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Original Research

Discriminating association of a common telomerase reverse transcriptase promoter polymorphism with telomere parameters in non-small cell lung cancer with or without epidermal growth factor receptor mutation

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rs2853669 single-nucleotide polymorphism (rs2853669 SNP);
Telomerase reverse transcriptase (TERT);
Telomere length;
Epidermal growth factor receptor

Abstract ***Background:*** The role of epidermal growth factor receptor (EGFR) pathways in regulating telomerase is increasingly being recognised. We analysed the impact of rs2853669 single nucleotide polymorphism (SNP) on telomere parameters and its prognostic value for non-small cell lung cancer (NSCLC) with or without EGFR mutation.
Methods: The association of rs2853669 with telomerase reverse transcriptase (TERT) mRNA level and relative telomere length (RTL) was analysed using resected tumour samples from 250 NSCLC patients. We also investigated the patients' clinical outcomes with a median follow-up of 57 months (2–99 months).
Results: The rs2853669 T/C allele was significantly associated with lower TERT mRNA expression (versus C/C and versus T/T; $p < 0.001$ for both) and shorter RTL (versus C/C and versus T/T; $p = 0.039$ and 0.023) in patients without EGFR mutation. Such difference was not observed in their counterparts harbouring EGFR mutation. When considering the

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mutation (EGFR);
Survival outcome

cohort as a whole, T/C allele was significantly associated with shortest overall survival compared with T/T or C/C allele (mean: 61.8, 80.9 and 88.7 months, $p_{\text{log-rank}} < 0.001$) and disease-free survival (mean: 78.3, 87.9 and 91.5 months, $p_{\text{log-rank}} = 0.019$). Stratification analyses showed that the negative prognostic effect of T/C on OS was constrained in patients without EGFR mutation.

Conclusion: Our study revealed significant associations of a common SNP within TERT promoter region on telomere parameters and survival in NSCLC patients without EGFR mutation. The result may help providing instruction for therapeutic interventions targeting telomerase and evidence for investigation of TERT-EGFR interacting mechanism in telomere biology.

1. Introduction

Non-small cell lung cancer (NSCLC) accounts for 85% of lung carcinoma, which is the leading cause of cancer-related death in both male and female worldwide [1]. Telomerase activity is considered as a diagnostic marker and as a therapeutic target for NSCLC [2]. Telomerase reverse transcriptase (TERT) is the key determinant of the enzymatic activity of human telomerase, and its transcriptional control is a major contributor to the regulation of telomerase activity in many types of human cells [2–7]. The regulatory mechanisms that impel telomerase reactivation in carcinogenesis are multifaceted and have only recently started to be revealed. The variants in promoter region of TERT is believed to be responsible for the transcriptional activity of the TERT mRNA by generating a consensus binding site for E-twenty-six/ternary complex transcription factors (Ets/TCFs) [4,8–10]. A TERT promoter polymorphic variant (rs2853669) within an endogenous Ets-2 transcription factor binding site was revealed to be associated with an escalation in telomerase activity from the T/T allele to the C/C allele [11,12]. However, the prognostic value of rs2853669 in NSCLC remained to be elucidated, and a multifaceted study of telomere parameter and clinical outcomes in a single cohort is needed to further understand the role of rs2853669 in telomere regulation and clinical outcomes in NSCLC patients.

Mutations in the epidermal growth factor receptor tyrosine kinase (EGFR-TK) domain are among the most common oncogenic mutations in lung adenocarcinoma (ADEC) and are present in approximately 35% of Asian patients [13]. EGFR mutations have been characterised as associated with enhanced EGFR signalling and have been shown to be driver mutations for NSCLC [14–16]. The role of EGFR pathways in regulating telomerase enzyme is increasingly being recognised [9,12–20]. It has been reported by Yoshiko et al. that EGF can activate telomerase through the direct activation of TERT transcription by Ets factor via the MAP kinase signalling pathway in established fibroblast cell lines and several cancer cell lines (vulvar cancer, breast cancer and cervical cancer), indicating a potential crosstalk between the two well-established markers for NSCLC development [14,21]. However, the clinical relationships between EGFR and TERT parameters in NSCLC patients have not been well characterised. In the present study, we sequenced the TERT promoter region and analysed the association of rs2853669 with telomere parameters (TERT mRNA level and relative telomere length [RTL]) and survival outcome by introducing EGFR mutation status to further elucidate the prognostic potential of the rs2853669 and its role in regulating telomere biology in NSCLC patients.

2. Materials and methods

2.1. Patients and tumour specimens

The study was conducted by using specimens resected from 250 patients with primary NSCLC who were diagnosed and treated at the First Affiliated Hospital of Zhejiang University between May 2010 and May 2017. These patients were selected for the following criteria: (1) No history of chemotherapy for potential change in telomere length; (2) stage I ~ III NSCLC patients who underwent successful R0 pulmonary resection; (3) patients with validate follow-up data (patients who are lost to follow-up were excluded from our cohort). Samples were collected with informed consent and approval from the Ethics Committees of the First Affiliated Hospital of Zhejiang University and were kept frozen in liquid nitrogen. The patients' demographic characteristics and clinical data were collected from medical records or by phone calls. Pathological information was collected from pathological reports. Tumour-node-metastasis stage was defined according to the eighth edition of the AJCC/UICC staging manual. Follow-up was conducted by phone calls to patients or their families.

