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Circulating mRNA Expression of Astrocyte-Elevated Gene-1 Associated with Treatment Response and Survival in Non-Small Cell Lung Cancer Patients Treated with Pemetrexed

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Abstract: Background: Astrocyte-elevated gene-1 (AEG-1) functions as an oncogene and regulates angiogenesis in non-small cell lung cancer (NSCLC). In this prospective study, we assessed the values of plasma AEG-1 mRNA expression by liquid biopsy associated with tumour response and survival in NSCLC patients treated with pemetrexed. Methods: Patients diagnosed with advanced NSCLC were enrolled to be treated with pemetrexed combined with platinum as first-line chemotherapy. All patients underwent blood sampling before any cancer treatment (C0) and at first response evaluation after two cycles (C2) of treatments. Response to chemotherapy and survival were assessed. Plasma mRNA was extracted from peripheral blood mononuclear cell (PBMC) and quantification of RNA was performed by real-time PCR. Results: A total of 50 patients with advanced NSCLC were included and 13 of 50 patients combined with bevacizumab. In patient groups of stable disease (SD) (n = 13) and progressive disease (PD) (n = 10), the plasma mRNA of AEG-1, thymidylate synthase (TS), and CK19 were elevated significantly at C2 compared to patients in treatment response group (PR, n = 27) (PR vs. SD or PD, AEG-1: 1.22 ± 0.80 vs. 4.51 ± 15.45 , $p = 0.043$). NSCLC patients who had elevated AEG-1 (AEG-1 ≥ 2) after two cycles of chemotherapy had shorter PFS and OS (high AEG-1 vs. low AEG-1, median, PFS: 5.5 vs. 11.9 months, $p = 0.021$; OS: 25.9 vs. 40.8 months, $p = 0.019$, respectively). In a Cox regression analysis, increased plasma mRNA expression of AEG-1 indicated poor prognosis in survival. Conclusions: Circulating mRNA concentration of AEG-1 could be a predictive and prognostic biomarker in NSCLC patients treated with pemetrexed. Increased expression of AEG-1 contributed to the chemoresistance and caused lung cancer progression.

Keywords: lung cancer; pemetrexed; angiogenesis; oncogene; liquid biopsy; astrocyte-elevated gene-1; thymidylate synthase

1. Introduction

Lung cancer is the world's leading cause of death from cancer [1], and it is usually diagnosed at an advanced or metastatic stage. Treatments for advanced stages are based on cytotoxic chemotherapy and other systemic therapies [2]. Response to treatment is assessed by computed tomography (CT) using the response evaluation criteria in solid tumors

(RECIST) criteria [3]. Although clinical image presentation is often delayed, indicating cancer progression, to date, there is no reliable biomarker for the evaluation of response to standard chemotherapy treatment in lung cancer. Therefore, there is a need for more prognostic factors to manage patients with advanced non-small cell lung cancer (NSCLC).

Expression of astrocyte-elevated gene-1 (AEG-1), a novel oncoprotein, is elevated in multiple cancers and plays a vital role in tumour cell growth, invasion, angiogenesis, and progression to metastasis [4,5]. High expression of AEG-1 could promote the carcinogenesis and lead to a poor clinical prognosis of NSCLC [6–8]. Recent findings suggest that AEG-1 contributes to broad-spectrum resistance to various chemotherapeutics [9,10]. Our previous data demonstrated that thymidylate synthase (TS) expression might be regulated by AEG-1 and that increased expression of these proteins contributes to lung cancer disease progression and may be associated with the development of resistance to pemetrexed [11]. AEG-1 could be used as a biomarker to identify subgroups of patients who require more intensive treatments. However, the acquisition of tumour tissue via biopsy may be very difficult to perform at the onset of resistance after chemotherapy due to small lesions or patients' poor conditions.

Liquid biopsy analysis is currently a rapidly expanding field in translational cancer research as it might be useful at different points of the diagnostic/therapeutic course of cancer patients [12]. Messenger RNAs (mRNA) possess a critical role in intracellular protein translation, and it is likely that extracellular mRNAs reflect the status of the intracellular process and are conceivably potential biomarkers for cancer diagnosis or therapeutic monitoring [13]. Recent studies reported various coding RNAs in plasma or serum from patients with cancer, and levels of circulating cell free mRNAs were found to be predictive of clinical outcome [14,15] and disease prognosis [16,17].

