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Galectin-9 in non-small cell lung cancer

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ABSTRACT

Objectives: Lung cancer has the highest incidence and fatality among all cancers. Our research analyzed the expression of galectin-9 on non-small cell lung cancer (NSCLC) tumor cells and tumor infiltrating lymphocytes (TILs).

Materials and methods: We analyzed the expression level of galectin-9 protein in 136 NSCLC primary tumor samples by immunohistochemistry (IHC).

Results: We tested 136 surgical resected primary NSCLC tumor tissues for galectin-9 from Medical University of Gdansk, Poland. We found that on tumor cells, galectin-9 level only had correlation with T cell immunoglobulin and mucin-domain containing-3 (TIM-3) level (Correlation Coefficient = 0.360, $p < 0.001$). On TILs, galectin-9 level had broad connections with other checkpoints including programmed cell death protein-1 (PD-1) (Correlation Coefficient = 0.332, $p < 0.001$), programmed cell death-ligand 1 (PD-L1) (Correlation Coefficient = 0.247, $p = 0.004$) and TIM-3 (Correlation Coefficient = 0.350, $p < 0.001$). Interestingly, galectin-9 level on TILs also had positive relation with PD-L1 level on tumor cells (Correlation Coefficient = 0.278, $p = 0.001$), galectin-9 level on tumor cells (Correlation Coefficient = 0.181, $p = 0.035$) and TIL percentage (Correlation Coefficient = 0.236, $p = 0.006$). High level of galectin-9 on TILs indicated shorter RFS (recurrence-free survival) (RFS 1.82 years, 95% CI 0.795–2.845 vs. 0.67 years, 95% CI 0.086–1.254, $P = 0.033$). Patients with galectin-9 positive tumor cells display longer overall survival (OS) (1.76 years, 95% CI 0.222–3.298 vs. 3.10 years, 95% CI 2.662–3.538, $P = 0.039$)

Conclusions: We found galectin-9 expression on both NSCLC tumor cells and TILs. Galectin-9 expression was found in all NSCLC pathological type. Galectin-9 level on TILs had correlation with TIM-3, PD-1 and PD-L1 level. On tumor cells, galectin-9 level had correlation with TIM-3 level. Patients with low galectin-9 level on tumor cells or high galectin-9 level on TILs were more likely to have poor prognosis.

1. Introduction

With the highest incidence and fatality among all cancers, lung cancer posts a great threat to the public health around the globe [1]. The most common pathologic type of lung cancer is non-small cell lung cancer (NSCLC). Although targeted therapy could prolong the survival of patients harboring driver genes, these population are only a small part of all NSCLC patients. Besides, relapse often occurs after several cycles of treatment [2,3]. Patients are craving for a generally applicable therapy with better outcome.

Immunotherapy has been used in solid tumors after its breakthrough in treating melanoma [4,5]. Recently, several immune checkpoint blockade therapies have been applied for treating NSCLC, such as anti-programmed cell death protein-1/ligand-1 (PD-1/PD-L1) and anti-cytotoxic T-lymphocyte associated protein-4 (CTLA-4) antibodies [6–8]. Unfortunately, our understanding towards tumor immune microenvironments is inadequate. Without an optimal biomarker to screen drug effective population, the overall response rate of

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immunotherapy is still far from satisfying [9]. There are unmet needs to enhance immunotherapy with new approaches.

Galectin-9 belongs to the galectin protein family which is a subset of lectin with carbohydrate recognition domain [10]. The role of galectin-9 in cancer is complicated. In breast cancer, it was reported that galectin-9 could induce tumor cell aggregation, making tumor cells less aggressive, preventing metastasis and improving patient survival [11,12]. A previous study confirmed that galectin-9 could induce apoptosis in hepatocellular carcinoma in vitro and vivo [13]. Paradoxically, back to immunology, galectin-9 is a ligand of T cell immunoglobulin and mucin-domain containing-3 (TIM-3). Combining with TIM-3, galectin-9 triggers the termination of T cell mediated immunity [14]. Galectin-9 also impairs innate immunity by down-regulating natural killer cells (NK cells) function [15]. However, former studies reported that in certain scenarios, galectin-9 could promote the function of some immune cells [16,17]. Moreover, clinical data of galectin-9 in different kind of tumors shows notable discordance. Here, we tested galectin-9 level in NSCLC tumor tissues with immunohistochemistry (IHC). The aim of this study was to describe galectin-9 status in NSCLC and revealing its correlation with immune checkpoints and patient survival.