2.2. Mutation analyses

All DNA extraction and sequencing were conducted by a same method and was described in our previous study [22]. The primers designed to amplify the exons for detecting EGFR mutations are shown in Supplementary Table 1. Polymerase chain reaction (PCR) amplification of the TERT promoter region (−27 to −286) and the 18 to 21 exons of EGFR were performed using a Phanta Max Master Mix (Vazyme Biotech, P515-02). After confirming the quality of the PCR products by gel electrophoresis, sequencing PCR was carried out using a BigDye Terminator v.3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The sequences generated were analysed using Lasergene (DNASTAR). All samples were checked in forward and reverse directions.

2.3. Quantification of TERT mRNA expression in lung tissue

TERT mRNA was measured by quantitative real-time PCR (RT-qPCR). Total RNA was extracted from resected tumours using a TRIzol Plus RNA Purification Kit (Life Technologies). The quality and concentration of purified RNA was confirmed using a NanoDrop 2000 (Thermo Scientific). For RT-qPCR, 2 μg of total RNA was reversely transcribed using HiScript II Q RT SuperMix (Vazyme Biotech, R222-01). The reactions were carried out in triplicate using AceQ qPCR SYBR Green Master Mix (Vazyme Biotech, Q111-02/03) in a PRISM 7900HT system (Life Technologies) with the GADPH gene as the internal control. Primer sequences for TERT gene amplification were generated by PrimerBank (https://pga.mgh.harvard.edu/primerbank).

2.4. Measurement of RTL

RTL in DNA from 250 tumour samples was analysed using the monochrome multiplex quantitative PCR (MMQ-PCR) method developed by Cawthon [23], By comparing telomere repeat copy number (T) to a single copy gene (SCG) copy number (S) (T/S ratio) in a single PCR reaction, MMQ-PCR is more accurate than telomere length measurement by singleplex Q-PCR. Each reaction was performed in a 96-well reaction set in a 20-μl volume using 10 μl qPCR SYBR Green Master Mix (AceQ®) and 15 ng DNA template. Four primers (see Supplementary Table 1) were used in each reaction to amplify telomere repeats and the SCG beta-globin (hbgu). A reference genomic DNA sample was diluted in a 3-fold serial fashion (from 1.85 ng to 150 ng) and was used in each Q-PCR plate to generate two standard curves for telomere and SCG products. Thermal cycling was performed as follows: 95°C 15 min, 2 cycles of 94°C/15 s, 49°C/15 s, followed by 32 cycles of 62°C/10 s, 74°C/15 s with signal acquisition for telomere, 84°C/10 s, 88°C/15 s with signal acquisition for hbgu. After the run was complete, CurveExpret Basic (version 1.40) was used to generate the standard curves for T and S, which were used to quantify the telomere repeats and SCG based on the respective Ct values. The T/S is therefore a relative and dimensionless value which is proportional to the average telomere length per cell. T/S $>$ 1.0 indicates an average telomere length greater than that of the standard DNA; T/S of $<$1.0 indicates an average telomere length shorter than that of the standard DNA. The merit of this MMQ-PCR is to avoid potential pipetting variation between wells presenting in monoplex quantitative PCR in which the telomere repeat and SCG were amplified separately, and the correlation of T/S with terminal restriction fragment length measured by blot was stronger with the multiplex QPCR method than with the monoplex method.

2.5. Statistical analysis

Continuous variables were summarised by mean and standard deviation and were analysed by t-test. Categorical data were summarised as frequencies and percentages and were analysed by chi-square test. The comparison of RTLs was performed using multivariable linear regression models adjusted for binary variables that showed significant differences ($p < 0.05$) in basic demographic analyses, including histology type, tumour location and T staging status. Literacy level reached to junior high school education or above were defined as educated. Overall survival (OS) was defined as the period between the time of surgery and cancer-specific death. Disease-free survival (DFS) was defined as the period between the time of surgery and tumour recurrence. Survival curves were estimated using the Kaplan–Meier and log-rank test. Univariate Cox proportional hazard regression models were used to determine the effects of covariates on patient survival. For multivariate survival analyses, the Cox proportional hazard regression models were adjusted for covariates that showed a significant impact in the univariate model ($p < 0.1$) and certain known factors. A one-to-one stacked bar graph was generated to illustrate the distribution of the RTL response to TERT mRNA expression. The strength of the relationship between RTL and TERT mRNA expression was assessed with Pearson correlation analysis. The size of the correlation coefficients can be interpreted as significant when $p < 0.05$. All analyses were performed using SPSS, version 24.0, (SPSS Inc., Chicago, IL).

3. Results

3.1. Distribution of rs2853669 polymorphism genotypes in NSCLC patients

Our cohort included 250 NSCLC patients who were genotyped for the promoter region of TERT.

