Pathology - Research and Practice 215 (2019) 885–892

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Genetic and epigenetic alterations of the *EGFR* and mutually independent association with *BRCA1*, *MGMT*, and *RASSF1A* methylations in Vietnamese lung adenocarcinomas

Quang Ngoc Nguyen[a,d,*,1], Linh Dieu Vuong[a,1], Van-Long Truong[b,1], To Van Ta[a], Nam Trung Nguyen[c,d], Hung Phi Nguyen[a,*], Ha Hoang Chu[c,d,**]

[a] Pathology and Molecular Biology Center, National Cancer Hospital K, 30 Cau Buou Street, Thanh Tri, Hanoi, Viet Nam
[b] Department of Smart Food and Drug, College of BNIT, Inje University, Gimhae, 50834, South Korea
[c] National Key Laboratory of Gene Technology, Institute Vietnam, Academy of Science and Technology, 18 Hoang Quoc Viet Street, Cau Giay, Hanoi, Viet Nam
[d] Biotechnology Department, Graduate University of Science and Technology, Academy of Science and Technology, 18 Hoang Quoc Viet Street, Cau Giay, Hanoi, Viet Nam

ARTICLE INFO

Keywords:
EGFR mutation
BRCA1
MGMT
RASSF1A
Methylation
Lung adenocarcinomas

ABSTRACT

Genetic and epigenetic alterations importantly contribute to the pathogenesis of lung cancer. In the study, we measured the frequency and distribution of molecular abnormalities of *EGFR* as well as the aberrant promoter methylations of *BRCA1*, *MGMT*, *MLH1*, and *RASSF1A* in Vietnamese lung adenocarcinomas. We investigated the association between genetic and epigenetic alteration, and between each abnormality with clinicopathologic parameters. Somatic *EGFR* mutation that was found in 49/139 (35.3%) lung adenocarcinomas showed a significant association with young age, female gender, and non-smokers. EGFR overexpression was identified in 82 tumors (59.0%) and statistical relationships with *EGFR* or *BRCA1* methylation but not *EGFR* mutation. In addition, *EGFR*, *BRCA1*, *MGMT*, *MLH1*, and *RASSF1A* methylations were found in 33 (23.7%), 41 (29.5%), 46 (33.1%), 28 (20.1%), and 41 (29.5%) cases of a total of 139 lung adenocarcinomas, respectively. The *RASSF1A* methylation was found to be linked to the smoking habit. Methylations in *MGMT* and *RASSF1A* were also found to correlate with metastasis status. Furthermore, the distribution of *EGFR* mutation and that of *BRCA1*, *MGMT* or *RASSF1A* methylation were significantly exclusive in lung adenocarcinomas. The main finding of our study demonstrate that epigenetic abnormalities might play a critical role for the lung tumorigenesis in patients with smoking history and metastasis, and partly affect the predictive value of *EGFR* mutations through blocking expression due to promoter *EGFR* hypermethylation. Mutually exclusive distribution of genetic and epigenetic alterations reflects differently biological characteristics in the etiology of lung adenocarcinomas.

1. Introduction

Lung cancer is the leading cause of cancer mortality in many countries among both men and women. It has been divided into two major histological types including small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC) [1]. Lung adenocarcinoma is the most common histologic subtype of NSCLC and predominant in Asians, and never-smokers [2]. It is widely accepted that genetic and epigenetic alterations play important roles in pathogenesis of the lung cancer through the activation of oncogenes and/or the blockage of tumor suppressor genes. Understanding precise molecular modifications would be of great value to have a better prognostic molecular signature to develop individualized therapeutic interventions for each tumor type.

Recently, genetic alterations in the *epidermal growth factor receptor* (*EGFR*) have been known to implicate in lung carcinogenesis and frequently correlate with clinicopathologic characteristics such as adenocarcinoma subtype, female gender, and no smoking history [3,4]. The activating mutations in the tyrosine kinase (TK) domain of *EGFR* are served as a therapeutic target for lung cancer harboring specific

Abbreviations: H&E, hematoxylin and eosin; IHC, immunohistochemistry; MS-PCR, methylation-specific polymerase chain reaction; NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma

