNPs (Supplementary Table 1). Adjustment for sex, age, smoking status and disease stages did not significantly affect respective HRs and *P* values.

4. Discussion

Considering the advanced NSCLC patients, especially patients with adenocarcinoma histology, could get great benefits from Gefitinib treatment, the current cohort only included lung adenocarcinoma patients. In 128 advanced lung adenocarcinoma patients treated with Gefitinib, we examined whether *EGFR* htSNPs affect survival. Although none of these *EGFR* htSNPs were significantly related to PFS,

Table 3
Association between *EGFR* htSNPs and overall survival of advanced lung adenocarcinoma patients treated with Gefitinib.

SNPs	All patients		Median OS (month)	95%CI (month)	Univariate model			Multivariate model[a]		
	n	%			HR	95%CI	*P*	HR	95%CI	*P*
rs4947492	128	100.0								
AA	55	43.0	14.9	6.9–22.8		Reference			Reference	
AG	60	46.9	11.8	3.6–20.0	1.04	0.67–1.61	0.870	0.86	0.53–1.39	0.538
GG	13	10.1	24.6	NA	0.49	0.19–1.24	0.486	0.29	0.10–0.83	0.021
rs11977388	127	99.2								
TT	50	39.1	13.2	6.1–20.3		Reference			Reference	
TC	64	50.0	16.8	7.2–26.4	0.80	0.51–1.25	0.321	1.26	0.80–2.00	0.322
CC	13	10.1	16.5	0.0–40.4	0.84	0.37–1.90	0.681	1.34	0.56–3.18	0.509
rs2075102	128	100.0								
CC	90	70.3	11.8	5.9–17.7		Reference			Reference	
CA	33	25.8	16.8	12.8–20.8	1.03	0.64–1.68	0.894	0.91	0.55–1.49	0.697
AA	5	3.9	29.4	NA	0.81	0.25–2.58	0.717	1.15	0.34–3.90	0.820
rs7809028	122	95.3								
GG	60	46.9	20.9	7.4–34.4		Reference			Reference	
GA	53	41.4	16.5	10.2–20.4	1.30	0.81–2.10	0.278	0.74	0.45–1.21	0.223
AA	9	7.0	2.0	1.0–3.1	2.14	0.98–4.65	0.056	0.52	0.22–1.21	0.127
rs2293347	128	100.0								
GG	59	46.1	21.0	14.0–27.9		Reference			Reference	
GA	59	46.1	15.0	8.3–21.8	1.54	0.96–2.47	0.073	1.75	1.08–2.86	0.025
AA	10	7.8	2.0	0.0–5.4	2.41	1.41–5.11	0.021	2.44	1.06–5.56	0.036
rs1154848	126	98.4								
AA	48	34.4	10.0	4.2–15.8		Reference			Reference	
AG	66	48.4	16.8	7.0–26.6	0.68	0.43–1.08	0.099	1.53	0.94–2.51	0.091
GG	21	15.6	29.4	4.9–53.9	0.63	0.31–1.28	0.200	1.84	0.86–3.95	0.117

Note: One hundred and thirty seven DNA samples from 128 patients were available for genotyping analyses of EGFR htSNPs (rs4947492, rs11977388, rs2075102, rs7809028, rs2293347, and rs1154848). A total of 122 samples of the 128 patients were evaluable for rs7809028, 126 samples of the 128 patients for rs1154848, 127 samples of the 128 patients for rs11977388, and all 128 data for rs4947492, rs2075102 and rs2293347.
Abbreviations: EGFR, epidermal growth factor receptor; SNP, single nucleotide polymorphism; htSNP, haplotype-tagging SNP; n, number of patients; HR, hazard ratio; 95%CI, 95% confidence interval.
[a] From separate Cox regression models, each was adjusted for sex, age, smoking status, EGFR mutation and stages.

