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Apolipoprotein E is a predictive marker for assessing non-small cell lung cancer patients with lymph node metastasis

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ABSTRACT

Background: Apolipoprotein E (APOE) modulates lipid homeostasis in the systemic circulation and induces inflammatory immune responses in the tumor microenvironment. We evaluated APOE expression in order to assess tumor progression in non-small cell lung cancer (NSCLC).

Methods: Immunohistochemical staining for APOE was performed on tissue microarray blocks from 148 patients who had undergone surgery for NSCLC. The staining intensity and the proportion of APOE-positive tumor cells (based on distinct membranous and cytoplasmic staining) were scored. The relationships between APOE expression and clinical (age, sex, and smoking history) and pathological (TNM stage and histological type) factors were evaluated.

Results: Positive APOE staining was observed in 93 (64.6%) patients. APOE expression patterns differed among NSCLC histological types (p-value = 0.016). Negative APOE expression was significantly associated with lymph node metastasis in NSCLC (p-value = 0.040). Both cases of N2 (stage IIIA) disease showed negative APOE expression.

Conclusions: APOE is a useful marker for assessing NSCLC patients with lymph node metastasis.

1. Introduction

Lung cancer is one of the most common cancers worldwide, and its incidence is increasing. Although the life expectancy of lung cancer patients is increasing mainly due to successful surgical interventions and the development of anticancer agents, there is still no specific marker to detect advanced lung cancers (N2 disease, stage IIIA). Recently, thoracic surgeons have expressed contradictory opinions regarding the best method of managing N2 disease: aggressive intraoperative node dissection or induction therapy without surgery [1]. Identification of a marker related to the occurrence of lymph node metastasis may provide improved options for patients with advanced non-small cell lung cancer (NSCLC).

Apolipoprotein E (APOE) is a 34 kDa small particle that plays an important role in lipid metabolism. APOE transports cholesterol, triglycerides, and phospholipids between peripheral tissues and the liver in the systemic circulation. Although plasma APOE is synthesized mainly in the liver, it is also produced in the brain, kidneys, spleen and lungs and by macrophages [2]. The role of APOE in cancer cells is unclear and limited studies have addressed it. Since the cholesterol is a metabolic precursor to make the structure of the plasma membrane, it may alter receptor tyrosine kinase (membrane-associated protein), reduce apoptosis, and increase tumor growth [3]. APOE, which regulate cholesterol homeostasis may play a significant role in cancer cell proliferation.

Regarding the lungs, APOE has been found to be related to asthma, lung cancer, emphysema, pulmonary hypertension, and normal lung parenchyma. To date, data regarding APOE expression in NSCLC are scarce. However, APOE has been known to impact on carcinogenesis *via* an autocrine or paracrine mechanism [4–6]. Alveolar macrophages (AMs) have been shown to promote the metastasis of breast cancer to the lungs by suppressing antitumor T cells [7]. AMs produce inflammatory cytokines to perform an immunosuppressive function in tumor-bearing hosts. In addition, macrophages stimulate angiogenesis, cell migration, invasion, and immunosuppression as the tumor progresses to malignancy [8]. Macrophage-derived APOE modifies the microenvironment by interacting with the extracellular matrix to retain lipoproteins in the vessel wall and to further isolate paracrine growth factors and cytokines [9]. In this study, we hypothesized that macrophage-derived APOE retained in the extracellular matrix participates in

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Table 1
Clinicopathological characteristics of the patients.

Characteristic	Number (%) (n = 148)
Mean age (years)	64.85 (31–77)
Male gender	125 (84.5)
Smoking history	97 (65.5)
Histologic type	
Squamous cell carcinoma	96 (64.9)
Well-differentiated	15
Moderately- differentiated	59
Poorly-differentiated	22
Adenocarcinoma	37 (25)
Acinar	15
Solid	6
Papillary	8
Micropapillary	3
Lepidic	3
Mucinous	2
Large cell neuroendocrine carcinoma	8 (5.4)
Others	7 (4.7)
T stage (pathological)	
1mi	2 (1.4)
1a	1 (0.7)
1b	19 (12.8)
1c	26 (17.6)
2a	45 (30.4)
2b	17 (11.5)
3	25 (16.9)
4	13 (8.8)
N stage	
0	103 (69.6)
1	42 (28.4)
2	3 (2.0)
3	0 (0)
M stage	
0	145 (98.0)
1a	3 (2.0)
1b	0 (0)
1c	0 (0)
Mean survival (range), months	37 (0–113)
Five-year survival	33 (22.3)

the interplay between inflammatory cytokines and antitumor T cells to modify the tumor microenvironment in order to generate metastatic potential. To confirm this finding in NSCLC, we investigated the correlation between APOE expression and clinical (age, sex, and smoking history) and pathological (histological type and pathological T, N, and M stages) factors in NSCLC patients.