It has been hypothesized that circulating mRNA of AEG-1 expression could be a predictive factor of tumour response and a good candidate for a prognostic factor. Liquid biopsy analysis is currently a rapidly expanding field in translational cancer research as it might be useful at different points of the diagnostic/therapeutic course of cancer patients. Some studies have shown that circulating mRNA concentration could be a predictive factor of tumour response to surgery and to radiation therapy in cancer [18,19], but very few studies have investigated circulating mRNA concentration as a predictive factor of tumour response to chemotherapy [20–22]. In this prospective study, we will assess the predictive and prognostic values of AEG-1, combined with TS and CK19 expression, on tumour response and survival according to circulating mRNA concentration by liquid biopsy in NSCLC patients treated with pemetrexed.

2. Material and Methods

2.1. Patients and Data Collection

This prospective study was approved by the investigational review boards of the National Taiwan University Hospital (NO. 201510131RINB), and all participating patients provided informed consent before chemotherapy. Patients with a diagnosis of locally advanced or metastatic non-squamous cell lung cancer, stage IIIB or IV (8th version of the TNM staging system for lung cancer of the International Association for the Study of Lung Cancer) [23], and those who were treated with pemetrexed plus platinum as first-line chemotherapy regimen in the National Taiwan University Hospital Yunlin Branch were potentially eligible for inclusion. The diagnosis of NSCLC was based on histological examination of biopsy specimens or on cytological analysis of samples aspirated with a fine needle. Molecular testing for epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase (ALK) rearrangements were investigated from the pathologic or cytological specimens. Patients' basic data and medication usage were retrieved from electronic medical records.

2.2. Evaluation of Treatment Responses and Survival

The primary objective was to identify associations between specific biomarkers and treatment response rate. The secondary objective was to identify biomarkers associated with patient survival. All patients underwent blood sampling before chemotherapy (C0) and after 2 cycles of chemotherapy (C2).

The patients were administered with cisplatin or carboplatin plus pemetrexed intravenously every three weeks in patients with wild-type EGFR and ALK mutation or patients harboring EGFR or ALK mutation with disease progression after target therapy. Furthermore, patients received 500 mg/m² pemetrexed intravenously every three weeks as continuation maintenance therapy if the disease did not progress after 4 cycles of the first-line treatment. All patients were adults with good performance status (Eastern Cooperative Oncology Group Performance Status: 0–1). Treatment was discontinued if there was evidence of progressive disease, or unacceptable toxicity.

Chest computed tomography was performed every 3 months as routine clinical practices, and as needed to confirm treatment response. Treatment responses were evaluated according to the Response Evaluation Criteria in Solid Tumours (RECIST), version 1.1 (3), and were defined as complete remission, partial response (PR), stable disease (SD), and progressive disease (PD). Response rate (RR) was defined as the percentage of patients who achieved a complete remission or partial response. Disease-control rate (DCR) was defined as the percentage of patients who achieved a complete remission, partial response, or stable disease.

Progression-free survival (PFS) was defined as the time from the first cycle of pemetrexed plus platinum to documented progression or death from any cause, and was censored at the date of the last follow-up visit for patients who were still alive and who had not exhibited progression. The overall survival (OS) was measured from the date of the start of the treatment to the date of the last follow-up. Patients were followed up until December 2018.

2.3. Isolation of Human PBMCs

We collected 4 mL of human venous blood sample in heparinised vials (BD biosciences, Franklin Lakes, NJ, USA) and mixed well by gently inverting the tube several times. We took 4 mL of Ficoll Histopaque in a 15 mL centrifuge tube and gently layered the blood on the top of Ficoll Histopaque. The layering should be undertaken very slowly so that blood and Ficoll Histopaque stay as two different layers. We centrifuged the tubes without any delay for 30 min at 1200 g in 4 °C in a swing-out bucket. Fixed angle rotors also can be used but would require more caution when separating cells in interphase. We aspirated the whitish buffy coat (about 1 mL) (PBMCs) formed in the interphase between histopaque and medium. Then, we washed the cells (centrifuge in 1000 × g for 10 min) twice with 10 mL of sterile PBS or sterile Dulbecco's modified eagle medium. The approximate yield of cells added 1 mL Trizol and were placed in the −80 °C refrigerator for freezing.