* Corresponding authors at: Pathology and Molecular Biology Center, National Cancer Hospital K, 30 Cau Buou Street, Hanoi, Viet Nam.
** Corresponding author at: Biotechnology Institute, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Street, Hanoi, Viet Nam.
E-mail addresses: quangk8s@gmail.com (Q.N. Nguyen), phihung134@gmail.com (H.P. Nguyen), chuhoangha@ibt.ac.vn (H.H. Chu).
[1] These authors are equally contributed to this work.

https://doi.org/10.1016/j.prp.2019.01.032
Received 19 November 2018; Received in revised form 5 January 2019; Accepted 25 January 2019
0344-0338/

mutations by its excellent clinical responsiveness to anticancer drugs (gefitinib and erlotinib) [5]. Treatment regimen based on *EGFR* mutations extends survival rate of patients suffering from NSCLC [6]. Therefore genetic alterations not only provide us important information about biological pathogenesis but also enable to predict the response to chemotherapy. Despite of a numerous of accumulated studies on *EGFR* mutations, the molecular abnormalities in lung adenocarcinoma with wild-type *EGFR* genotype, which is possibly associated with smoking habit and clinicopathologic factors toward poor prognosis have not been completely investigated.

Aberrant promoter methylation, which often downregulates gene expression, has been known as one of the important mechanisms in the pathogenesis of many human cancers. Analysis of DNA methylation in lung cancer is a vital step in order to understand oncogenic pathway, screen for cancer predisposition and primary diagnostics, monitor of disease progression and detect recurrence early, and develop more targeted treatment strategies to improve clinical management [7,8]. Frequent methylated genes such as *deleted in lung and oesophageal cancer 1* (*DLEC1*), *cadherin-13* (*CDH13*), *retinoic acid receptor beta* (*RARβ*), *adenomatous polyposis coli protein* (*APC*), *breast cancer susceptibility gene 1* (*BRCA1*), *O^6-methyltransferase-DNA methylguanin* (*MGMT*), *MutL homolog 1* (*MLH1*), *Ras associated domain-containing protein* (*RASSF1A*), and *checkpoint with forkhead and ring finger domains* (*CHFR*) had been observed in lung cancer [9–11]. These DNA methylation was closely related to smoking status in lung adenocarcinoma [12]. In addition, the promoter methylation of several genes such as *CDH13*, *RASSF1A*, and *APC* has been reported to be associated with high risk of early recurrence [13]. Although both genetic and epigenetic abnormalities frequently occur in lung cancer, the relationship among them remains poorly understood. The missing link between DNA methylation and environmental factors or genetic alterations contributes to limit the applications of methylated signature genes in predicting appropriate therapies. Therefore, analyzing the relationship between genetic and epigenetic alterations provides useful information for understanding biological mechanisms and considerations about adjuvant treatment.

In this study, we analyzed molecular abnormalities of *EGFR* and aberrant promoter methylation of *BRCA1*, *MGMT*, *MLH1*, and *RASSF1A* in 139 lung adenocarcinomas to measure the frequency as well as distribution of each abnormality and the association with clinicopathologic parameters. In addition, the relationship between genetic and epigenetic alteration of *EGFR* and hypermethylation of candidate genes was also investigated.

2. Materials and methods

2.1. Patients and tissue collection

A total of 139 lung adenocarcinoma specimens were collected and histologically diagnosed at Pathology and Molecular Biology Center in National Cancer Hospital K, Vietnam from 2015 to 2017. The study was approved by the guidelines of Hospital K ethics committee in Vietnam. Written informed consent was obtained from all the patients. None of the patients had been undergone chemotherapy and/or radiotherapy prior to biopsy or surgery. Tissue samples were obtained by needle aspiration (89/139) and surgical procedures (50/139). Clinical characteristics of the patients including age, gender, smoking history, histological subtypes, and staging were obtained from surgical and pathological records. All hematoxylin and eosin (H&E) and immunohistochemistry (IHC) stained sections were examined by two independent Hospital K pathologists to define the histological diagnosis of lung adenocarcinoma according to WHO-2015 histologic classification [1].

