

Prognostic role of TPL2 in early-stage non-small cell lung cancer

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Received July 19, 2016; Accepted November 9, 2016

DOI: 10.3892/mmr.2017.6430

Abstract. Non-small cell lung cancer (NSCLC) accounts for ~70% of all lung cancer-associated mortalities worldwide. The serine/threonine protein kinase tumor progression locus 2 [TPL2/MAP3 kinase 8 (MAP3K8)] may impact oncogenic events; however the role of TPL2 in lung carcinogenesis remains unclear. The present study was focused on the potential prognostic role of TPL2 in 101 patients with early-stage NSCLC. Since TPL2 is a potential target of *miR-21*, the association between TPL2 and *miR-21* expression was also examined. *TPL2* and *miR-21* mRNA expression was quantified using reverse transcription quantitative polymerase chain reaction (RT-qPCR). TPL2 protein levels were evaluated by immunohistochemistry (IHC). The present study identified that the mRNA expression of *TPL2* was low in 52/101 (51%) cases and high in 49/101 (49%) cases. IHC analysis of TPL2 protein expression often demonstrated identical mRNA results. No statistically significant associations were observed between the mRNA expression of *TPL2* and the predominant clinicopathological characteristics of the patients with NSCLC, as well as identifying no association between *TPL2* and *miR-21*. *TPL2* mRNA expression was significantly higher in patients with NSCLC with good prognosis (disease-free interval $P=0.009$; overall survival $P=0.024$), when compared with those of poor prognosis. Focusing on the difference in mRNA expression of *TPL2* among the adenocarcinomas in affected patients, *TPL2* was more highly expressed in lepidic adenocarcinomas compared with in the other subtypes ($P=0.012$). The present study is the first examination, to the best of our knowledge, of TPL2 in early-stage NSCLC in relation to *miR-21*, and in different adenocarcinoma subtypes. Future studies must clarify the mechanism by which TPL2 is involved in lung carcinogenesis due to its important translational implications.

Introduction

Although surgical intervention is effective for the majority of early-stage non-small cell lung cancer (NSCLC), ~20-30% of patients experience relapses (1). Therefore, the identification of prognostic markers and novel therapeutic targets is of crucial importance. The serine/threonine protein kinase tumor progression locus 2 [TPL2/MAP3 kinase 8 (MAP3K8)] acts as an essential signal for the expression of pro-inflammatory mediators in several cell systems, including peripheral macrophages (2), adipocytes (3), hepatic stellate cells (4), airway epithelial cells (5) and glial cells (6). Inflammatory molecules have emerged as significant in the development of cancer, with roles in events leading to the formation, growth and metastasis of tumors. TPL2 has been suggested to enhance tumor growth and metastatic progression in distinct types of human cancer (7-9). Overexpression of TPL2 has been reported in human gastric/colon adenocarcinomas (10), large granular T-cell neoplasias (11) and breast cancer (12,13). However, a detailed evaluation of TPL2 expression and function in human malignancies and normal tissues remains to be performed, and the role of TPL2 in carcinogenesis remains unclear. TPL2 may stimulate oncogenic events, including the cellular response to therapy and immune control of cancer growth (14). However, in certain cases, TPL2 may function as a tumor suppressor (15). Little information regarding the prognostic role of TPL2 in the oncogenesis of NSCLC exists to assist with identifying those at high-risk of relapse following surgical resection.

Several previous studies have identified micro (mi) RNAs as critical regulators of immune responses, including inflammation (16,17). Mechanistically, miRNAs are small, non-coding RNA molecules that pair with partially complementary sequences in their target mRNAs and regulate their stability and/or translation. Extracellular signals, differentiation and oncogenic transformation bring about alterations in the expression of miRNAs, allowing miRNAs to regulate these processes. Previous studies have suggested that *miR-21* overexpression may be an independent negative prognostic factor in the overall survival of patients with NSCLC (18), and that *miR-21* targets numerous genes involved in cancer cell phenotypes (19). Using three miRNA target identification programs (miRNAorg, MicroCosm Targets and PITA), *TPL2* was identified among the potential targets of *miR-21*. Therefore, the present study was based on the analysis of the

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Key words: tumor progression locus 2, non-small cell lung cancer, lung adenocarcinoma subtype, *miRNA-21*, prognosis

potential association between *TPL2* and *miR-21* in cases of early-stage NSCLC.