2.4. Plasma mRNA Extraction and Quantitative Real-Time PCR

Total RNA (from PBMCs) was extracted using a QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany). First-strand complementary DNA (cDNA) synthesis was performed using 2 µg of total RNA using Invitrogen Super Script Reverse Transcriptase (Thermo Fisher Scientific, Carlsbad, CA, USA).

Total RNA was quantified by Nanodrop and then transcribed into complementary DNA (cDNA). Real-time PCR was performed according to the manufacturer's protocol for Applied Biosystems Power SYBR[®] Green PCR Master Mix, and the relative AEG-1 (Astrocyte elevated gene-1, Metadherin, MTDH), TS (Thymidylate synthetase, TYMS), CK19 (Keratin19) mRNA expression levels were determined as a ratio to that of Actin mRNA expression.

The mRNA expression level including AEG-1, TS and CK19 before chemotherapy was indicated as baseline as 1.0 for comparisons of these biomarker variables change after 2 cycles of chemotherapy. Therefore, healthy control samples were not needed in this study.

2.5. Statistical Methods

Variables as plasma mRNA level including AEG-1, TS and CK19 were presented as mean $\pm$ standard deviation. For comparisons of these biomarker variables, we used the independent t-test and paired t-test, respectively. Categorical variables are expressed as percentages. The median value of plasma AGE-1, TS, and CK19 mRNA expression were captured as cut-off levels. Correlation of patients' clinical factor and treatment response was estimated by using the Pearson Chi-Square. For survival analysis, we used the Kaplan–Meier method and median survival (and corresponding 95% confidence intervals) were compared using the log-rank test. Then, we used a Cox regression model (enter method) in order to assess variables associated with survival. If $p < 0.05$ was considered significant. Statistical analysis was performed using MedCalc (Version 19.6.1).

3. Results

3.1. Clinical Evaluation of Treatment Efficacy

From the year 2015 to 2018, a total of 50 patients (29 male and 21 female) with advanced non-squamous NSCLC received pemetrexed/platinum-based doublets chemotherapy. The median of age was 65 years old (range: 37 to 92 years old). Twenty-two of them harboured EGFR mutation and three of them were ALK positive. Chemotherapy regimens included 36 cisplatin and 14 carboplatin with pemetrexed, 13 of 50 patients combined with bevacizumab (Table 1).

Table 1. Pemetrexed plus platinum with and without bevacizumab for NSCLC: Basic Characteristics and Treatment Response.

	Overall	PC (n = 37)	PCB (n = 13)
Age, years old, (median, range)	65 (37–92)	68 (37–92)	62 (47–76)
Gender			
Male	29 (58.0%)	25 (67.6%)	4 (30.8%)
Female	21 (42.0%)	12 (32.4%)	9 (69.2%)
Smoking status			
Current or ever	21 (42.0%)	18 (48.6%)	3 (23.1%)
Never	29 (58.0%)	19 (51.4%)	10 (76.9%)
Mutation			
EGFR	22 (44.0%)	16 (43.2)	6 (46.2%)
ALK	3 (6.0%)	2 (5.4%)	1 (7.6%)
Wild type	25 (50.0%)	19 (51.4%)	6 (46.2%)
Platinum			
Cisplatin	36 (62.0%)	28 (75.7%)	8 (61.5%)
Carboplatin	14 (28.0%)	9 (24.3%)	5 (38.5%)
Treatment Response			
PR	26 (52.0%)	18 (48.6%)	8 (61.5%)
SD	14 (24.0%)	10 (27.0%)	4 (30.8%)
PD	10 (20.0%)	9 (24.3%)	1 (7.7%)
Response rate (%)	52%	48.6%	61.5%
Disease control rate (%)	80%	75.7%	92.3%

NSCLC: non-small cell lung cancer; PC: pemetrexed/platinum; PCB: pemetrexed/platinum/bevacizumab; EGFR: epidermal growth factor receptor; ALK: anaplastic lymphoma kinase; PR: partial response; SD: stable disease; and PD: progressive disease.