The frequencies for the rs2853669 minor alleles (T/C and C/C) were 42.4% (106 cases) and 14.4% (36 cases), respectively, and 108 patients (43.2%) were wild-type allele (T/T) carriers. The baseline characteristics of the patients by histology classification are shown in Table 1. In ADEC patients, the T/C allele was significantly associated with advanced T stage ($p = 0.018$) and EGFR mutation ($p = 0.006$) but not with age at diagnosis, gender, smoking cigarette, alcohol consumption history or other identifiable clinical features. In squamous cell carcinoma (SCC) patients, no significant association between rs2853669 and baseline characteristics was found.

3.2. Association between rs2853669 and TERT mRNA transcription level

We quantified the TERT mRNA transcription level of high-quality RNA from 250 NSCLC tumour samples using real-time PCR. Overall, we found T/C allele was associated with lower TERT mRNA transcription level both in ADEC and SCC NSCLC patients (χ2 test, $p = 0.002$ and $p = 0.033$, Table 1). Stratified analysis by EGFR status revealed that the impact of rs2853669 on TERT mRNA transcript level was confined in tumours without EGFR mutation. Significant difference was found in between-group analyses by student's t-test ($p < 0.001$, $p < 0.001$ and $p = 0.011$. Fig. 1A). However, the rs2853669 showed no impact on TERT mRNA transcript level in tumours with EGFR mutation (Fig. 1A). Stratification analyses by pathologic classification and EGFR status were summarised in Supplementary Table 2, as expected, we found T/C allele carriers transcript significant lower TERT mRNA in ADEC and SCC patients without EGFR mutation ($p < 0.001$ and $p = 0.033$).

3.3. Association between rs2853669 and RTL (T/S value)

TERT has an important role in telomere lengthening; thus, we hypothesised a potential impact of rs2853669 on RTL in NSCLC patients because of the result of TERT mRNA analyses above. Overall, we found that T/C allele was significantly associated with shorter RTL in quantified samples from ADEC patients (χ^2 test, $p = 0.025$, Table 1). Similar to TERT mRNA level, between-group comparison analyses by Student's t-test in stratified cohort by EGFR status revealed such significant association of rs2853669 with RTL was more common in patients without EGFR mutation ($p = 0.024$, $p = 0.757$ and $p = 0.039$. Fig. 1B). An additional stratification of pathologic classification further revealed that such association was only present in ADEC patients ($p = 0.033$, Supplementary Table 2). Further validating analysis by a multiple linear regression model adjusting for potentially associated variables (gender, age, smoke history and drink history) was performed. As excepted, the significant association of RTL with rs2853669 polymorphism allele was found to be exist in NSCLC patients without EGFR mutation, specifically in ADEC patients (Table 2).

Table 1
Clinical characteristics of 250 non-small cell lung cancer patients by histology.

	rs2853669 polymorphism													
	Adenocarcinoma							Squamous cell carcinoma						
	T/T		T/C		C/C			T/T		T/C		C/C		
	No	%	No	%	No	%	*P* value	No	%	No	%	No	%	*p* value
All patients	63	100	77	100	27	100	–	45	100	29	100	9	100	–
Age at diagnosis, years	58.3 ± 11.2		61.6 ± 9.3		59.7 ± 9.7		0.164	62.3 ± 8.7		60.6 ± 7.9		64.1 ± 6.6		0.465
Sex, male	27	42.9	36	47.4	16	57.1	0.452	38	84.4	26	89.7	6	66.7	0.253
Literacy, educated	18	28.6	34	44.7	10	35.7	0.143	20	44.4	16	55.2	2	22.2	0.215
Cigarette	20	31.7	28	36.8	8	28.6	0.697	37	82.2	22	75.9	5	55.6	0.216
Alcohol consumption	9	14.3	16	21.1	3	10.7	0.365	15	33.3	11	37.9	4	44.4	0.793
Tumour size, cm	3.3 ± 1.7		3.2 ± 1.5		2.8 ± 1.1		0.297	4.1 ± 1.9		3.8 ± 1.6		3.9 ± 0.8		0.739
Grade II (versus III&IV)	30	47.6	29	38.2	12	42.9	0.532	24	53.3	12	41.4	7	77.8	0.154
T stage T1&T2 (versus T3&T4)	53	84.1	66	86.8	28	100.0	0.018	28	62.2	24	82.8	7	77.8	0.147
Lymph node permeate (yes versus no)	16	25.4	25	32.9	8	28.6	0.624	12	26.7	7	24.1	3	33.3	0.861
Disease stage, I&II (versus III&IV)	47	74.6	54	71.1	21	75	0.867	32	71.1	22	75.9	6	66.7	0.836
Resect margin (positive versus negative)	5	7.9	3	3.9	1	3.6	0.524	2	4.4	2	6.9	0	0.0	0.690
TERT mRNA expression	−14.2 ± 1.7		−14.8 ± 1.8		−13.4 ± 1.9		0.002	−14.1 ± 3.4		−14.5 ± 2.5		−13.4 ± 5.2		0.033
Relative telomere length (T/S)	0.92 ± 0.40		0.81 ± 0.22		0.96 ± 0.33		0.047	0.9 ± 0.34		0.8 ± 0.25		1.0 ± 0.52		0.339
EGFR mutation (no. patients)	19	30.2	37	48.7	5	17.9	0.006	2	4.4	2	6.9	1	11.1	0.723
Local recurrence	4	28.6	7	9.2	3	10.7	0.739	1	2.2	1	3.4	0	0.0	0.834
Distant metastasis	2	3.2	1	1.3	0	0.0	0.525	2	4.4	0	0.0	0	0.0	0.421

Continuous data were summarised with means ± standard deviations.
Grade II/III/IV, poorly/moderate/well differentiated; T/S, the ratio of telomere repeat copy number to single copy gene number; EGFR, epidermal growth factor receptor; TERT, telomerase reverse transcriptase.