2.2. DNA isolation and bisulfite modification of genomic DNA

Before DNA extraction, tumors underwent a light microscope-assisted macrodissection quality-control process to enrich tumor cells in specimen. Genomic DNA was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's standard protocol. The quality of specimen DNA was examined by Polymerase Chain Reaction (PCR) targeting housekeeping gene, *β-globin*. Sodium bisulfite modification of genomic DNA was performed using the Epitect Bisulfite Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

2.3. Immunohistochemistry analysis

Immunohistochemical staining for EGFR was carried out using BenchMark XT System (Ventana Medical System, Tucson, Arizona, USA). The pattern of EGFR staining was scored using a 4-tier grading system based on membrane staining intensity as described: 0 = no staining; 1+ = faint membranous staining; 2+ = moderate membranous staining; 3+ = strong membranous staining. Tumors scored 2+ and 3+ were considered as positive for overexpression.

2.4. Methylation analysis

The methylation status of *EGFR*, *BRCA1*, *MGMT*, *MLH1*, and *RASSF1* was examined by methylation-specific PCR (MS-PCR) method. Briefly, sodium bisulfite-treated DNA was used as a template of MS-PCR using oligonucleotide primers to specifically amplify either the methylated or unmethylated promotor sequence of targeting genes. Primer sequences for each gene are provided in the Supplementary Table 1.

2.5. EGFR mutation analysis

EGFR mutations were detected using Therascreen *EGFR* RGQ PCR kit (Qiagen, Valencia, CA, USA) for four exons from exon 18 to 21 of the tyrosine kinase domain of *EGFR*. The therascreen *EGFR* RGQ PCR Kit utilizes two technologies including ARMS (Astrazeneca) and Scorpions (Qiagen) to detect 29 somatic mutations in the *EGFR* cancer-related gene using polymerase chain reaction (PCR) on the Rotor-Gene Q24 instrument.

2.6. Statistical analysis

Statistical analysis was carried out using SPSS software (IBM Corporation, New York, NY, USA). Correlation was analyzed using either the χ^2 test or Fisher's exact test. Statistical significance for all analyses was set at $p < 0.05$.

3. Results

3.1. Patient characteristics

One hundred thirty nine patients with lung adenocarcinomas including 94 males (67.6%) and 45 female (32.4%) were enrolled in this study. The patients ranged in age from 25 to 80 (average, 57.4 years). Seventy-nine patients were smokers composed of 76 males and 3 females, and the remaining 60 patients were non-smokers. Histological diagnosis revealed 79 (56.8%) acinar adenocarcinomas (A), 22 (15.8%) papillary adenocarcinomas (P), 3 (2.2%) micro-papillary adenocarcinomas (MP), 34 (24.5%) solid adenocarcinomas (S), and 1 (0.7%) mixed type of A and P. One hundred and four (74.8%) samples were collected from primary tumors and thirty five (25.2%) samples were obtained from metastatic tumors. Pathologic stages showed 12 (8.6%) patients in stage II, 55 (39.6%) patients in stage III, and 72 (51.8%) patients in stage IV. In this study, we found that smoking status did not exhibit a significant association with other clinicopathologic parameters, except that smoking was more common in male gender (Supplementary Table 2).

3.2. Genetic and epigenetic alterations of the EGFR and the correlation with clinicopathologic parameters in lung adenocarcinomas

EGFR mutations were identified in 49 (35.3%) tumors of 139 lung adenocarcinomas with a total of 55 mutations distributed in exons from 18 to 21. The 55 mutations consisted of 32 missense mutations (including 5 G719X in exon 18, 3 T790 M and 3 S768I in exon 20, and 19 L858R and 2 L861Q in exon 21), 22 in-frame deletion mutations in exon 19, and one insertion mutation in exon 20. Among the 49 tumors with mutations, 81.6% cases were detected either exon 19 in-frame deletion or the exon 21 L858R point, which are the most common mutations in NSCLC and are known to be predictive of response to the EGFR TKIs. *EGFR* mutation was associated with a number of clinicopathologic features such as age ($p = 0.023$), gender ($p < 0.001$) and smoking status ($p = 0.005$). The prevalence of *EGFR* mutations was more frequent in younger patients (44.8%), female gender (57.8%), and non-smokers (50.0%) than in older patients (26.4%), male gender (24.5%), and smokers (25.3%). In addition, the rate of *EGFR* mutations was less common in solid adenocarcinoma subtype (20.6%) than in the other histologic subtypes (40.0%) ($p = 0.04$). However, *EGFR* mutation was not found to correlate with other clinicopathologic characteristics such as metastasis and pathologic stage (Table 1).