2. Materials and methods

2.1. Case selection

A total of 148 patients who had undergone surgery for NSCLC at Gyeongsang National University Hospital, Jinju, South Korea, between January 2002 and December 2009 were enrolled. Representative hematoxylin and eosin-stained slides from 148 consecutive patients were reviewed by two experienced pathologists. Electronic medical records were reviewed, and the clinical and pathological data, including age, sex, smoking history, histological type, T stage, N stage, M stage, mean survival time, and five-year survival rate, were collected (Table 1). The stages of cancer were determined according to the eighth edition of the AJCC guidelines. This study was approved by the institutional review board of Gyeongsang National University Hospital (GNUH-2019-02-017).

2.2. Tissue microarray and immunohistochemistry

Representative hematoxylin and eosin-stained glass slides containing samples of intratumoral lesions from the 148 NSCLC specimens were collected. A core was obtained from the invasive tumor front of each representative paraffin block and transplanted into the recipient TMA block. Immunohistochemical staining was performed on 4-μm sections of the TMA block samples. After attachment to glass slides, sections were deparaffinized, rehydrated, and incubated in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Then, sections were heated for 20 min in 10 mM citrate buffer (pH 6.0) in a microwave oven (700 W). After incubation with Ultra V block (LabVision Corporation, Fremont, CA, USA) for 7 min at room temperature to block background staining, slides were incubated with a primary monoclonal antibody specific for APOE (1:500 dilution, ab1906, Abcam, Cambridge, MA, USA according to the manufacturer's recommendations for visualization. An ultraView Universal DAB detection kit was used (760-500, Ventana, Tucson, AZ, USA). 3, 3′-Diaminobenzidine was used to detect reactivity. Sections were counterstained with hematoxylin.

2.3. Assessment of APOE expression

The immunohistochemical staining patterns of APOE were evaluated in 144 of the 148 (4 cases could not be evaluated due to the loss of tissue specimens) TMA blocks. Distinct membranous and cytoplasmic staining for APOE was considered positive. The intensity of the stained tumor cells was scored as follows: unstained, 0; weak, 1+; moderate, 2+; and strong, 3+. A score of 0 was considered negative, whereas scores of 1+, 2+, and 3+ were considered positive. The proportion of stained tumor cells was scored as follows: unstained, 0; 1%–24%, 1+; 25%–50%, 2+; 50%–75%, 3+; and greater than 75%, 4+. A proportion of less than 25% was considered negative, whereas a proportion of $\geq$25% was considered positive. Alveolar macrophages were used as internal controls for APOE immunohistochemical staining, as APOE can be produced by macrophages.

2.4. Statistical analysis

The correlation between APOE expression and clinicopathological characteristics was evaluated (Table 2) using the chi-square test. Values of p less than 0.05 were considered statistically significant. SPSS ver. 24.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

3. Results

3.1. Patient characteristics

A total of 148 patients with NSCLC were enrolled in this study. The clinical and pathological information of the patients is summarized in Table 1. The mean age of the patients was 64.85 years. Of the patients, 125 (84.5%) were male, and 86 (65.2%) had a smoking history. The histopathological types of the tumors were as follows: 96 (64.9%) specimens showed squamous cell carcinoma (SCC), 37 (25%) showed adenocarcinoma (ADC), 8 (5.4%) showed large cell neuroendocrine carcinoma (LCNEC), and 7 (4.7%) showed other types, including pleomorphic and mucoepidermoid carcinoma. The distribution of pathological T stages was as follows: 1mi, 2 (1.4%); 1a, 1 (0.7%); 1b, 19 (12.8%); 1c, 26 (17.6%); 2a, 45 (30.4%); 2b, 17 (11.5%); 3, 25 (16.9%); and 4, 13 (8.8%). The distribution of N stages was as follows: 0, 103 (69.6%); 1, 42 (28.4%); 2, 3 (2.0%); and 3, 0 (0%). The distribution of M stages was as follows: 0, 145 (98.0%); 1a, 3 (2.0%); 1b, 0 (0%); and 1c, 0 (0%). The mean survival time was 37 months (range: 0–113 months), and the five-year survival rate was 33%.