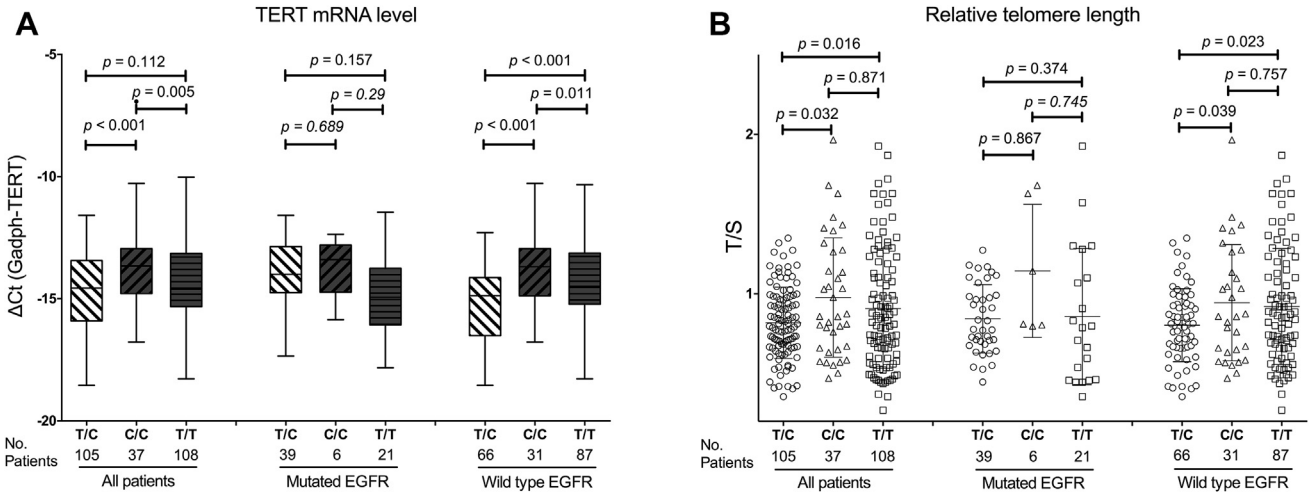

Fig. 1. **Association between rs2853669 and telomere parameters among all quantified NSCLC samples (n = 250)**. Comparison of mean TERT mRNA transcription level ($\Delta Ct = Ct_{GADPH}\text{-}Ct_{TERT}$) (A) and mean relative telomere length (T/S value) (B) against a background of EGFR mutation status by t-test. T/S, the ratio of telomere repeat copy number to single copy gene number; EGFR, epidermal growth factor receptor; TERT, telomerase reverse transcriptase; NSCLC, non-small cell lung cancer.

3.4. TERT mRNA transcription level and RTL in NSCLC patients

Correlation analysis between RTL and TERT mRNA transcript level in patients against the background of EGFR mutation status was performed. A tendency of progressive increasing in TERT mRNA transcription level towards longer RTL was observed in a one-to-one stacked bar (Fig. 2). In addition, Pearson correlation analysis showed a pronounced coefficient effect between TERT mRNA transcript level and RTL in tumours without EGFR mutation (Person r = 0.776, $p < 0.001$). However, this effect was not obtained in tumours with EGFR mutations (Person r = 0.034, $p = 0.782$).

3.5. Impact of rs2853669 polymorphism on NSCLC patients survival, association with EGFR mutation

One hundred and one (40.4%) patients were followed-up until death, and 46 patients (18%) were followed-up until tumour recurrence. Mean follow-up was 66.0 ± 26.3 months (median: 66; range: 1–99). Kaplan–Meier survival analysis and log-rank comparison revealed that heterozygous carriers (T/C) had the worst OS (mean ± SD: T/C: 63.6 ± 3.1; T/T: 79.4 ± 2.5; C/C: 77.3 ± 1.8; log-rank $p = 0.001$; Fig. 3A) and DFS (mean ± SE: T/C: 78.3 ± 3.3; T/T: 88.0 ± 2.4; C/C: 91.5 ± 3.1; log-rank $p = 0.019$; Fig. 3B).

In line with the Kaplan–Meier estimates, univariate Cox regression analyses revealed a significant impact of T/C on OS ($HR_{T/T} = 0.417$; 95% confidence interval [CI], 0.273–0.636; $p < 0.01$; $HR_{C/C} = 0.304$; 95%CI, 0.15–0.618; $p = 0.001$, T/C as reference) and DFS ($HR_{T/T} = 0.536$; 95%CI, 0.289–0.993; $p = 0.049$; $HR_{C/C} = 0.259$; 95%CI, 0.078–0.859; $p = 0.027$, T/C as reference) (Table 3). To clarify whether rs2853669 status has an independent prognostic quality, multivariate Cox regression models were performed by adjusting for covariates that showed a significant impact in the univariate model ($p < 0.1$) and certain known factors,

Table 2
Comparison analyses of RTL in 250 non-small cell lung cancer patients.