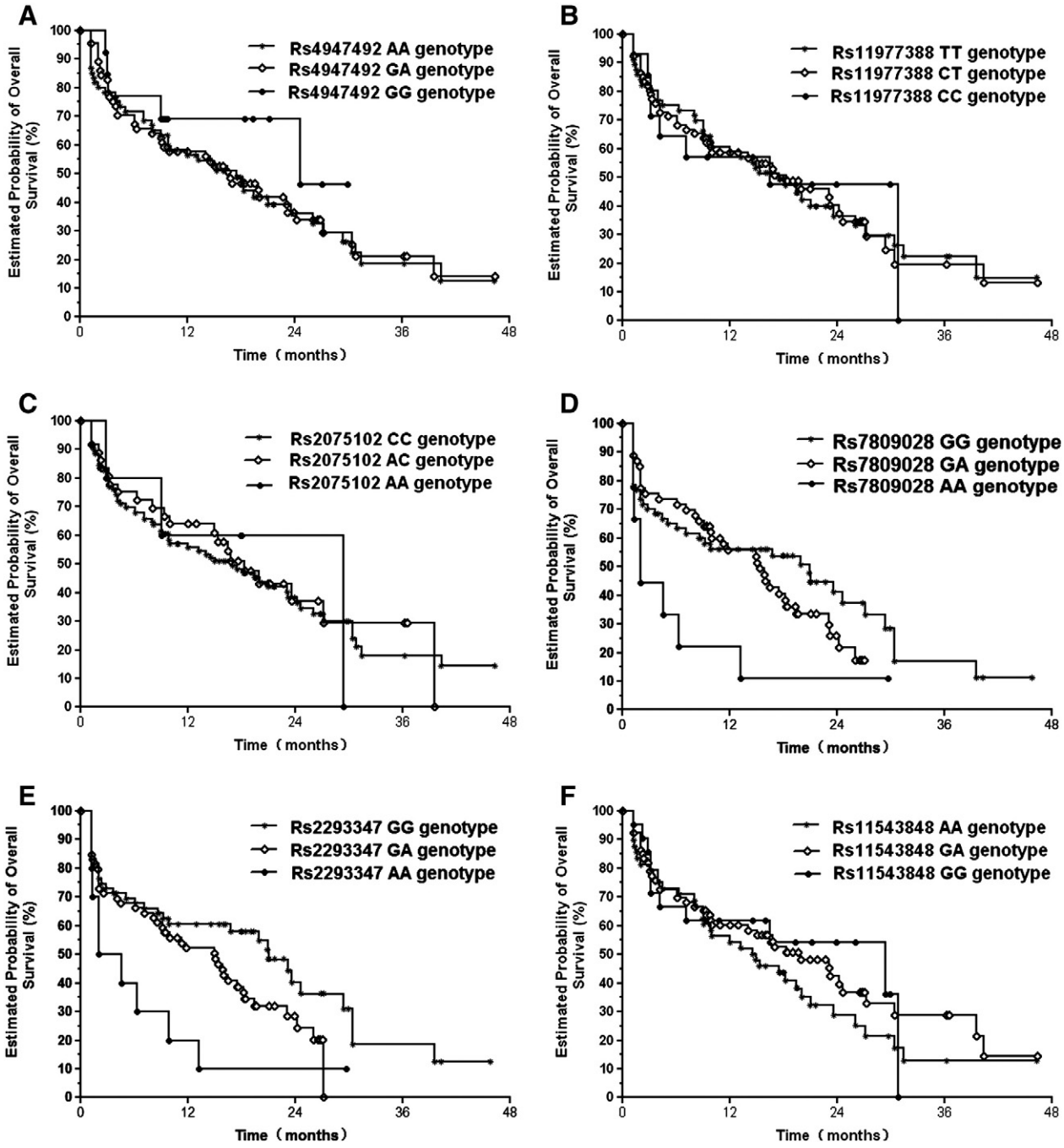

Fig. 1. Kaplan–Meier curves of OS for the patients with different genotypes of *EGFR* tag-SNPs. (A), rs4947492; (B), rs11977388; (C), rs2075102; (D), rs7809028; (E), rs2293347; (F), rs1154848.

significant association between the *EGFR* rs4947492 and rs2293347 polymorphisms and OS were observed.

A 19 month difference of median OS between rs2293347GG carriers and rs2293347AA carriers indicates that the rs2293347 variant might be a potential prognostic marker among Chinese lung adenocarcinoma patients treated with Gefitinib. This observation is biologically plausible since rs2293347 is a synonymous SNP (D994D) located in exon 25, which encodes part of the regulatory domain (EGFR regulatory region is encoded by exons 25–28) (Mitsudomi and Yatabe, 2010). Also, it has been reported that synonymous SNPs may influence mRNA stability, translational kinetics, and splicing (Kimchi-Sarfaty et al., 2007 and Sauna et al., 2007). Interestingly, rs4947492 polymorphism, which locates in the potential gene regulatory region, was also associated with OS of patients. This might be due to altered expression of EGFR caused by A-to-G change in the rs4947492 polymorphic site. However, further studies on the role of these genetic variants in EGFR expression or their biological function are warranted.

There are several limitations of this study, such as its moderate sample size. Results from this study should not be over-interpreted before they are validated by well-designed and larger prospective studies. In addition, because significant regional difference of *EGFR* rs2293347 genotypes was observed in Chinese, the correlation between *EGFR* rs2293347 and OS may be sub-population specific and, thus, need to be confirmed in independent Chinese cohorts in the future.

In all, to our knowledge, this is the first report to demonstrate that SNPs in the *EGFR* gene were significantly associated with patients' OS

to Gefitinib therapy in a southern Chinese population. Our findings may potentially have several clinical implications, including that the rs2293347 genotypes may act as prognostic tools for patients' OS to Gefitinib therapy in advanced lung adenocarcinoma.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2012.12.087.

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