2. Patients and methods

2.1. Samples

We collected one hundred and thirty-six surgical resected primary lung cancer specimens from Medical University of Gdansk, Poland (ethical number 15–235), which we mentioned in our published paper [18]. The patients had not undergone radiation or chemotherapy before surgery. All participants were competent to provide their consent.

2.2. IHC by Ventana benchmark XT®

IHC was performed by Ventana benchmark XT® [18] Primary antibodies (Galectin-9, NBP2-45619, Novusbio) were applied.

2.3. Select the IHC cutoff value

The pathologic tests were completed by two experienced pathologists independently. All samples were reviewed. Galectin-9 positive on TILs was confirmed only when there was more than 30% staining. But all positive stains were considered meaningful on tumor cells. We tested different cutoffs with survival analysis to decide the best cutoff, when the statistical differences of RFS (recurrence-free survival) and OS (overall survival) were maximized simultaneously [18,19].

2.4. Evaluation of TILs

According to previous study, we calculated the number of lymphocytes within each histospots [20]. The fraction of TILs was divided into three level: < 30% was low, 30%–60% was medium and > 60% was rather high. Any spot categorized into different level were re-checked by two pathologists together until a consensus was reached [21].

2.5. Data analysis

The relationship between galectin-9 level and clinical pathological parameters was evaluated by Chi-square tests. Expression level of galectin-9 and other checkpoints were tested by Spearman's correlation tests, in order to analyze the association between checkpoints. We also performed logistic regression for prediction of galectin-9 expression by using factors including age, gender, smoking history, lung cancer stage, grade, PD-1, PD-L1 and TIM-3 level. The survival distributions of different groups were compared using the Kaplan-Meier method. We also performed univariate and multivariate Cox regression. The odds ratios (ORs) and their 95% confidence intervals (95%CIs) were computed after adjusting by age, gender, smoking status, T stage, N stage, M stage, lung cancer stage and grade. All P-values were 2-sided, and statistical significance was defined as $P < 0.05$. Statistical analysis was performed using SPSS statistical software package (version 17.0; SPSS, Inc.; Chicago, IL).

Table 1
Characteristics of the 136 patients from Medical University of Gdansk.

Characteristic	Total
Gender, n (%)	
Male	108(79.4%)
Female	28(20.6%)
Age, median	
< 70	102(75.0%)
≧70	34(25.0%)
Smoking status, n (%)	
Non-Smoker	6(4.4%)
Smoker	130(95.6%)
Surgery, n (%)	
Wedge	2(1.5%)
Segmentectomy	3(2.2%)
Lobectomy	72(52.9%)
Bilobectomy	8(5.9%)
Pneumonectomy	45(33.1%)
Sleeve lobectomy	6(4.4%)
T stage, n (%)	
1–2	103(75.7%)
3–4	33(24.3%)
N stage, n (%)	
0	75(54.4%)
1	62(45.6%)
M stage, n (%)	
0	129(94.9%)
1	7(5.1%)
Lung cancer staging, n (%)	
I–II	92(67.6%)
III– IV	44(32.4%)
Pathology, n (%)	
SCC	80(58.8%)
Adenocarcinoma	38(27.9%)
Large cell carcinoma	4(2.9%)
NSCLC NOS/Mixed	12(8.8%)
NSCLC others	2(1.5%)
Grade, n (%)	
G1	16(11.8%)
G2	55(40.4%)
G3	46(33.8%)
Unknown	19(14.0%)
Surgical margin, n (%)	
Complete	112(82.4%)
Macroscopic positive	19(7.4%)
Unknown	14(10.3%)

3. Results

3.1. Clinical characteristics of patients

From April 2010 to August 2011, Medical University of Gdansk, Poland provided us with 136 surgical resected NSCLC specimens along with their clinical data. In these patients, 108 (79.4%) were male and 28 (20.6%) were female. Average age was 64. Six (4.4%) patients had no smoking history. Nighty-two (67.6%) patients had stage I or II NSCLC and 44 (32.4%) had stage III or IV. Thirty-eight (27.9%) patients had adenocarcinoma, and 80 (58.8%) had squamous cell carcinoma

Table 2
Relationship between galectin-9 and clinical data (samples from Medical University of Gdansk).