T/S Mean difference	Adenocarcinoma		Squamous cell carcinoma
	Wild-type EGFR	Mutated EGFR	Wild-type EGFR
Gender (male versus female)	−0.01 (−0.14 to 0.11), $p = 0.816$	−0.07 (−0.24 to 0.09), $p = 0.388$	−0.21 (−0.41 to −0.02), $p = 0.028$
Age (≥61 versus <61)	−0.05 (−0.16 to 0.08), $p = 0.471$	−0.08 (−0.24 to 0.08), $p = 0.329$	−0.08 (−0.23 to 0.07), $p = 0.307$
Cigarette (yes versus no)	0.03 (−0.16 to 0.10), $p = 0.695$	−0.03 (−0.21 to 0.15), $p = 0.733$	−0.02 (−0.20 to 0.16), $p = 0.812$
Alcohol consumption (yes versus no)	0.09 (−0.07 to 0.25), $p = 0.259$	−0.13 (−0.36 to 0.10), $p = 0.257$	0.12 (−0.04 to 0.28), $p = 0.13$
rs2853669 (multiple liner regression model)			
T/C (T/T as ref)	−0.16 (−0.3 to −0.03), $p = 0.021$	−0.01 (−0.18 to 0.17), $p = 0.921$	−0.01 (−0.08 to 0.05), $p = 0.695$
T/C (C/C as ref)	−0.13 (−0.27 to 0.009), $p = 0.066$	0.04 (−0.03 to 0.12), $p = 0.236$	−0.05 (−0.16 to 0.05), $p = 0.302$
T/T (C/C as ref)	−0.03 (−0.15 to 0.22), $p = 0.725$	−0.2 (−0.66 to 0.25), $p = 0.362$	−0.04 (−0.14 to 0.06), $p = 0.421$

Values given are mean differences with standard deviation, with 95% confidence interval
RTL, relative telomere length; T/S, the ratio of telomere repeat copy number to single copy gene number; EGFR, epidermal growth factor receptor.

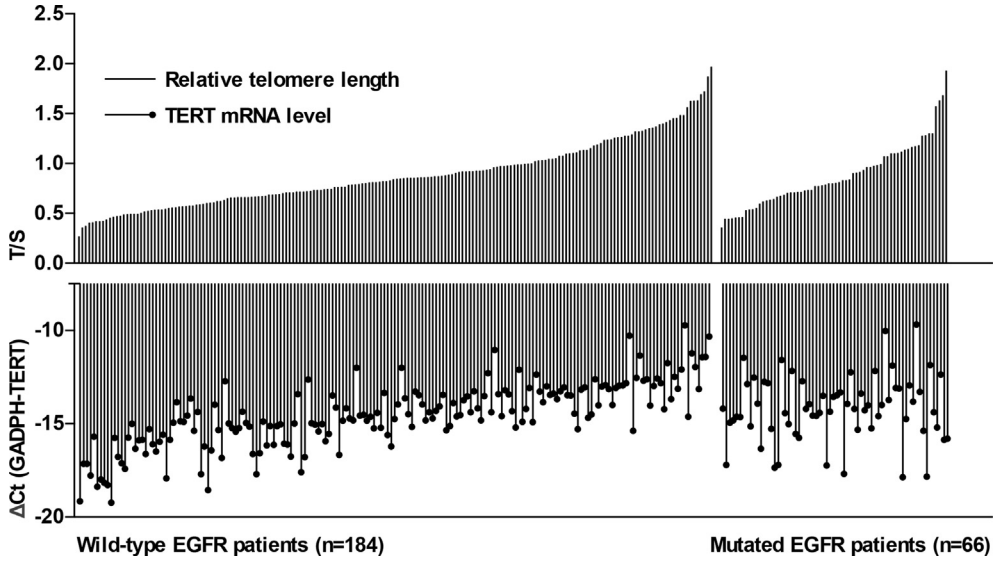

Fig. 2. **A one-to-one stacked bar graph to illustrate the association between TERT mRNA transcript level and RTL in all quantified NSCLC samples (n = 250).** A tendency of progressive increasing in TERT mRNA transcription level towards longer RTL was observed in patients without EGFR mutation. RTL, relative telomere length; EGFR, epidermal growth factor receptor; TERT, telomerase reverse transcriptase; NSCLC, non-small cell lung cancer.

including alcohol consumption status, resection margin status and postoperative treatment (Table 3). The results revealed that the rs2853669 T/C allele was associated with a 1.5-fold and 2.2-fold increase in the risk of death referring to T/T ($p < 0.001$) and C/C allele ($p = 0.001$). Consistently, T/C allele also confers 1-fold and 3-fold increase in the risk of tumour recurrence than T/T ($p = 0.049$) and C/C allele ($p = 0.028$).