Aberrant promoter methylation of *EGFR* was detected in 33 (23.7%) of a total of 139 lung adenocarcinomas. No significant association between *EGFR* methylation and clinicopathologic variables was observed (Table 1).

3.3. EGFR expression and the association with genetic and epigenetic alterations in lung adenocarcinoma

The EGFR expression was detected in 107 out of 139 tumors (77.0%). When adopting a cut-off value of < 10% positive cells (2+ and 3+), 82 tumors (59.0%) was found to exhibit EGFR overexpression. No statistical significance regarding clinicopathologic data such as age, gender, smoking status, histologic subtype, metastasis status, or pathologic stage was observed (Table 1). Notably, the EGFR overexpression exhibited no significant association with *EGFR* mutation status ($p = 0.973$) but statistically correlated with *EGFR* methylation status ($p = 0.002$), where the EGFR was overexpressed in the unmethylated cases of *EGFR* (85.4%), compared to the methylated *EGFR* (14.6%) (Table 2). The interrelationship among the *EGFR* mutation, the *EGFR* methylation, and the EGFR overexpression are shown in Fig. 1, and Table 6. The prevalence of EGFR overexpression significantly decreased in the cases with promoter *EGFR* methylation but not with *EGFR* mutation, either one alteration or both. These results indicate that promoter *EGFR* hypermethylation results in the down-regulation of EGFR expression.

Table 2
Correlation of EGFR expression with *EGFR* alterations and *BRCA1*, *MGMT*, *MLH1*, and *RASSF1A* methylations.

		EGFR overexpression		*P-value*
		Negative (%)	Positive (%)	
N	**139**	**57 (41.0)**	**82 (59.0)**	
EGFR mutation				*0.973*
Mutation	**49**	20 (40.8)	29 (59.2)	
Wild-type	**90**	37 (41.1)	53 (58.9)	
EGFR methylation				*0.002*
Yes	**33**	21 (63.6)	12 (36.4)	
No	**106**	36 (34.0)	70 (66.0)	
BRCA1 methylation				*0.019*
Yes	**41**	23 (56.1)	18 (43.9)	
No	**98**	34 (34.7)	64 65.3)	
MGMT methylation				*0.434*
Yes	**46**	21 (45.7)	25 (54.3)	
No	**93**	36 (38.7)	57 (61.3)	
MLH1 methylation				*0.279*
Yes	**28**	14 (50.0)	14 (50.0)	
No	**111**	43 (38.7)	68 (61.3)	
RASSF1A methylation				*0.408*
Yes	**41**	19 (46.3	22 (53.7)	
No	**98**	38 (38.8)	60 (61.2)	

χ^2 test;

Table 1
Genetic and epigenetic alterations of the *EGFR* gene and the correlation with clinicopathologic parameters.