Materials and methods

Patients. A total of 101 patients with NSCLC at peripheral stage I (T1N0M0) underwent surgical resection in the Unit of Thoracic Surgery in the Department of Surgical, Medical, Molecular Pathology and Critical Care at Pisa University (Pisa, Italy), and were retrospectively selected. Histological diagnoses were formulated by two pathologists independently (G.F. and A.S.), in accordance with the World Health Organization classification (20,21). Clinicopathological characteristics were noted in all cases. Written informed consent was obtained from each patient for tissue collection and molecular analysis.

Target prediction. Alignment of miRNAs with the *TPL2* target gene was predicted using the microRNA target prediction programs microRNA.org (Harvard Medical School, Boston, MA, USA), MicroCosm Targets 5.0 (The European Bioinformatics Institute) and PITA (Weizmann Institute of Science).

RNA isolation. Following standard deparaffinization and manual tumor macrodissection of the areas with prevalent adenocarcinomas, the miRNeasy formalin-fixed and paraffin-embedded (FFPE) kit (Qiagen GmbH, Hilden, Germany) was used to isolate total RNA, including miRNAs, from 5 μ m sections of FFPE tissues, according to the manufacturer's protocol. A total of 600 ng of total RNA was used to synthesize cDNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a reaction volume of 20 μ l.

Expression of *TPL2* mRNA and *miR-21* using the reverse transcription quantitative-polymerase chain reaction (RT-qPCR). Quantification of the mRNA expression of *TPL2* was performed in triplicate using the Rotor Gene SYBR Green PCR kit (Qiagen GmbH) on a Rotor Gene 6000 (Qiagen GmbH). The following primers used for RT-qPCR are as follows: *TPL2*, forward: 5'-TAATCCACAAGCAAGCAC CTC-3' and reverse: 5'-TGATTGGGGTTTCTCTCCAG-3'; β -actin, forward: 5'-CCAACCGCGAGAAGATGA-3' and reverse: 5'-CCAGAGGCGTACAGGGATAG-3'. Specific TaqMan® MicroRNA assays (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used to amplify *miR-21* and *RNU6B*, according to the manufacturer's protocol. In addition, the threshold cycle and baselines were determined in an identical manner. The expression was calculated by relative quantification using β -actin and *RNU6B* as reference controls for *TPL2* and *miR-21*, respectively. Fold expression changes were determined by the $2^{-\Delta\Delta C_q}$ method (22) using a pool of 12 non-cancerous tissues as a calibrator group. The analysis was performed using the DataAssist™ software (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Protein expression of *TPL2*. The protein expression of *TPL2* was assessed in FFPE tissue samples using immunohistochemistry (IHC). The Cot (M-20) anti-*TPL2* rabbit polyclonal antibody (dilution, 1:50; cat. no. sc-720, Santa Cruz

Biotechnology, Inc., Dallas, TX, USA) was incubated at 37°C for 36 min to detect the protein. The avidin-biotin peroxidase method was used by developing the immunoreaction with diaminobenzidine. Simultaneous staining of a known *TPL2*-positive case was used as a positive control. Incubation of parallel slides omitting the first antibody was performed as a negative control. The number of *TPL2* immunoreactive cells was determined by scoring a minimum of five high-power fields (magnification, x40) and counting the number of immunoreactive cells out of the total epithelial cells analyzed in each field of view.

A proportion score (PS) was assigned by representing the estimated proportion of positively stained cells, and was divided into three groups: negative (score 0), 1-60% (score 1) and >60% (score 2). An intensity score (IS) was also assigned and divided into four classes: None (score 0), weak (score 1), intermediate (score 2) and strong (score 3). A total score was obtained from the sum of the PS and IS, and was graded into two classes: High (4-5, with a higher expression of *TPL2*) and low (0-3, with a lower or negative *TPL2* expression).