EGFR is a well-studied prognostic factor in NSCLC patients. However, the statistical power of its prognostic effect only present inconclusive significance (p = 0.055) in the multivariate Cox regression model for DFS (Table 3). Thus, we assume a potential association between the rs2853669 and EGFR mutation in patients' survival. We re-examined the role of rs2853669 in OS and DFS against the background of EGFR mutation status. Consistently, we found that prognostic function of rs2853669 for OS is conditioned to NSCLC patients with wild-type EGFR (Fig. 4. A, B). Although statistical comparison analyses stratified by EGFR status did not present a significant difference between rs2853669 genotypes, T/C allele is more likely to illustrate a worst DFS-curve in patients with wild-type EGFR than in patients with mutated EGFR (Fig. 4. B, D).

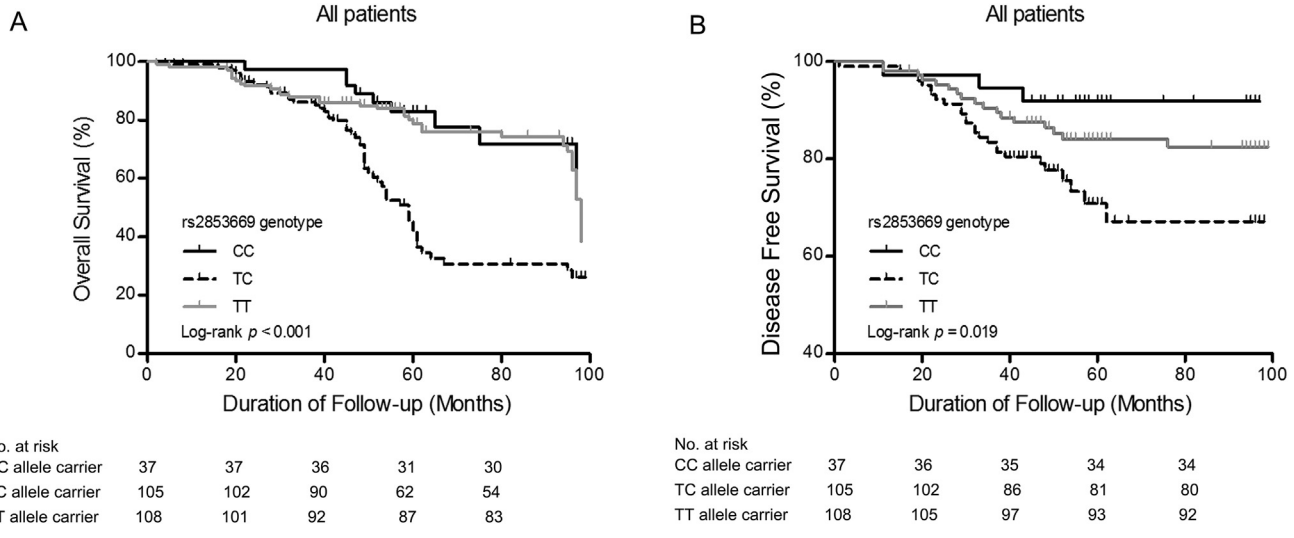

Fig. 3. **Kaplan–Meier estimates of overall survival and disease-free survival in NSCLC patients (n = 250) among rs2853669 genotypes**. (A) Those carrying T/C alleles had shortest overall survival. (B) Those carrying T/C allele had shortest disease-free survival. NSCLC, non-small cell lung cancer.

Table 3
Univariate and multivariable Cox predictors for death risk in 250 patients with NSCLC.

	Overall survival analysis				Disease-free survival analysis			
	Univariate		Multivariable		Univariate		Multivariable	
Variables	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
Age, <61 (≥61 as ref)	0.815 (0.444–1.496)	0.716	0.979 (0.643–1.492)	0.922	1.017 (0.379–2.732)	0.973	0.863 (0.455–1.635)	0.651
Sex, Female (male as ref)	0.938 (0.511–1.722)	0.742	1.962 (0.949–4.057)	0.069	1.039 (0.578–1.869)	0.898	0.765 (0.327–1.790)	0.537
Cigarette, No (yes as ref)	0.664 (0.365–1.205)	0.178	0.555 (0.270–1.141)	0.109	1.167 (0.651–2.091)	0.604	0.643 (0.266–1.551)	0.326
Alcohol, No (yes as ref)	0.746 (0.602–0.924)	0.007	0.627 (0.370–1.063)	0.083	0.996 (0.494–2.008)	0.992	0.695 (0.294–1.642)	0.407
Educated, yes (no as ref)	0.750 (0.506–1.110)	0.150	0.761 (0.514–1.127)	0.173	0.614 (0.344–1.094)	0.098	0.613 (0.344–1.093)	0.097
Histology, SCC (ADEC as ref)	0.833 (0.454–1.530)	0.757	1.044 (0.632–1.727)	0.866	2.138 (1.031–4.432)	0.041	2.281 (1.000–5.201)	0.050
Margin, negative (positive as ref)	0.596 (0.213–1.666)	0.151	0.589 (0.269–1.291)	0.186	0.292 (0.131–0.654)	0.003	0.368 (0.152–0.893)	0.027
Grade, II (III and IV as ref)	0.984 (0.544–1.778)	0.275	0.983 (0.645–1.499)	0.936	0.536 (0.296–0.970)	0.019	0.472 (0.252–0.884)	0.019
EGFR, mutated (WT as ref)	1.926 (0.812–4.565)	0.708	1.327 (0.793–2.220)	0.282	0.555 (0.304–1.012)	0.055	0.836 (0.423–1.653)	0.607
RTL, T/S < 0.818 (≥0.818 as ref)	0.629 (0.344–1.149)	0.548	1.413 (0.921–0.600)	0.705	0.876 (0.490–1.564)	0.653	0.930 (0.502–1.722)	0.817
Treatment, Surgery + Chemo (surgery alone as ref)	0.372 (0.245–0.565)	<0.001	0.463 (0.293–0.733)	<0.001	0.555 (0.407–0.755)	<0.001	0.326 (0.17–0.626)	0.001
rs2853669, T/T allele (T/C as ref)	0.417 (0.273–0.636)	<0.001	0.443 (0.281–0.669)	<0.001	0.536 (0.289–0.993)	0.047	0.527 (0.278–0.999)	0.049
rs2853669, C/C allele (T/C as ref)	0.304 (0.150–0.618)	0.001	0.380 (0.183–0.790)	0.001	0.259 (0.078–0.859)	0.027	0.251 (0.073–0.864)	0.028