The initial response rate of chemotherapy included 26 partial responses, 12 stable diseases, and 10 progressive diseases. The treatment response rate and disease-control rate of pemetrexed/platinum doublet chemotherapy were 48.6% (18/37) and 75.7% (28/37), respectively. The treatment response rate and disease-control rate of bevacizumab-combined chemotherapy were 61.5% (8/13) and 92.3% (12/13), respectively (Table 1).

There was no significant difference of treatment response rate (RR) and disease-control rate (DCR) between pemetrexed/platinum doublet (PC) and bevacizumab-combined chemotherapy (PCB) (RR: $p = 0.200$ and DCR: $p = 0.197$, respectively). Patients treated with bevacizumab-combined chemotherapy had significant longer progression-free survival (PFS) and overall survival (OS) (PCB vs. PC, PFS, median, 15.4 vs. 7.7 months, $p = 0.01$; OS, median, 40.8 vs. 28.9, months, $p = 0.02$) (Figure 1).

Figure 1. Kaplan–Meier survival analysis illustrated chemotherapy combination affects (A) progression-free survival (PFS) and (B) overall survival (OS). Non-small cell lung cancer (NSCLC) patients treated with bevacizumab-combined pemetrexed/platinum doublet chemotherapy (PCB) had significant longer PFS and OS compared to pemetrexed/platinum doublet (PC) (PCB vs. PC, PFS, median, 15.4 vs. 7.7 months, $p = 0.01$; OS, median, 40.8 vs. 28.9, months, $p = 0.02$).

3.2. The Association of Plasma Biomarkers and Clinical Treatment Response

Plasma biomarkers including AEG-1, thymidylate synthase (TS), and CK19 were analysed before chemotherapy (C0) and after two cycles of chemotherapy (C2). In patient groups of SD (n = 14) and PD (n = 10), the plasma mRNA of AEG1, TS, and CK19 were elevated significantly at C2 compared to patients in treatment response group (PR, n = 26) (PR vs. SD or PD, AEG-1: 1.22 ± 0.80 vs. 4.51 ± 15.45 , $p = 0.043$; TS: 1.76 ± 1.77 vs. 10.22 ± 23.95 , $p = 0.003$; CK9: 3.05 ± 7.86 vs. 245.03 ± 884.14 , $p = 0.005$, respectively) (Figure 2). Plasma AEG-1 mRNA expression was lower in patients treated with bevacizumab at C2 but not significantly (with bevacizumab vs. without, AEG-1: 1.25 ± 0.75 vs. 3.25 ± 12.20 , $p = 0.560$). Moreover, the change of plasma mRNA level as AEG-1, TS, and CK19 between different chemotherapy regimens including either cisplatin or carboplatin was not significant.

Figure 2. Cont.

Figure 2. Bar graph with horizontal lines and marks for mean $\pm$ standard deviation demonstrated plasma mRNA expression change of (A) astrocyte-elevated gene-1 (AEG-1), (B) thymidylate synthase (TS), and (C) CK19 in non-small cell lung cancer (NSCLC) patients before chemotherapy (C0) and after two cycles of chemotherapy (C2). Patients with stable disease (SD) or progressive disease (PD) had significant increased plasma mRNA expression of AEG-1, TS and CK19, compared to those with partial response (PR) after two cycles of chemotherapy.

3.3. The Elevation of Circulating AEG-1 mRNA Expression Indicated Poor Prognosis

NSCLC patients with elevated plasma AEG-1 mRNA expression ($\text{AEG-1} \geq 2$) after two cycles of chemotherapy had significantly shorter PFS (high AEG-1 vs. low AEG-1, PFS, median, 5.5 vs. 11.9 months, $p = 0.021$). Elevated plasma TS ($\text{TS} \geq 5$) and CK19 ($\text{CK19} \geq 50$) mRNA expression after two cycles of chemotherapy also had shorter PFS (High TS vs. low TS, PFS, median, 5.8 vs. 11.9 months, $p = 0.501$; high CK19 vs. low CK19, PFS, median, 5.5 vs. 11.9 months, $p = 0.474$, respectively) (Figure 3).

Figure 3. Cont.