		EGFR mutation		*P-value*	*EGFR* methylation		*P-value*	EGFR overexpression		*P-value*
		Mutation (%)	Wild-type (%)		Yes (%)	No (%)		Negative (%)	Positive (%)	
N	**139**	**49 (35.3)**	**90 (64.7)**		**33 (23.7)**	**106 (76.3)**		**57 (41.0)**	**82 (59.0)**	
Age (57.4 ± 10.8)				*0.023*			*0.718*			*0.599*
≤57.4	**67**	30 (44.8)	37 (55.2)		15 (22.4)	52 (77.6)		29 (43.3)	38 (56.7)	
< 57.4	**72**	19 (26.4)	53 (73.6)		18 (25.0)	54 (75.0)		28 (38.9)	44 (61.1)	
Gender				*< 0.001*			*0.893*			*0.191*
Male	**94**	23 (24.5)	71 (75.5)		22 (23.4)	72 (76.6)		35 (37.2)	59 (62.8)	
Female	**45**	26 (57.8)	19 (42.2)		11 (24.4)	34 (75.6)		22 (48.9)	23 (51.1)	
Smoking status				*0.005*			*0.616*			*0.627*
Smoker	**79**	20 (25.3)	59 (74.7)		20 (25.3)	59 (74.7)		31 (39.2)	48 (60.8)	
Non-smoker	**60**	29 (48.3)	31 (51.7)		13 (21.6)	47 (78.3)		26 (43.3)	34 (56.7)	
Histological subtypes										
A	**79**	32 (40.5)	47 (59.5)	*0.155*	16 (20.3)	63 (79.7)	*0.267*	27 (34.2)	52 (65.8)	*0.060*
P	**22**	7 (31.8)	15 (68.2)	*0.811*	5 (22.7)	17 (77.3)	*0.903*	9 (40.9)	13 (59.1)	*0.992*
MP	**3**	2 (66.7)	1 (33.3)	*0.284*	2 (66.7)	1 (33.3)	*0.077*	2 (66.7)	1 (33.3)	*0.361*
S	**34**	7 (20.6)	27 (79.4)	*0.042*	10 (29.4)	24 (70.6)	*0.371*	18 (52.9)	16 (47.1)	*0.104*
A&P	**1**	1 (100)	0 (0.0)	*0.353*	0 (0.0)	1 (100.0)	*0.575*	1 (100.0)	0 (0.0)	*0.229*
Tumors				*0.787*			*0.751*			*0.147*
Primary	**104**	36 (34.6)	68 (65.4)		24 (23.1)	80 (76.9)		39 (37.5)	65 (62.5)	
Metastasis	**35**	13 (37.1)	22 (62.9)		9 (25.7)	26 (74.3)		18 (51.4)	17 (48.6)	
Stages				*0.263*			*0.189*			*0.572*
I & II	**12**	6 (50.0)	6 (50.0)		1 (8.3)	11 (91.7)		4 (33.3)	8 (66.7)	
III & IV	**127**	43 (33.9)	84 (66.1)		32 (25.2)	95 (74.8)		53 (41.7)	74 (58.3)	

χ^2 test (excluding Fisher's exact test for histological subtypes); A: Acinar adenocarcinomas; P: Papillary adenocarcinomas; MP: Micro-papillary adenocarcinomas; S: Solid adenocarcinomas; A&P: Mixed type of A and P.

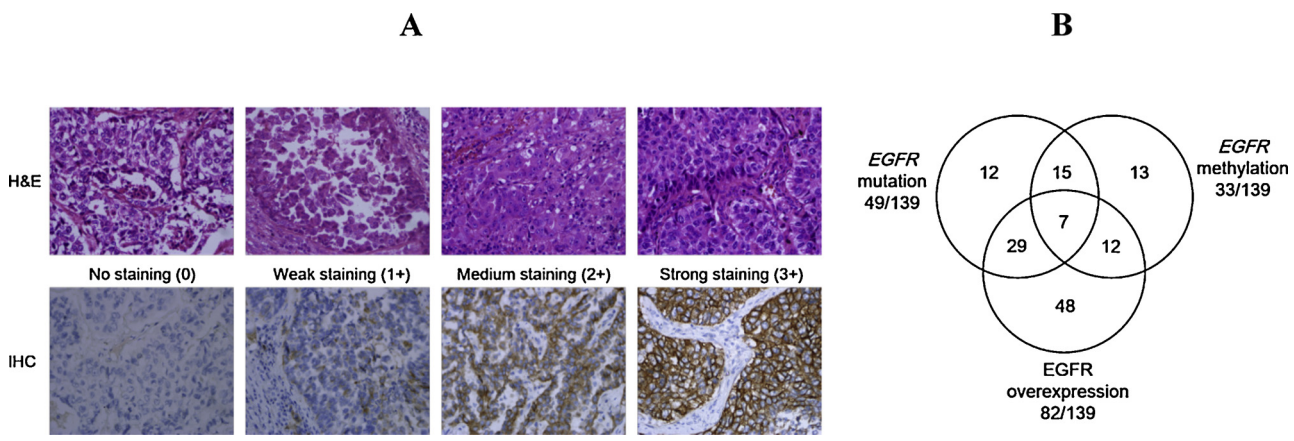

Fig. 1. (A) H&E staining (upper panel) and EGFR IHC staining analysis (lower panel). Photographs were taken at ×200 magnification. Brown color represents positive staining for EGFR. (B) The Venn diagram illustrating relationship among *EGFR* mutations, *EGFR* methylation and EGFR overexpression in Vietnamese patients with lung adenocarcinoma (n = 139).