3.2. Correlation between APOE expression and clinicopathological characteristics

Among the clinical and pathological factors listed previously (age; sex; smoking history; histological type; and pathological T, N, and M stages), the histological type showed a statistically significant

Table 2
The relationship between APOE expression and clinicopathological characteristics (n = 144 cores).

Characteristic	APOE expression					
	Intensity			Proportion		
	Negative	Positive	p-value	< 25%	≥25%	p-value
Age			0.889			0.808
< 65	22 (15.3)	39 (27.1)		37 (25.7)	24 (16.7)	
≥65	29 (20.1)	54 (37.5)		52 (36.1)	31 (21.5)	
Sex			0.685			0.920
Male	42 (29.2)	79 (54.9)		75 (52.1)	46 (31.9)	
Female	9 (6.3)	14 (9.7)		14 (9.7)	9 (6.3)	
Smoking			0.492			0.382
Non-smoker	20 (13.9)	29 (20.1)		29 (20.1)	20 (13.9)	
Smoker	31 (21.5)	63 (43.8)		60 (41.7)	34 (23.6)	
Histologic type			**0.016**			**0.001**
Squamous cell carcinoma	42 (29.2)	52 (36.1)		69 (47.9)	25 (17.4)	
Adenocarcinoma	7 (4.9)	29 (20.1)		16 (11.1)	20 (13.9)	
Large cell neuroendocrine	1 (0.7)	6 (4.2)		2 (1.4)	5 (3.5)	
Others	1 (0.7)	6 (4.2)		2 (1.4)	5 (3.5)	
T stage			0.964			0.626
< 2b	32 (22.2)	58 (40.3)		57 (39.6)	33 (22.9)	
≥2b	19 (13.2)	35 (24.3)		32 (22.2)	22 (15.3	
N stage			**0.040**			0.156
< 1	30 (20.8)	70 (48.6)		58 (40.3)	42 (29.2)	
≥1	21 (14.6)	23 (16.0)		31 (21.5)	13 (9.0)	
M stage			0.939			0.861
< 1	50 (34.7)	91 (63.2)		87 (60.4)	54 (37.5)	
≥1	1 (0.7)	2 (1.4)		2 (1.4)	1 (0.7)	

Significance of bold value: p-value less than 0.05.