	Gal-9 expression on tumor cells			Gal-9 expression on TILs		
	Negative	positive	P value	Negative	positive	P value
Age, n (%)						
< 70	59(57.8%)	43(42.2%)	0.548	96(94.1%)	6(5.9%)	0.69
≧70	22(64.7%)	12(35.3%)		31(91.2%)	3(8.8%)	
Gender, n (%)						
Female	19(67.9%)	9(32.1%)	0.39	26(92.9%)	2(6.7%)	1
Male	62(57.4%)	46(42.6%)		101(93.5%)	7(6.5%)	
Smoking status, n (%)						
Non-Smoker	3(50.0%)	3(50.0%)	0.686	5(83.3%)	1(16.7%)	0.342
Smoker	78(60.0%)	52(40.0%)		122(93.8%)	8(6.2%)	
Lung cancer staging, n (%)						
Stage I–II	50(54.3%)	42(45.7%)	0.093	86(93.5%)	6(6.5%)	1
Stage III–IV	31(70.5%)	13(29.5%)		41(93.2%)	3(6.8%)	
Pathology, n (%)						
AD	24(63.2%)	14(36.8%)	0.698	91(92.9%)	7(7.1%)	1
Non-AD	57(58.2%)	41(41.8%)		36(94.7%)	2(5.3%)	
Grade, n (%)						
G1	11(68.8%)	5(31.3%)	0.422	16(100%)	0(12.5%)	1
G2-3	57(56.4%)	44(43.6%)		97(96.0%)	4(5.8%)	
TILs percentage, n (%)						
< 30 (1+)	35(56.5%)	27(43.5%)	0.599	60(96.8%)	2(3.2%)	0.181
≥30 (2+ – 3+)	46(62.2%)	28(37.8%)		67(90.5%)	7(9.5%)	
Metastasis, n (%)						
Negative	76(58.9%)	53(41.1%)	0.701	120(93.0%)	9(7.0%)	1
Positive	5(71.4%)	2(28.6%)		7(100%)	0(0%)	

(SCC) (Table 1).

3.2. Association between galectin-9 and clinical parameters

In all tested samples, 55 (40.4%) had positive galectin-9 expression on tumor cells, 9 (6.6%) had galectin-9 positive TILs. No significant difference of galectin-9 level on tumor cells was detected comparing adenocarcinoma (AD) with non-adenocarcinoma (non-AD) ($p = 0.698$). Same result was found on TILs ($p = 1.000$) (Table 2).

3.3. Correlation between galectin-9 and other checkpoints

We use the Spearman's correlation test to describe the connection between the level of galectin-9 expression and other checkpoints. The IHC scores was evaluated as grade variables. We found that on tumor cells, galectin-9 level only had correlation with TIM-3 level (Correlation Coefficient = 0.360, $p < 0.001$). However, on TILs, galectin-9 level had broad connections with other checkpoints including PD-1 (Correlation Coefficient = 0.332, $p < 0.001$), PD-L1 (TILs) (Correlation Coefficient = 0.247, $p = 0.004$) and TIM-3 (Correlation Coefficient = 0.350, $p < 0.001$). Interestingly, galectin-9 level on TILs also had positive relation with PD-L1 level on tumor cells (Correlation Coefficient = 0.278, $p = 0.001$), galectin-9 level on tumor cells (Correlation Coefficient = 0.181, $p = 0.035$) and TIL percentage (Correlation Coefficient = 0.236, $p = 0.006$) (Table 3).

3.4. Logistic regression for galectin-9 expression

With the logistic regression model, we performed univariate and multivariate analysis. After adjusting for age, gender, smoking status, pathologic type and tumor stage, the OR for galectin-9 expression on TILs was 5.843 (95% CI: 1.138–30.002; $p = 0.0034$) when samples showed PD-L1 positive on tumor cells compared with those showed negative on tumor cells (Tables S1 and S2).