3.4. Aberrant promoter methylations of BRCA1, MGMT, MLH1, and RASSF1A and the correlation with clinicopathologic parameters in lung adenocarcinoma

As shown in Table 3, aberrant promoter methylations of *BRCA1*, *MGMT*, *MLH1*, and *RASSF1A* were detected in 41 (29.5%), 46 (33.1%), 28 (20.1%), and 41 (29.5%) tumors of a total of 139 lung adenocarcinomas, respectively. Methylation status of at least one of the five genes was identified in 100 of 139 tumors (71.9%); methylation of at least two genes in 54/139 cases (38.8%); three genes in 24/139 (17.3%); four genes in 9/139 (6.7%); and 2/139 cases (1.4%) were methylation of all five genes. A statistically significant association between methylation and smoking status was observed, where the prevalence of *RASSF1A* methylation in smokers (36.7%) was higher than that in non-smokers (20.0%) ($p = 0.032$). Furthermore, aberrant *MGMT* and *RASSF1A* methylations were associated with metastasis status ($p = 0.025$ and 0.045, respectively). No association between the methylation of the five genes and age of diagnosis, gender, histologic subtype, or pathologic stage was found (Table 3). In more detail, we analyzed the relationship between methylation of each set of two out of five genes. The results showed that *RASSF1A* methylation was correlated with *BRCA1* ($p < 0.001$) and *MLH1* ($p < 0.001$) (Table 4).

Table 4
P-values for pairwise correlation of *EGFR*, *BRCA1*, *MGMT*, *MLH1*, and *RASSF1A* methylations.

	EGFR P-value	*MLH1 P-value*	*RASSF1A P-value*	*MGMT P-value*
BRCA1	*0.580*	*0.419*	*< 0.001*	*0.571*
MGMT	*0.378*	*0.093*	*0.175*	
RASSF1A	*0.907*	*< 0.001*		
MLH1	*0.096*			

Table 3
EGFR, *BRCA1*, *MGMT*, *MLH1*, and *RASSF1A* methylations and correlations with clinicopathologic characteristics.

		BRCR1 Methylation		*P- value*	*MGMT* methylation		*P- value*	*MLH1* methylation		*P- value*	*RASSF1A* methylation		***P- value***
		Yes (%)	No (%)		Yes (%)	No (%)		Yes (%)	No (%)		Yes (%)	No (%)	
N	**139**	**41 (29.5)**	**98 (70.5)**		**46 (33.1)**	**93 (66.9)**		**28 (20.1)**	**111 (79.9)**		**41 (29.5)**	**98 (70.5)**	
Age				*0.512*			*0.062*			*0.527*			*0.930*
≤57.4	**67**	18 (26.9	49 (73.1)		17 (25.4)	50 (74.6)		12 (17.9)	55 (82.1)		20 (29.9)	47 (70.1)	
< 57.4	**72**	23 (31.9)	49 (68.1)		29 (40.3)	43 (59.7)		16 (22.2)	56 (77.8)		21 (29.2)	51 (70.8)	
Gender				*0.193*			*0.731*			*0.673*			*0.613*
Male	**94**	31 (33.0)	63 (67.0)		32 (34.0)	62 (66.0)		18 (19.1)	76 (80.9)		29 (30.9)	65 (69.1)	
Female	**45**	10 (22.2)	35 (77.8)		14 (31.1)	31 (68.9)		10 (22.2)	35 (77.8)		12 (26.7)	33 (73.3)	
Smoking status				*0.311*			*0.299*			*0.971*			*0.032*
Smoker	**79**	26 (32.9)	53 (67.1)		29 (36.7)	50 (63.3)		16 (20.3)	63 (79.7)		29 (36.7)	50 (63.3)	
Non-smoker	**60**	15 (25.0)	45 (75.0)		17 (28.3)	43 (71.7)		12 (20.0)	48 (80.0)		12 (20.0)	48 (80.0)	
Histological subtypes													
A	**79**	21 (26.6)	58 (73.4)	*0.387*	26 (32.9)	53 (67.1)	*0.958*	15 (19.0)	64 (81.0)	*0.696*	22 (27.8)	57 (72.2)	*0.625*
P	**22**	7 (31.8)	15 (68.2)	*0.795*	6 (27.3)	16 (72.7)	*0.527*	5 (22.7)	17 (77.3)	*0.742*	7 (31.8)	15 (68.2)	*0.795*
MP	**3**	1 (33.3)	2 (66.7)	*0.883*	2 (66.7)	1 (33.3)	*0.212*	1 (33.3)	2 (66.7)	*0.476*	1 (33.3)	2 (66.7)	*0.883*
S	**34**	11 (33.4)	23 (67.6)	*0.674*	12 (35.3)	22 (64.7)	*0.754*	7 (20.6)	27 (79.4)	*0.941*	11 (32.4)	23 (67.6)	*0.674*
A&P	**1**	1 (100.0)	0 (0.0)	*0.121*	0 (0.0)	1 (100.0)	*0.480*	0 (0.0)	1 (100.0)	*0.614*	0 (0.0)	1 (100.0)	*0.521*
Tumors				*0.772*			*0.024*			*0.341*			*0.045*
Primary	**104**	30 (28.8)	74 (71.2)		29 (27.9)	75 (72.1)		19 (18.3)	85 (81.7)		26 (25.0)	78 (75.0)	
Metastasis	**35**	11 (31.4)	24 (68.6)		17 (48.6)	18 (51.4)		9 (25.7)	26 (74.3)		15 (42.9)	20 (57.1)	
Stages				*0.721*			*0.533*			*0.753*			*0.760*
I & II	**12**	3 (25.0)	9 (75.0)		3 (25.0)	9 (75.0)		2 (16.7)	10 (83.3)		4 (33.3)	8 (66.7)	
III & IV	**127**	38 (29.9	89 (70.1)		43 (33.9)	84 (66.1)		26 (20.5)	101 (79.5)		37 (29.1)	90 (70.9)	