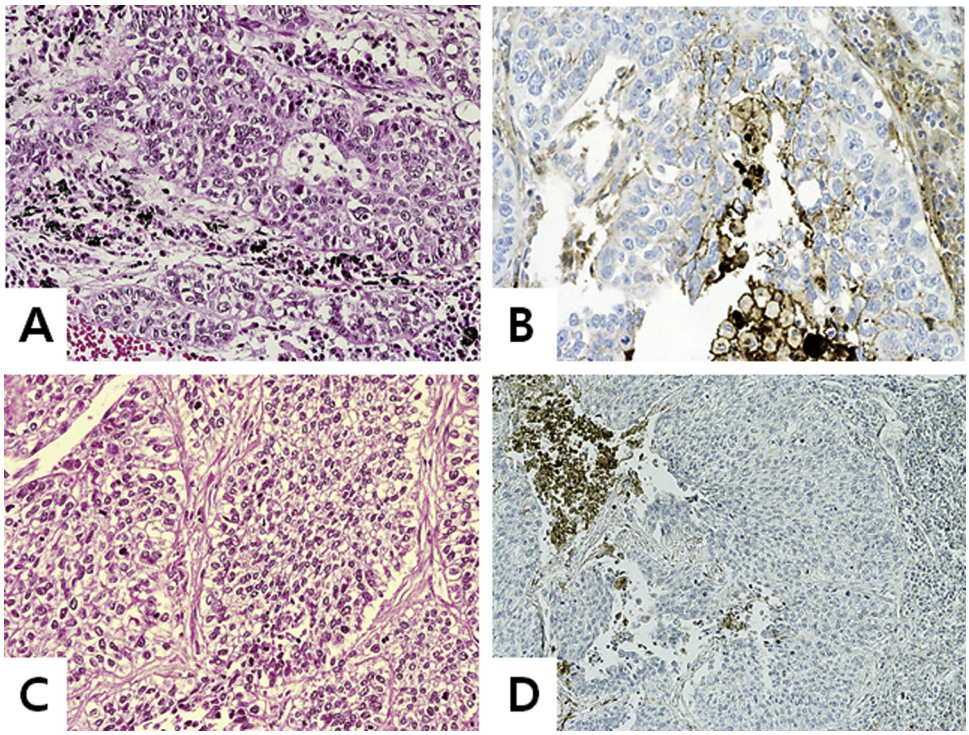

Fig. 1. APOE expression in squamous cell carcinoma. Tumor cells with positive APOE expression (A, B) showed a more pleomorphic and vesicular chromatin pattern with a higher nuclear to cytoplasmic ratio than those with negative APOE expression (C, D).

correlation with the APOE staining intensity (p-value = 0.016) and positive proportion (p-value = 0.001) (Table 2). In cases of ADC, there was a tendency for the intensity rather than the proportion to differ, whereas in SCC, there was a tendency for the proportion rather than the intensity to differ. In addition, patients with positive APOE expression (positive staining intensity in more than 25% of the whole core) showed different histopathologic findings from those with negative APOE expression (a negative intensity with a negative proportion). In SCC, tumor cells with positive APOE staining (Fig. 1A, B) showed more pleomorphic and vesicular chromatin patterns with a high nuclear to cytoplasmicratio than those in APOE-negative samples (Fig. 1C, D). For ADC, tumor cells with positive APOE staining (Fig. 2A, B) demonstrated more hyperchromatic nuclei with a vigorous desmoplastic reaction than those with negative APOE staining (Fig. 2C, D).

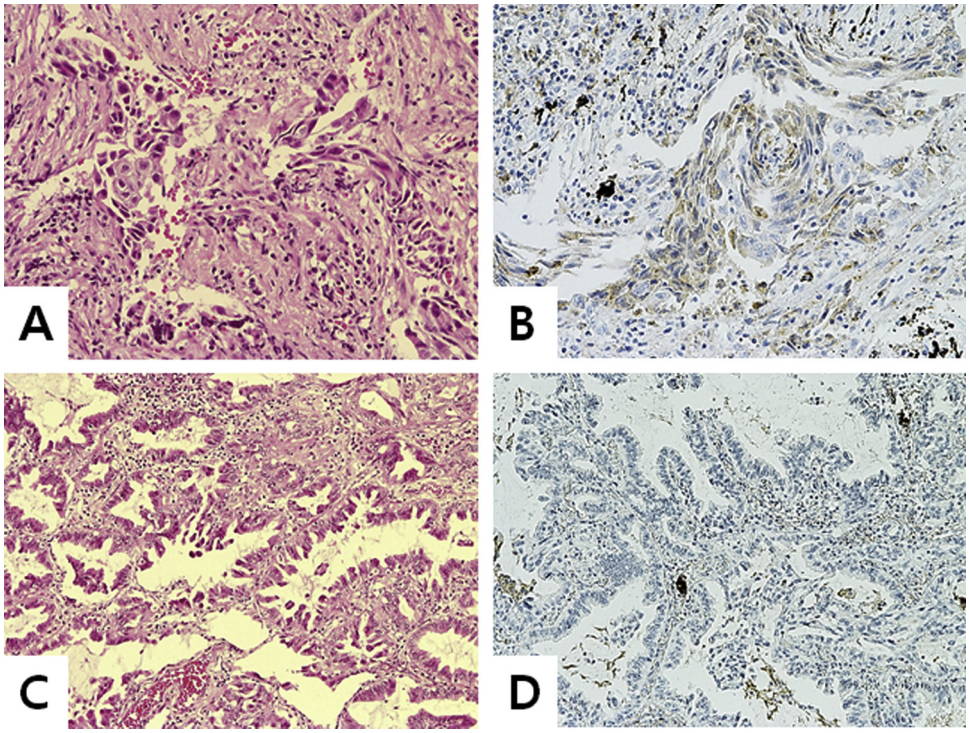

Fig. 2. APOE expression in adenocarcinoma. Tumor cells with positive APOE expression (A, B) demonstrated more hyperchromatic nuclei with a vigorous desmoplastic reaction than those with negative APOE expression (C, D).

3.3. The relationship between APOE expression and lymph node metastasis

N stage showed a statistically significant correlation with negative APOE staining intensity (p-value = 0.040). Of the 148 NSCLC patients, 2 patients had N2 disease (stage IIIA). Both experienced recurrence, and one died. Both patients showed negative APOE staining intensity (p-value = 0.054) (Table 3).