Table 3
Relationship between expression level of galectin-9 and other checkpoints.

	Gal-9 on Tumor cells		Gal-9 on TILs	
Other checkpoints	Correlation Coefficient	p value	Correlation Coefficient	p value
Gal-9 on Tumor cells	/	/	0.181	**0.035**
TIM-3 on Tumor cells	0.360	**< 0.001**	0.110	0.202
TIM-3 on TILs	0.131	0.130	0.350	**< 0.001**
PD-L1 on Tumor cells	0.126	0.145	0.278	**0.001**
PD-1 on TILs	0.012	0.894	0.332	**< 0.001**
PD-L1 on TILs	0.057	0.511	0.247	**0.004**
TIL	−0.076	0.378	0.236	**0.006**

3.5. Association between galectin-9 expression and survival in NSCLC patients

High level of galectin-9 on TILs indicated shorter RFS (RFS 1.82 years, 95% CI 0.795–2.845 vs. 0.67 years, 95% CI 0.086–1.254, $P = 0.033$). Interestingly, patients with galectin-9 positive tumor cells display markedly longer overall survival (OS 1.76 years, 95% CI 0.222–3.298 vs. 3.10 years, 95% CI 2.662–3.538, $P = 0.039$) (Fig. 1).

3.6. Cox regression for survival analysis

We computed the odds ratios of clinical parameters and checkpoint expression levels using univariate and multivariate cox regression. Cancer stage was able to predict both RFS ($P = 0.009$, Odds Ratio 2.613 95%CI 1.277–5.345) and OS ($P = 0.002$, Odds Ratio 3.055, 95% CI 1.518–6.149). Galectin-9 positive on TILs indicated shorter RFS ($P = 0.017$, Odds Ratio 2.482 95% CI 1.178–5.227) (Tables 4 and 5).