Statistical analysis. One-way analysis of variance and chi-squared tests were used to determine the association between the expression of *TPL2*, *miR-21* levels and the clinicopathological parameters. Survival analyses were performed using the Kaplan-Meier method, along with the log-rank test and the Cox proportional hazard model. Statistical analyses were performed using the JMP10 software (SAS Institute, Inc., Cary, NC, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. The present study was performed using 101 patients with early-stage NSCLC (71 males and 30 females), including 54 patients with adenocarcinoma (ADC) and 47 patients with squamous cell carcinoma (SCC). Regarding their histological classification, different histological subtypes of ADC were identified: Acinar (19/54, 35.2%), lepidic (18/54, 33.3%), mucinous (9/54, 16.7%), papillary (6/54, 11.1%) and solid (2/54, 3.7%). The median age at diagnosis was 67 years (range, 49-81; mean, 67.03 years), and the median follow-up was 63 months (range, 6-161 months). In terms of tumor grade, five tumors were G1 phase, 71 tumors were G2 phase and 25 were G3 phase. Disease progression and mortality from lung cancer were observed in 27 patients with ADC (26.7%) and 20 with SCC (19.8%) of the 101 patients with NSCLC, respectively. The median progression-free survival and the overall survival were 54 months (95% CI: 6-161) and 68 months (95% CI: 11-161), respectively. Regarding smoking habits of the entire patient group, there were 15 non-smokers, 44 former smokers and 42 current smokers (Table I).

***TPL2* expression and clinicopathological characteristics.** *TPL2* mRNA expression was quantified in 101 NSCLC tissues and in 12 non-cancerous tissues, and was normalized against the β -actin housekeeping gene, using RT-qPCR. The samples were divided into high and low expression groups based on the median fold-change value (0.815 for *TPL2*). *TPL2* mRNA expression was low in 52/101 (51%) cases and

Table I. Correlation between the mRNA expression of tumor progression locus 2 and the predominant clinicopathological characteristics of patients with early-stage non-small cell lung cancer.

Characteristic	TPL2 expression		P-value
	Low %	High %	
Age, years			0.368
≤67	24 (47.1)	27 (52.9)	
>67	28 (56.0)	22 (44.0)	
Gender			0.809
Male	36 (50.7)	35 (49.3)	
Female	16 (53.3)	14 (46.7)	
Histology			0.471
Adenocarcinoma	26 (48.1)	28 (51.9)	
Squamous cell carcinoma	26 (55.3)	21 (44.7)	
Smoking history			0.512
Never	6 (40.0)	9 (60.0)	
Former	25 (56.8)	19 (43.2)	
Current	21 (50.0)	21 (50.0)	
Tumor grade			0.661
G1	3 (60.0)	2 (54.5)	
G2	38 (53.5)	33 (46.5)	
G3	11 (44.0)	14 (56.0)	

high in 49/101 (49%) cases (data not shown). The present study determined whether *TPL2* expression was correlated with the predominant clinicopathological characteristics. No statistically significant associations were observed between *TPL2* mRNA expression and any of the predominant clinicopathological characteristics of the patients with NSCLC (Table I). However, focusing on the difference in *TPL2* levels among the different subtypes of the 54 adenocarcinomas, it was identified that *TPL2* mRNA expression was high in 6/19 (31.6%) acinar, 13/18 (72.3%) lepidic, 5/9 (55.6%) mucinous, 3/6 (50%) papillary and 1/2 (50%) solid subtypes, with a significantly higher level in lepidic when compared with all other subtypes (Chi-squared test, $P=0.012$; data not shown).

Using the 2-level (high/low) immunohistochemical scoring system, *TPL2* protein expression was low in 45/101 (44%) and high in 56/101 (56%) cases (Fig. 1). Even if the correlation between the level of *TPL2* mRNA and the score of its protein was not significant (chi-squared test, $P=0.36$), samples with a low *TPL2* protein score expression typically exhibited lower mRNA levels (2.33-fold change value ± 1.55) when compared with samples with high *TPL2* score (4.0 \pm 1.2). In addition, a high score for *TPL2* protein was identified in the lepidic adenocarcinoma subtype ($P=0.003$), as aforementioned for *TPL2* mRNA expression.