4. Discussion

APOE is fundamental in sustaining lipid and cholesterol homeostasis. APOE modulates lipid transport between liver and peripheral tissues in the systemic circulation through exogenous and endogenous pathways and reverses cholesterol transport [2]. It is primarily synthesized in the liver and is also produced in the brain, spleen, and kidneys and by macrophages and adipocytes. Among APOE from these sources, macrophage-derived APOE plays an important role in reverse cholesterol transport. It modifies the microenvironment by interacting with the extracellular matrix to retain lipoproteins in the vessel wall and to further isolate paracrine growth factors and cytokines [9]. The action of APOE is not limited to lipid metabolism; APOE is also involved in various pathological processes. By influencing mitochondrial dysfunction and endoplasmic reticulum stress, APOE induces inflammatory immune responses [10]. In addition, APOE has been found to have a protective function against infectious microorganisms, such as hepatitis C virus and bacteria [11–13].

The role of APOE in cancer cells is unclear, and limited studies have addressed it. Previous studies revealed that overexpression of APOE is associated with malignant behavior in colorectal cancer [14,15], glioblastoma [16], stomach cancer [17], and anaplastic thyroid cancer [18]. In particular, APOE silencing arrests the cell cycle and increases apoptosis in ovarian cancer cells (OVCAR3) [9]. Through an endogenous pathway, cytosolic APOE maintains cell growth and prevents apoptosis through an autocrine mechanism. Regarding lung cancers, a previous study of small cell lung cancer revealed that APOE expression was not correlated with TNM stage [19]. In this study, we evaluated APOE expression in order to predict tumor progression in NSCLC. To our knowledge, this study is the first such study in NSCLC.

APOE from exogenous sources mediates endocytosis and induces signal transduction by binding to proteins of the LDL receptor family on the cell membrane, whereas endogenous APOE is mainly associated with the Golgi apparatus in the cytosol [14]. In this study, NSCLC cases with positive APOE expression showed cytoplasmic and membranous staining patterns, suggesting that the APOE in these cancer cells probably originated from both exogenous and endogenous sources. Cases with positive APOE expression showed different histopathologic findings from those with negative APOE expression: an increase in pleomorphic and vesicular chromatin patterns with a high NC ratio in SCC and an increase in hyperchromatic nuclei with a vigorous desmoplastic reaction in ADC (Figs. 1 and 2). We assumed that cancer cells with positive APOE expression acquire enhanced cell proliferation and survival through an autocrine mechanism, endocytosis, and signal transduction (Fig. 3A).

Unlike tumor-associated macrophages (TAMs), which are widely

Table 3
The patients' characteristics of N2 disease (Stage IIIA) (n = 2).

sex/age	T stage	N stage	death event	APOE intensity	APOE proportion	DFS (months)	DSS (months)	Recurrence
M/65	2b	2	no	0	0	11	107	yes
M/62	2a	2	yes	0	0	3	4	yes

DFS: disease-free survival, DSS: disease-specific survival.