SCC, squamous cell carcinoma; ADEC, adenocarcinoma; Grade II/III/IV, poorly/moderate/well differentiated; RTL, relative telomere length; T/S: the ratio of telomere repeat copy number to single copy gene number; HR, hazards ratio, CI, confidence interval; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer.

4. Discussion

Because TERT has been accepted as a crucial marker for cell survival and cancer progression, the prognostic effect of rs2853669 in cancers has been investigated in several cohort and molecular epidemiology studies for its association with telomere parameters, whereas the result are inconsistent. A recent study found that NSCLC patients carrying the C allele of rs2853669 had prolonged survival time in breast cancer patients [10]. On the contrary, Spiegl-Kreinecker showed that rs2853669 C/C allele was associated with inferior survival outcome among patients with acute myeloid leukaemia and glioblastoma [24]. In the meantime, some researches still failed to detect ground evidence of association between rs2853669 and OS among hepatocellular carcinoma patients, bladder cancer patients and clear cell renal cell carcinoma patients [25–27]. The inconsistent result might largely contribute to different cancer types and human races. As for NSCLC, a genetic association study published by Wei et al. revealed that telomerase and telomere function may be essential for carcinogenesis and progression of EGFR-mutated NSCLC by altering TERT mRNA expression in both normal and tumour tissue [28]. In our study, tumours with T/C allele transcript lower TERT mRNA and were significantly associated with shorter RTL. They are significantly associated with shorter OS and DFS compared with T/T and C/C allele. This is in line with the established concept that malignant cells have shorter telomeres than their normal or less advanced counterparts [29]. However, Wu supported that long telomere of peripheral leucocyte as a predictor for increased risk of recurrence in early-staged (I and II) lung cancer [30]. We speculate that our different stage-population (26% of stage III and IV disease) may attribute to the inconsistent result. More importantly, limited studies had evaluated the difference of telomere length in tumour and in blood, besides, either of the study suggested dynamic changes in telomere biology during tumour progression. Elucidating the underlying mechanism would help better understand telomere biology and its role in tumour development.

By investigating EGFR mutation status and telomere parameters in our single consecutive cohort, we have tried to further elucidate the pattern by which rs2853669 alters telomerase activity and reveal its prognostic value for NSCLC. It is worth noting that the prognostic function of rs2853669 in NSCLC is conditioned to patients with wild-type EGFR. Similarly, molecular analyses of TERT mRNA and RTL based on such polymorphic variant within endogenous Ets-2 transcription factor binding site showed significant association only in patient without EGFR mutation. The Ets-2 is known to be a transcriptional repressor or activator or both to regulate expression of telomerase [31–33], its binding motif located in TERT promoter region is a major target of EGFR signalling for transactivation of TERT [34]. Considering the intricate role of EGFR in aetiology and development in NSCLC. It is reasonable to hypothesise a TERT-altering dependent/independent mechanism for telomere maintenance according to EGFR mutation status. Besides, these lines of evidence together also prompt us to wonder the causal