Target prediction and correlation between *TPL2* and *miR-21* expression. Alignment of miRNAs with the *TPL2* target mRNA was predicted by the miRNA target prediction programs miRBase, MicroCosm Targets and PITA. The

present study identified the *TPL2* gene as a potential target of *miR-21* (Fig. 2). Firstly, the patients with NSCLC were divided into *miR-21* high and low expression groups based on the median fold change values, which resulted as 1.7. Following this, the present study determined whether *TPL2* expression was correlated with *miR-21* levels, but no statistically significant associations were observed between groups (Chi-squared test, $P=0.66$).

Survival analysis. To evaluate the potential association between *TPL2* expression and the prognosis of the patients with NSCLC, a survival analysis by the Kaplan-Meier method using the disease-free interval (DFI) and the overall post-operative survival (OS) as endpoints was performed. It was noted that the tumors with a high *TPL2* mRNA expression demonstrated a significantly longer mean DFI and OS compared with the patients with low expression of this mRNA ($P=0.009$ and $P=0.024$, respectively; Fig. 3 and Table II). In the multivariate analysis, *TPL2* mRNA expression continued to be a good prognostic factor for DFI and OS ($P=0.0005$ and $P=0.005$; Table II). Tumor grading, a classical prognostic factor, was also associated with the DFI in the univariate ($P=0.0019$) and multivariate analyses ($P=0.0008$; Table II), as well as with the OS ($P=0.0009$ and $P=0.0002$, respectively). No significant differences were identified for other demographics or clinical characteristics (Table II). Furthermore, the median DFI and OS were similar in examined patients with either low or high *miR-21* expression (data not shown).

Following this analysis, the survival difference between the histological cell types was investigated. No significant difference was observed between SCC and ADC (Table II). However, based on the difference of the *TPL2* levels reported above, the NSCLCs were divided into three prognostic groups: Squamous cell carcinoma and the two predominant adenocarcinoma growth patterns (acinar and lepidic subtypes). The group with the most favorable prognosis was the lepidic predominant subtype, which was demonstrated earlier in the study to possess a higher level of *TPL2*, with a median DFI of 85 months and a median OS of 90 months. SCC and acinar ADC, both NSCLC tumors with lower *TPL2* levels, presented a worse prognosis with a mean DFI of 53 and 68 months and with a mean OS of 65 and 68 months, respectively ($P=0.02$ and $P=0.03$; Fig. 4).

Discussion

TPL2 is involved in numerous intracellular signaling pathways, with a prominent role in the regulation of inflammatory signal transduction (2-6). *TPL2* may be able to engage a plethora of signaling pathways and to act in concert with other kinases and signaling molecules. A major role of *TPL2* is in the activation of the ERK/MAPK pathway through the direct phosphorylation of MEK, an extracellular signal-regulated kinase, which is a pro-inflammatory mediator. Conversely, in certain disease models, *TPL2* deficiency exacerbates the inflammatory response (23). The diverse and often contradictory effects of *TPL2* may be due to the utilization of different kinases and signal transduction pathways in different cell types. Similar contradictory data concerning the role of *TPL2* in oncogenic signal transduction exist in the literature.