Figure 3. Expression level of plasma biomarker affects progression-free survival (PFS) plots by Kaplan–Meier survival analysis. (A) Patients with low and high levels of astrocyte-elevated gene-1 (AEG-1) expression are illustrated. PFS for patients with low AEG-1 expression was significantly longer (low vs. high, 11.9 vs. 5.5 months, $p = 0.02$, log rank test). (B) Patients with low and high levels of thymidylate synthase (TS) and, (C) CK19.

NSCLC patients with elevated plasma AEG-1 mRNA expression ($\text{AEG-1} \geq 2$) after two cycles of chemotherapy led to poor overall survival (OS) (high AEG-1 vs. low AEG-1, OS, median, 25.9 vs. 40.8 months, $p = 0.019$). Elevated plasma TS ($\text{TS} \geq 5$) and CK19 ($\text{CK19} \geq 50$) mRNA expression after two cycles of chemotherapy also had poor overall survival (High TS vs. low TS, OS, median, 34.0 vs. 59.0 months, $p = 0.853$; high CK19 vs. low CK19, OS, median, 29.3 vs. 34.0 months, $p = 0.880$, respectively) (Figure 4).

In a Cox regression multivariate analysis, increased circulating mRNA expression of AEG-1 can be a poor prognostic mark in survival (Hazard ratio (HR): 9.68, 95% CI: 1.69–55.57, $p = 0.01$), but not TS and CK19 (Figure 5).

Figure 4. Overall survival (OS) for patients with expression level of plasma biomarker plots by Kaplan–Meier survival analysis. (A) OS for patients with low AEG-1 expression was significantly longer (low vs. high, 40.8 vs. 25.9 months, $p = 0.019$, log rank test). (B) Patients with low and high levels of thymidylate synthase (TS) and (C) CK19.

treatment response. However, the role of CK19 expression was not significant in survival. This may be explained by the small sample size of the present study.

AEG-1 was confirmed to have functions as an oncogene and regulates angiogenesis [4,5]. AEG-1 expression directly correlates with increased expression of angiogenesis markers including angiopoietin-1 (Ang1), matrix metalloproteinase (MMP)-2, and HIF1- α and supporting a potential role of AEG-1 in tumour angiogenesis [43]. AEG-1 mRNA expression upregulation indicated a significantly positive correlation with vascular endothelial growth factor (VEGF) expression and increased intratumoural microvessel density (iMVD, labelled by CD105) counting [44]. Several studies have shown that over-expression of AEG-1 significantly associates with tumour aggressiveness and poor prognosis in NSCLC [6,45]. These results suggested that AEG-1 may play important roles in malignant transformation and tumour angiogenesis in NSCLC, and anti-AEG-1 mRNA expression may be a novel potential strategy for anti-angiogenic therapy of NSCLC. In our study, bevacizumab-combined chemotherapy decreased more plasma AEG-1 mRNA expression, also had higher response rate and longer PFS and OS. However, the differences were not significant. This may be limited by the small sample size of the present study.

This study has several limitations. First, our investigation was a prospective, observational study without randomization; the selection bias might be influenced by clinical physicians and uneven patient characteristics. Patients with good ECOG PS without contraindications of anti-angiogenesis therapy were more likely to receive first-line platinum-based chemotherapy combined with bevacizumab. Second, this study only recruited patients from one institute, and the limited provider and patient source may result in presentation with caution. Third, detailed subgroup analysis was difficult to be executed owing to the limited sample size. Further large-scale prospective studies are warranted to validate these findings.

5. Conclusions

AEG-1 functions as an oncogene and regulates angiogenesis in NSCLC. AEG-1 also promotes TS expression contributing to the development of resistance to pemetrexed. Bevacizumab-combined chemotherapy inhibits AEG-1 expression and could increase treatment benefits and survivals. Plasma AEG-1 mRNA level could be used a predictive and prognostic biomarker in NSCLC patients treated with pemetrexed. Increased expression of AEG-1 led to chemoresistance and caused lung cancer progression, which could be used for clinical evaluation combined with image studies.

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Informed Consent Statement: Patients with lung cancer provided written informed consent for the use of blood specimens and clinical data for research prior to undergoing chemotherapy.

Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within the article.

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