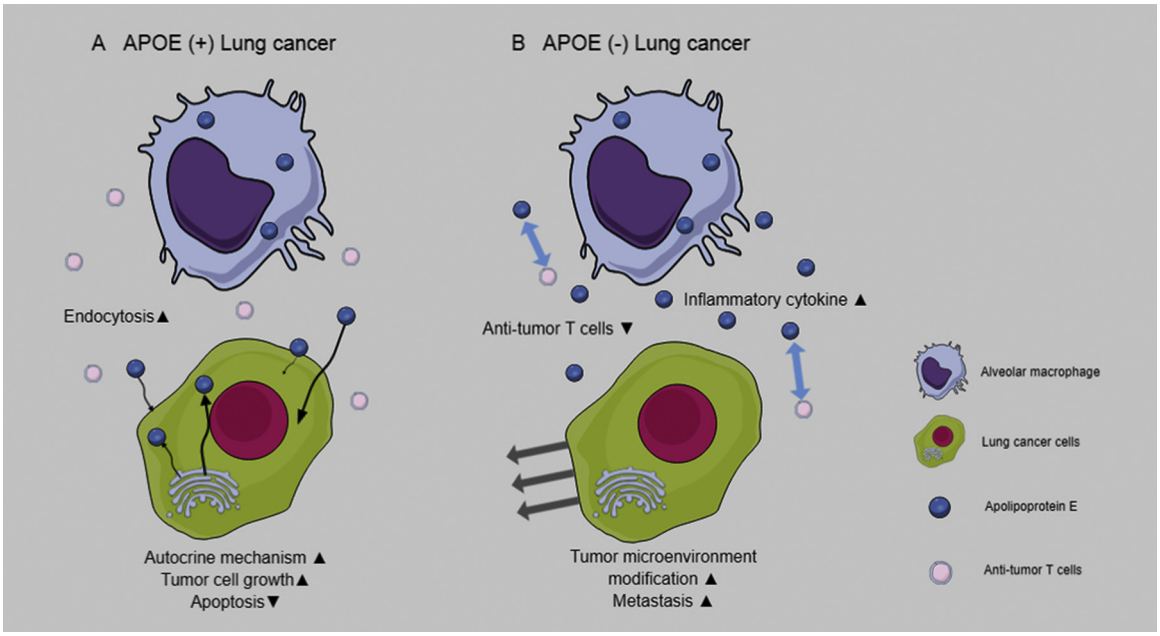

Fig. 3. A schematic of the impact of APOE expression. (A) Cancer cells with positive APOE expression acquire enhanced cell proliferation and survival through an autocrine mechanism, endocytosis, and signal transduction. (B) Macrophage-derived APOE retained in the extracellular matrix participates in the interplay between inflammatory cytokines and antitumor T cells to modify the tumor microenvironment. Cancer cells with negative APOE expression, with neither an endogenous nor exogenous pathway, may acquire metastatic potential through an immunomodulatory environment.

known as suppressors of antitumor immunity, little is known about tissue-resident macrophages, for example, AMs. Sharad et al. suggested that AMs promote the metastasis of breast cancer to the lungs (a tissue-specific niche) by suppressing antitumor T cells [5]. Using a breast cancer mouse model, these researchers proved that because they are regulated by TGF-beta, AMs produce inflammatory cytokines in order to perform an immunosuppressive function in tumor-bearing hosts. Binzhi Qian and Jeffrey W. Pollard reported that macrophages stimulate angiogenesis, cell migration, invasion, and immunosuppression as a tumor progresses to malignancy [6].

Our clinical data of patients with NSCLC showed that lymph node metastasis was significantly associated with negative staining intensity of APOE. Although 21 of the 51 patients with negative APOE staining intensity had lymph node metastasis, those numbers were statistically meaningful (p-value = 0.040). In addition, both patients with N2 disease showed negative APOE staining. We deduced that macrophage-derived APOE (from either TAMs or AMs) possibly interacted with inflammatory factors and anticancer T cells, thus modulating the tumor microenvironment (Fig. 3B). Therefore, we suggest that a negative APOE staining intensity in preoperative small biopsies may suggest treatment options for advanced NSCLC with lymph node metastasis or N2 disease.

The limitation of this study is that we could not prove a statistically significant relationship between APOE expression and survival (disease-free and disease-specific survival). Future *in vitro* and *in vivo* studies focusing on the interactions between macrophage-derived APOE and inflammatory factors are anticipated. Additionally, evaluation of APOE expression in a larger population of patients with advanced NSCLC is needed for better insight.

Since negative APOE staining was significantly associated with lymph node metastasis and N2 disease in NSCLC, we suggest that APOE might be a marker of nodal metastasis and may provide clinicians with versatile options. Therapy targeting the immunological effect of APOE in the regulation of tumor cell metastasis might be helpful. In conclusion, APOE is a useful marker for assessing NSCLC patients with lymph node metastasis.

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Authors' contribution

HJ An: Project development, Data analysis, Manuscript writing.
HM Koh: Data management, Manuscript editing.
DH Song: Manuscript editing, Data analysis, management, and Supervisor.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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References