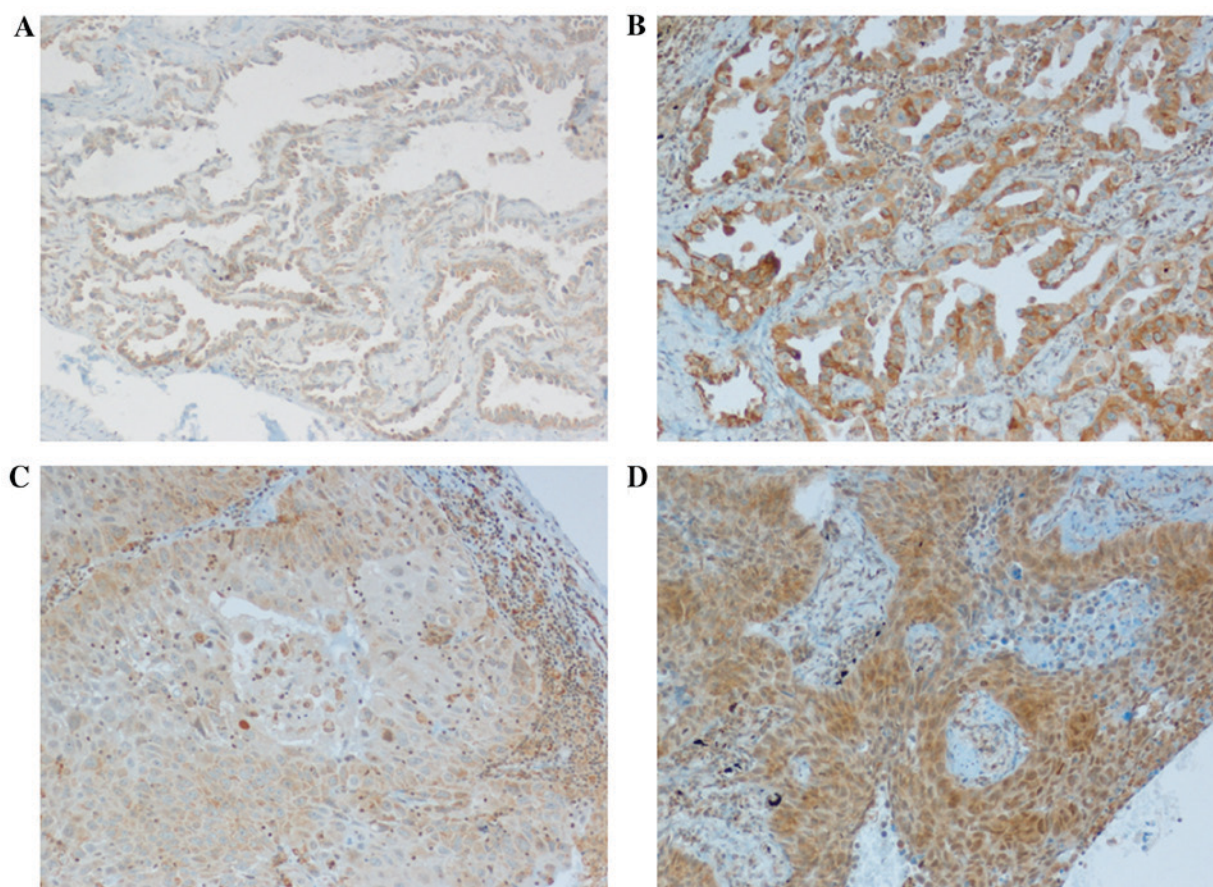


Figure 1. Immunohistochemical staining of tumor progression locus 2 in adenocarcinomas with (A and B) lepidic prevalent pattern and (C and D) squamous cell carcinoma, with low level positive staining shown on the left and high level positive staining on the right (magnification, x10).

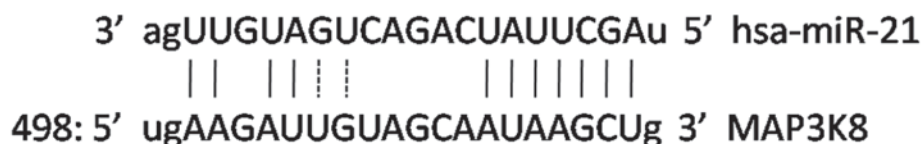


Figure 2. *miR-21* alignment with tumor progression locus 2 (*MAP3K8*) mRNA. The alignment of *miR-21* with tumor progression locus 2 was determined using the miRBase target prediction program.