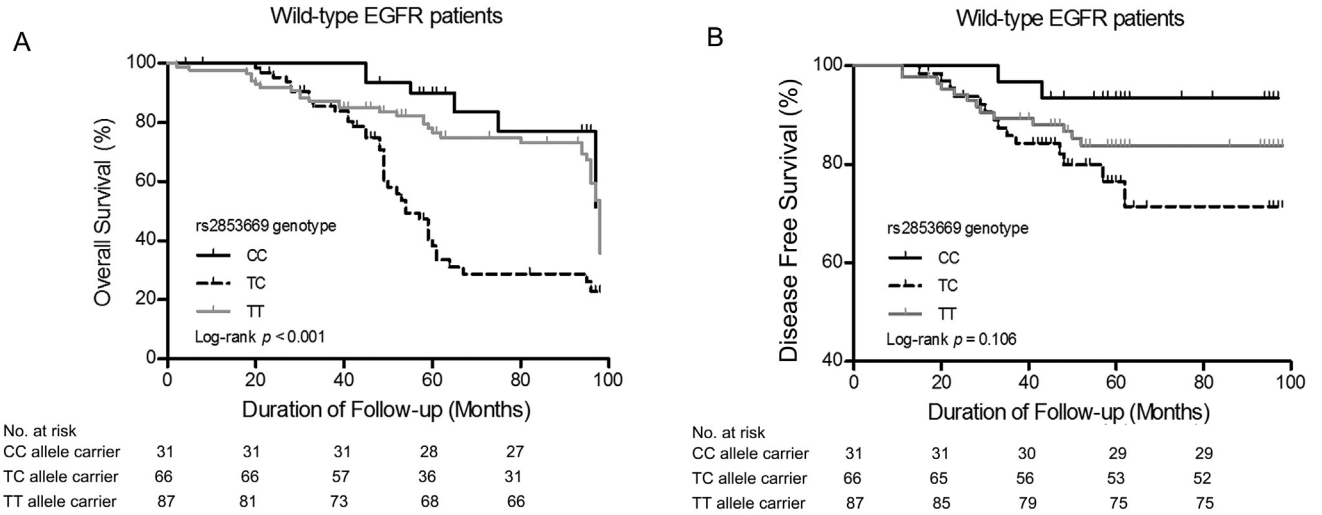

Fig. 4. **Kaplan-Meier estimates of overall survival and disease-free survival in NSCLC patients without (A, B n = 184) and with (C, D n = 66) EGFR mutation.** (A, C) Prognostic function of rs2853669 for overall survival is conditioned to patients with wild-type EGFR. (B, D) No statistical difference was found in disease-free survival among T/C, T/T and C/C allele of rs2853669 in stratification analyses by EGFR mutation. T/C allele are more likely to illustrate a worst disease-free survival curve in patients with wild-type EGFR than in patients with mutated EGFR. NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor.

relationship between EGFR mutation and telomerase dysregulation in NSCLC. In our ongoing preliminary study, inducting and correction of EGFR mutation in lung ADEC cell lines by CRISPR-point mutation showed a consequent alteration in transcription factor/TERT promoter region binding efficiency and TERT transcription level (data not published), suggesting that telomerase disorder is possibility one of a terminal events following a complex molecular response by EGFR mutation. Further experimental is required to characterise the cellular phenomena, efforts on intensive data are also needed for elucidating and validating our hypothesis of the underlying mechanism.

In addition, Multivariate regression model for RTL analyses further revealed RTL differ significantly in ADEC patients. Together with published result by Wu et al. [30], who reported that telomere length serves as a predictor in women patients with ADEC, we speculate that genetic variant in TERT promoter region probably plays a more dominant role in lung ADEC than other histology types. These hypotheses will also need to be validated in further molecular search focusing on ADEC.

Our results might also at least in part explain the inconsistent results of therapeutic strategies targeting telomerase in clinical trials for cancer patients. An antitelomerase agent (imetelstat, GRN163L) only showed promising results in patients with longer telomeres in a phase I clinical trial (NCT00510445) [35]. Supportively, another representative telomerase peptide (GV1001), showed promising results in a phase

I/II NSCLC study [36]. However, a phase III trial combining GV1001 with gemcitabine and capecitabine in locally advanced or metastatic pancreatic cancer did not improve OS [37]. The discrepant results of telomerase inhibitor in cancer treatment might be explained by inconsonant association of TERT mRNA and RTL in our cohort under different EGFR status. Telomere length of tumour tissues is reported to be an independent prognostic factor in NSCLC patients. Our results indicated that tumour burden and biology possibly become less dependent on telomerase activity in the presence of EGFR mutations in some cases, and these cases might have limited response to telomerase inhibitor in some registered trails.

In conclusion, our study revealed significant association of a common single polymorphism within TERT promoter region on telomere parameters and survival in NSCLC patients and we detected a conditional regulation of rs2853669 in regulating telomerase and telomere biology relating to EGFR mutation. Limitations of the present study should be noted. We used TERT mRNA transcription level to demonstrate telomerase activity. Although it has been well recognised to be associated with telomerase activity, there might still be some discrepancies compared with a more direct investigation of telomerase activity. Besides, although we attempted to balance the apparent biases by multivariate Cox regression model in survival analyses, selection bias for historical patients that have performed well cannot be overlooked because of the retrospective nature of this study. Nevertheless, this is a molecular epidemiology study conducted in a large cohort with a sufficient follow-up for survival analysis. Our results may help understanding the association of telomere biology and genetic variations for better cancer prognosis as well as for designing of future oncology clinical trial targeting telomerase. Besides, our study to an extend may also help providing evidence for molecular investigation of TERT-EGFR interacting mechanism in telomere biology.

5. Highlights

Our study revealed significant associations of a common single polymorphism (rs2853669) within TERT promoter region on telomere parameters and survival in NSCLC patients and detected a conditional regulation of rs2853669 in regulating telomerase and telomere biology relating to EGFR mutation. The results may help understanding the association of telomere biology and genetic variations for better cancer prognosis as well as for designing of future oncology clinical trial targeting telomerase. Besides, our study to some extend may also help providing evidence for molecular investigation of TERT-EGFR interacting mechanism in telomere biology in NSCLC.

Conflict of interest statement

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

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