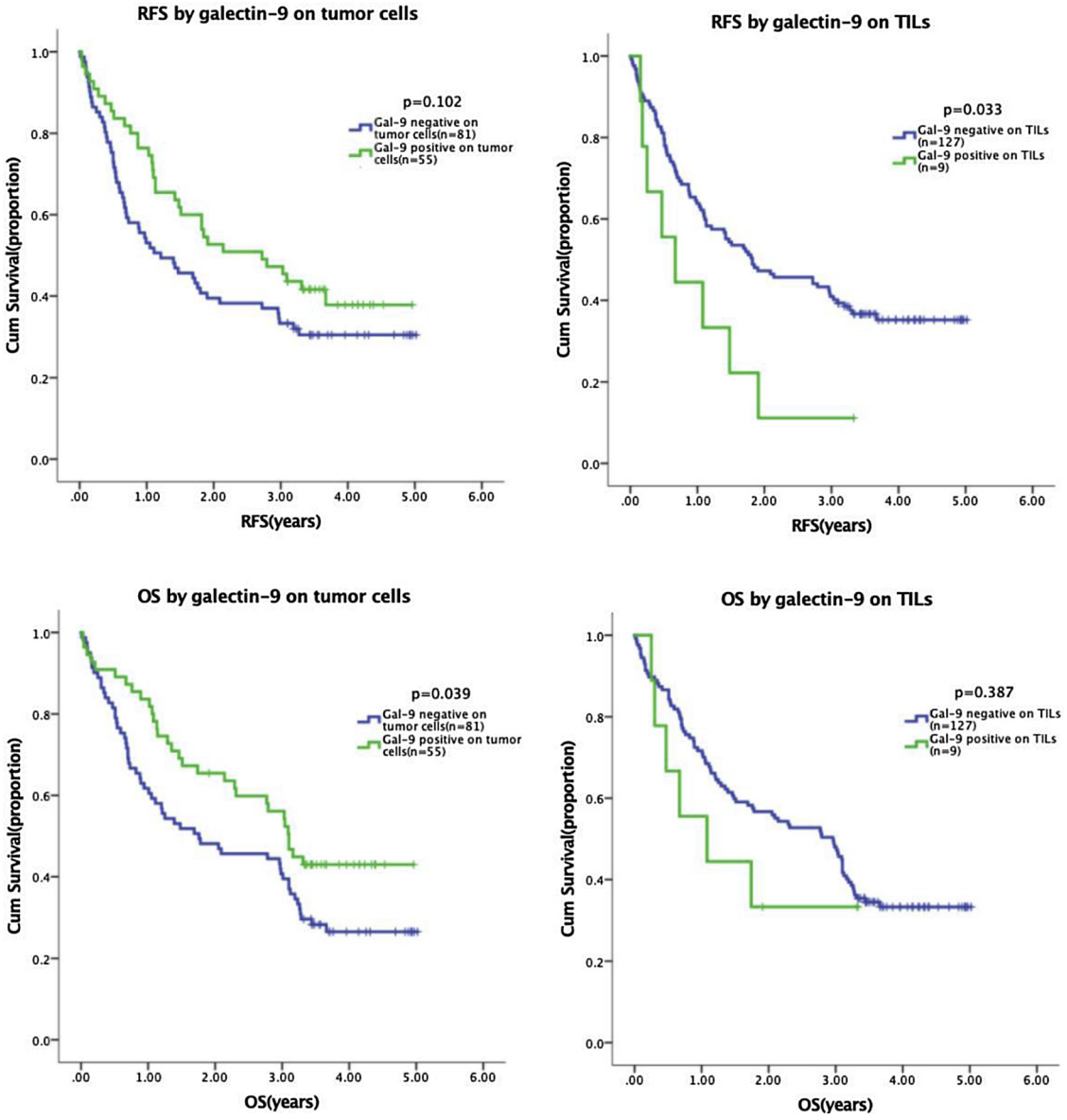

Fig. 1. RFS and OS by galectin-9 status.

4. Discussion

In this study, our aim was to describing galectin-9 expression status in NSCLC. The results of our analysis suggested that galectin-9 was expressed in all pathologic type of NSCLC. Clinical features of patients did not seem to effect galectin-9 level. However, we found that galectin-9 level was associated with the level of other checkpoints including PD1, PD-L1 and TIM-3. The most intriguing finding was that galectin-9 expression on tumor cells and TILs would lead to completely different clinical outcome. Elevated galectin-9 level on tumor cells led to longer

Table 4
COX regression analysis of RFS.

	Univariate			Multivariate		
Variables	Odds Ratio	95% CI	P	Odds Ratio	95% CI	P
Age (< 70 vs. ≥ 70)	0.789	0.480-1.299	0.351			
Gender (Female vs. Male)	1.727	0.974-3.061	0.061			
Smoking status (Non-smoker vs. Smoker)	0.611	0.252-1.536	0.303			
T (1-2 vs. 3-4)	1.811	1.149-2.855	**0.011**	1.205	0.717-2.027	0.481
N (0 vs. 1-2)	1.945	1.278-2.960	**0.002**	1.057	0.577-1.936	0.857
M (0 vs. 1)	4.393	1.974-9.772	**< 0.001**	2.498	1.050-5.942	0.038
Stage (I-II vs. III-IV)	3.156	2.045-4.871	**< 0.001**	2.613	1.277-5.345	**0.009**
Grade (1 vs. 2-3)	0.715	0.393-1.303	0.273			
Gal-9 on tumor cells (negative vs. positive)	0.7	0.455-1.077	0.105			
Gal-9 on TILs (negative vs. positive)	2.167	1.043-4.500	**0.038**	2.482	1.178-5.227	**0.017**

Table 5
COX regression analysis of OS.