Gkirtzimanaki *et al* (15) demonstrated a tumor suppressor function for TPL2 in lung cancer. However, in contrast, elevated levels of TPL2 have been reported in several tumor types (10-13). TPL2 inhibitors are currently in advanced pharmaceutical development for the treatment of myeloma (24). The effects of inhibiting different MAP kinases may have different consequences in a cell-type-dependent manner, and therefore, any therapeutic use of kinase inhibitors must be carefully considered.

Considering these discrepancies, the aim of the present study was to elucidate the role of TPL2 in lung cancer, specifically in early-stage NSCLC. NSCLC accounts for ~70% of all lung cancer-associated mortalities worldwide (25). The current standard of care for stage I NSCLC is surgery; however, 20-30% of these early-stage patients will experience recurrence (1), reflecting the urgent requirement to develop adjunctive, specific markers that can aid in the prognosis of lung cancer. In the present report, it was demonstrated that

low *TPL2* mRNA levels correlated with reduced survival in TNM stage I lung cancer patients, suggesting loss of its expression was an early event in the development of the disease. Immunohistochemical analysis of TPL2 protein expression confirmed the corresponding mRNA results, even if not fully. There are presumably several reasons for a weak correlation between mRNA and protein levels, including mRNA post-transcriptional modifications, translational efficiency, miRNA actions and errors in both mRNA and protein measuring techniques. In addition, due to subcellular localization, TPL2 protein abundance may not represent its biological activity.

It was later hypothesized that *TPL2* may be correlated with miRNAs, which are small, non-coding RNAs that regulate target gene expression (26,27). Several miRNAs are aberrantly expressed in the majority of human cancer types (19). Oncogenes and tumor suppressor genes exert their activity, in part, by regulating the expression of specific miRNAs (28).

Table II. Factors associated with disease-free survival in patients with early-stage non-small cell lung cancer.

Characteristic	Univariate analyses (P-value)	Multivariate analyses (P-value)
Age (≤ 67 vs. > 67 years)	0.7107	0.7141
Gender (male vs. female)	0.0866	0.4067
Histology (ADC vs. SCC)	0.1030	0.9908
Smoking (current/former vs. never)	0.5915	0.5477
Tumor grading (G1 vs. G2/G3)	0.0019	0.0008
TPL2 mRNA level (low vs. high)	0.0009	0.0005

ADC, adenocarcinoma; SCC, squamous cell carcinoma; TPL2, tumor progression locus 2.