[1] Gaetano Rocco, Katie Nason, Alex Brunelli, Gonzalo Varela, Thomas Waddell, David R. Jones, Management of stage IIIA (N2) non-small-cell lung cancer: a transatlantic perspective, Eur. J. Cardiothorac. Surg. 49 (2016) 1025–1027.
[2] J. Dose, P. Huebbe, A. Nebel, G. Rimbach, APOE genotype and stress response a mini review, Lipids Health Dis. 15 (2016) 121.
[3] L. Zhuang, J. Kim, R.M. Adam, K.R. Solomon, M.R. Freeman, Cholesterol targeting alters lipid raft composition and cell survival in prostate cancer cells and xenografts, J. Clin. Invest. 115 (4) (2005) 959–968.
[4] Z. Trost, J. Marc, M. Sok, D. Cerne, Increased apolipoprotein E gene expression and protein concentration in lung cancer tissue do not contribute to the clinical assessment of non-small cell lung cancer patients, Arch. Med. Res. 39 (2008) 663–667.
[5] J. Luo, J. Song, P. Feng, Y. Wang, W. Long, M. Liu, et al., Elevated serum apolipoprotein E is associated with metastasis and poor prognosis of non-small cell lung cancer, Tumour Biol. 37 (2016) 10715–10721.
[6] W.P. Su, Y.T. Chen, W.W. Lai, C.C. Lin, J.J. Yan, W.C. Su, Apolipoprotein E expression promotes lung adenocarcinoma proliferation and migration and as a potential survival marker in lung cancer, Lung Cancer 71 (2011) 28–33.
[7] Sharad K. Sharma, Navin K. Chintala, Surya Kumari Vadrevu, Jalpa Patel, Magdalena Karbowniczek, Maciej M. Markiewski, Pulmonary alveolar macrophages contribute to the premetastatic niche by suppressing antitumor t cell responses in

the lungs, J. Immunol. 194 (2015) 5529–5538.
[8] Binzhi Qian, Jeffrey W. Pollard, Macrophage diversity enhances tumor progression and metastasis, Cell 141 (2010) 39–51.
[9] Yu-Chi Chen, Gudrun Pohl, Tian-Li Wang, Patrice J. Morin, Bjo¨rn Risberg, Gunnar B. Kristensen, Apolipoprotein e is required for cell proliferation and survival in ovarian cancer, Cancer Res. 65 (2005) 331–337.
[10] K. Zhang, R.J. Kaufman, From endoplasmic-reticulum stress to the inflammatory response, Nature 454 (2008) 455–462.
[11] H. Fujioka, C.F. Phelix, R.P. Friedland, X. Zhu, E.A. Perry, R.J. Castellani, G. Perry, Apolipoprotein E4 prevents growth of malaria at the intraerythrocyte stage: implications for differences in racial susceptibility to Alzheimer's disease, J. Health Care Poor Underserv. 24 (2003) 70–78.
[12] M.A. Wozniak, R.F. Itzhaki, E.B. Faragher, M.W. James, S.D. Ryder, W.L. Irving, Apolipoprotein E-epsilon 4 protects against severe liver disease caused by hepatitis C virus, Hepatology 36 (2002) 456–463.
[13] M.A. Wozniak, E.B. Faragher, J.A. Todd, K.A. Koram, E.M. Riley, R.F. Itzhaki, Does apolipoprotein E polymorphism influence susceptibility to malaria? J. Med. Genet. 40 (2003) 348–351.
[14] Yun Tian, Jirong Wang, Ying Ye, Liqun Sun, Yingrui Fan, Li Wang, et al., Apolipoprotein e polymorphism and colorectal neoplasm: results from a meta-analysis, PLoS One 9 (2014) e102477.
[15] Zhixun Zhao, Shuangmei Zou, Xu Guan, Meng Wang, Zheng Jiang, Zheng Liu, et al., Apolipoprotein e overexpression is associated with tumor progression and poor survival in colorectal cancer, Front. Genet. 9 (2018) 650.
[16] Mistuni Ghosh, Robert O. Ryan, ApoE enhances nanodisk-mediated curcumin delivery to glioblastoma multiforme cells, Nanomedicine (Lond.) 9 (2014) 763–771.
[17] Ranran Kang, Ping Li, Tingting Wang, Xinxiu Li, Zichen Wei, Zhenlian Zhang, et al., Apolipoprotein E epsilon 2 allele and low serum cholesterol as risk factors for gastric cancer in a Chinese Han population, Sci. Rep. 6 (2016) 19930.
[18] Y. Ito, T. Takano, A. Miyauchi, Apolipoprotein e expression in anaplastic thyroid carcinoma, Oncology 71 (2006) 388–393.
[19] Jian Shi, Huichai Yang, Xiaoyang Duan, Lihua Li, Lulu Sun, Qian Li, et al., Apolipoproteins as differentiating and predictive markers for assessing clinical outcomes in patients with small cell lung cancer, Yonsei Med. J. 57 (2016) 549–556.