	Univariate			Multivariate		
Variables	Odds Ratio	95% CI	P	Odds Ratio	95% CI	P
Age (< 70 vs. ≥ 70)	0.835	0.512-1.362	0.471			
Gender (Female vs. Male)	2.159	1.174-3.970	**0.013**	1.845	0.992-3.43	0.053
Smoking status (Non-smoker vs. Smoker)	0.615	0.249-1.520	0.292			
T (1-2 vs. 3-4)	2.112	1.344-3.317	**0.001**	1.26	0.761-2.085	0.369
N (0 vs. 1-2)	1.89	1.247-2.866	**0.003**	0.994	0.541-1.827	0.984
M (0 vs. 1)	4.367	1.966-9.705	**< 0.001**	1.808	0.764-4.279	0.178
Stage (I-II vs. III-IV)	3.831	2.489-5.898	**< 0.001**	3.055	1.518-6.149	**0.002**
Grade (1 vs. 2-3)	0.782	0.421-1.453	0.437			
Gal-9 on tumor cells (negative vs. positive)	0.635	0.411-0.981	**0.041**	0.711	0.456-1.108	0.132
Gal-9 on TILs (negative vs. positive)	1.439	0.627-3.303	0.391			

OS, while higher galectin-9 on TILs led to shorter RFS.

Galectin-9 was first discovered in human Hodgkin's lymphoma, it has been studied in multiple discipline since then [22]. The cellular biological function of galectin-9 is complex. Galectin-9 in malignant tumors had multifaced role in the development and aggression of cancer. In breast cancer, galectin-9 could improve the adhesion of cultured cancer cells [11]. Researchers also found that patients with high galectin-9 tumors displayed lower chance of metastasis [12]. Galectin-9 exhibited direct anti-tumor effect as well. In hepatocellular carcinoma, recombinant mutant forms of galectin-9 could induce tumor cell apoptosis in vitro and vivo [13]. Similar result was also found in hematological malignancies such as myeloma and chronic myelogenous leukemia [23,24]. Paradoxically, via interacting with its receptor TIM-3, galectin-9 could also impair anti-tumor immunity by functionally terminating effective T helper 1 cells [14]. In innate immunity, galectin-9 downregulated genes that engaged in the cytotoxic activity of NK cells thus reduce interferon production [15]. Meanwhile, clinical data that using galectin-9 as a prognosis indicator for malignant tumors showed discordance as well. In melanoma, hepatocellular carcinoma, urothelial carcinoma and several various malignancies, patients with high galectin-9 level tended to have longer survival. Nevertheless, the opposing result was detected in clear-cell renal cell carcinoma, reporting that expression of galectin-9 result in shortened survival and early relapse [25]. There is an unmet need to describe galectin-9 status in NSCLC prospectively.

Our results indicated that galectin-9 was widely existed in NSCLC tissue on both tumor cells and TILs. There was no connection between galectin-9 level and clinical parameters. However, we discovered a co-expression pattern of galectin-9, TIM-3, PD-1 and PD-L1 on TILs. For the first time, we found that patients with high galectin-9 level on their TILs were more likely to have early recurrence. According to previous study, PD-1(+) TIM-3(+) CD8(+) phenotype lymphocytes was considered the most exhausted T-effector cell phenotype [26]. Our finding indicated that galectin-9 might also be involved in T-cell exhaustion. Moreover, galectin-9 was also reported to have interaction with TNF receptors including 4-1BB (CD137) and DR3, which effected T cell and NK cell activities and the development of immune-suppressive T regulatory cells [27,28]. These finding suggested the therapeutic potential of galectin-9 in immunotherapy.

Meanwhile, galectin-9 was co-expressed with TIM-3 on tumor cells. Patients with galectin-9 positive tumor cells had conspicuously longer survival than those with no galectin-9 on tumor cells. This finding was consistent with most of former researches and a recent meta-analysis, supporting the prognostic value of galectin-9 in NSCLC [29]. However, the galectin-9 receptor that induce cancer apoptosis has not been identified yet and its pharmacokinetic parameters in human is not clear [30]. In our view, using galectin-9 in cancer treatment should be considered upon a fair balance of its anti-cancer effect and immune-suppressive function.

To summarize, we discovered galectin-9 expression on NSCLC cancer cells and TILs by IHC. We also found a co-expression pattern of galectin-9 and immune checkpoints. Furthermore, galecin-9 level on tumor cells was notably correlated with prolonged survival time and galecin-9 level on TILs was notably correlated with early postoperative recurrence. More exploration on galecin-9 needs to be conducted to reveal the metabolic mechanism behind its multifaced biological functions.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.08.014.

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