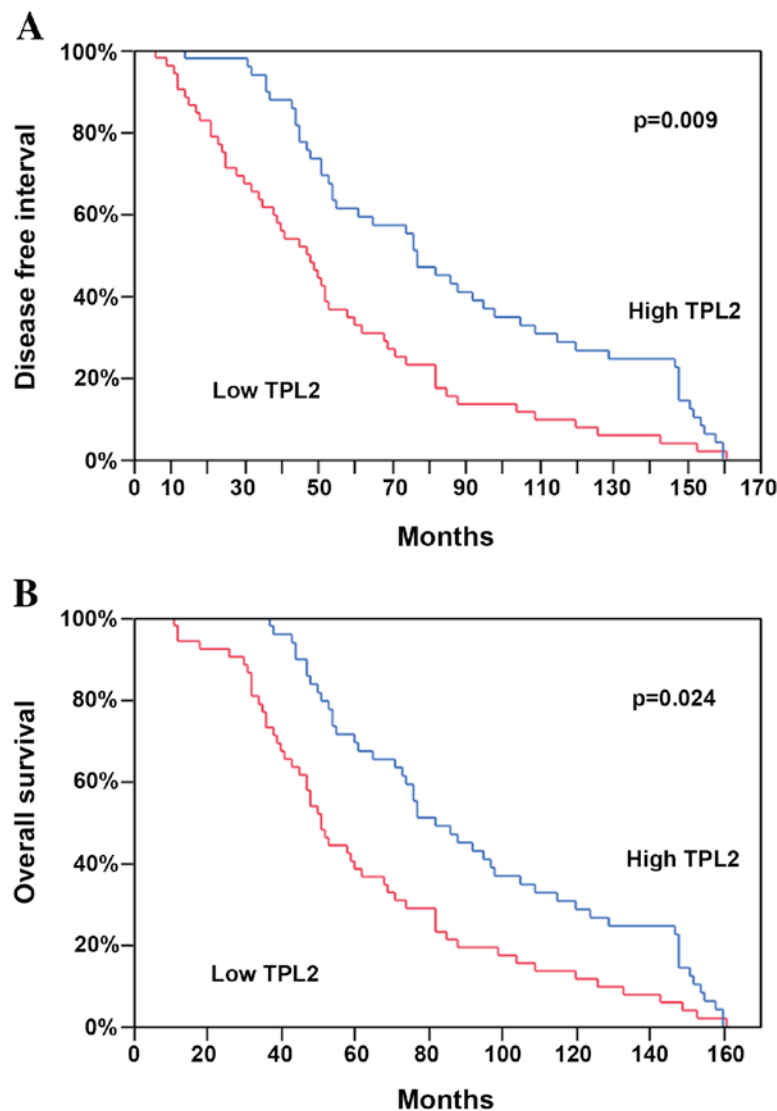


Figure 3. Kaplan-Meier curves of patients with early-stage non-small cell lung cancer. Kaplan-Meier curves for the survival analysis of (A) DFI and (B) OS in 101 patients with early-stage non-small cell lung cancer with high (blue line) or low (red line) *TPL2* expression. High *TPL2* mRNA expression demonstrated a significantly longer mean DFI and OS when compared to the patients with low expression of this mRNA ($P=0.009$ and $P=0.024$ vs. low *TPL2* mRNA expression). TPL2, tumor progression locus 2; DFI, disease free interval; OS, overall survival.

miR-21 has proven to be a useful prognostic indicator in numerous cancer types, including NSCLC (29,30). Using the miRNA target prediction programs miRBase, MicroCosm Targets and PITA, the present study identified *TPL2* as a

potential target of *miR-21*. As a result, the study evaluated *miR-21* expression in early-stage NSCLC and its association with *TPL2* levels by combining *miR-21* expression with *TPL2* levels. The present study is the first, to the best of our

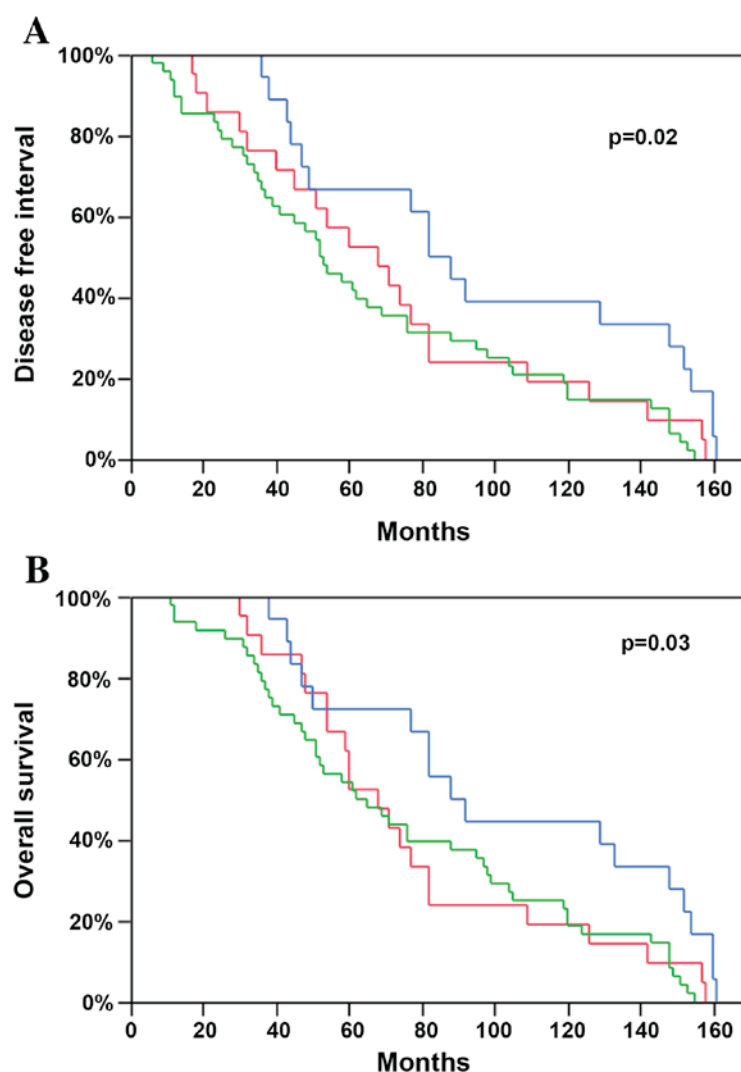


Figure 4. Kaplan-Meier curves of patients with SCC, acinar ADC and lepidic ADC subtype. Kaplan-Meier curves for the survival analysis of (A) DFI and (B) OS in patients with SCC (green line), acinar ADC (red line) and lepidic (blue line) ADC subtypes. The lepidic subtype possessed a higher median DFI and OS than the acinar subtype and SCC ($P=0.02$ and $P=0.03$ vs. SCC). DFI, disease free interval; OS, overall survival; SCC, squamous cell carcinoma.

knowledge, to test the hypothesis that *miR-21* may cooperate with *TPL2*. It was revealed that *TPL2* levels were not correlated with *miR-21*, suggesting no association between *miR-21* and *TPL2* in lung carcinogenesis. In addition, no difference in the disease prognosis was observed for *miR-21* in the present cases, as also previously reported by the authors (31). The prognostic role of this miRNA is controversial, whilst certain studies have demonstrated that *miR-21* overexpression is associated with poor NSCLC survival (18,32,33), other studies have indicated a converse or insignificant association (34,35). In view of the present results, the prognostic role of *TPL2* is independent from the expression of *miR-21*.

Furthermore, histological subtyping has been proposed as a potential screening tool to identify the patients with lung cancer at a high risk for recurrent disease and an unfavorable prognosis (36). In the comparisons between the patients with adenocarcinoma and squamous cell carcinoma, ADCs with a lepidic pattern exhibited significantly improved survival. Although this prognosis of lepidic adenocarcinoma (37-39), and the more aggressive course of the disease and poor prognosis in the SCC histotype (40) have been well-documented,

the finding of the lepidic ADC pattern correlation with high *TPL2* expression has been not previously reported. Squamous cell lung carcinoma, even if clearly biologically different from the adenocarcinoma histotype, may behave as an adenocarcinoma arising from a non-terminal respiratory unit that is centrally originated, solid in morphology, poorly differentiated and often necrotic (41).

TPL2 appears to have divergent roles in different cells and tissues, serving as an oncogene as well as a tumor suppressor gene. In addition, how *TPL2* acts in carcinogenesis remains to be elucidated. Gkirtzimanaki *et al* (15) hypothesized that *TPL2* may lead to increased accumulation and activation of p53, resulting in an accelerated cell death and reduced malignant transformation. This hypothesis is corroborated by the good prognosis of lung cancer patients bearing tumors with elevated levels of *TPL2*, as it was identified in early-stage NSCLCs. Downregulation of *TPL2*, as a result of a genetic or epigenetic alteration, including LOH, overexpression of miRNAs, or oncogenic RAS signaling, depending upon the histological subtype, may have an impact on the early stages of human lung disease similar to

the effects of TPL2 ablation on lung cancer initiation in the mouse (15).

In conclusion, the present study is the first examination, to the best of our knowledge, of the association between *TPL2* and early-stage lung carcinogenesis, underscoring its tumor suppressor gene role. In the present analysis, *miR-21* expression did not impact on *TPL2* expression. Future studies must involve an analysis of *TPL2* in advanced lung cancer stages and its downstream signaling. In addition, studies must correlate findings with other miRNA expression profiles to clarify the mechanism by which *TPL2* is involved in lung carcinogenesis, taking into consideration all histological subtypes, and any important translational implications.

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