χ^2 test (excluding Fisher's exact test for histological subtypes); A: Acinar adenocarcinomas; P: Papillary adenocarcinomas; MP: Micro-papillary adenocarcinomas; S: Solid adenocarcinomas; A&P: Mixed type of A and P.

Table 5
Correlation of *EGFR* alterations and *EGFR*, *BRCA1*, *MGMT*, *MLH1*, and *RASSF1A* methylations.

		EGFR mutation		*P-value*	*EGFR* methylation		*P-value*
		Mutation (%)	Wild-type (%)		Yes (%)	No (%)	
N	**139**	**49 (35.3)**	**90 (64.7)**		**33 (23.7)**	**106 (76.3)**	
BRCA1 methylation				*0.034*			*0.580*
Yes	**41**	9 (22.0)	32 (78.0)		11 (26.8)	30 (73.2)	
No	**98**	40 (40.8)	58 (59.2)		22 (22.4)	76 (77.6)	
MGMT methylation				*0.049*			*0.378*
Yes	**46**	11 (23.9)	35 (76.1)		13 (28.3)	33 (71.7)	
No	**93**	38 (40.9)	55 (59.1)		20 (21.5)	73 (78.5)	
MLH1 methylation				*0.408*			*0.096*
Yes	**28**	8 (28.6)	20 (71.4)		10 (35.7)	18 (64.3)	
No	**111**	41 (36.9)	70 (63.1)		23 (20.7)	88 (79.3)	
RASSF1A methylation				*0.004*			*0.907*
Yes	**41**	7 (17.1)	34 (82.9)		10 (24.4)	31 (75.6)	
No	**98**	42 (42.9)	56 (57.1)		23 (23.5)	75 (76.5)	

χ^2 test.

3.5. Correlation of EGFR alterations with aberrant promoter methylations of BRCA1, MGMT, MLH1, and RASSF1A genes in lung adenocarcinoma

The methylation frequencies of *BRCA1*, *MGMT*, *MLH1*, and *RASSF1A* were 9 (18.4%), 11 (22.4%), 8 (16.3%), and 7 (14.3%) out of a total 49 cases positive for *EGFR* mutation, respectively. The distribution of *EGFR* mutations and that of *BRCA1*, *MGMT*, or *RASSF1A* methylation were significantly exclusive in lung adenocarcinomas. An inverse association was found between *EGFR* mutation and methylated status of *BRCA1* ($p = 0.034$), *MGMT* ($p = 0.049$), or *RASSF1A* ($p = 0.004$), but not hypermethylation of *MLH1* ($p = 0.408$) (Table 5). It is noteworthy that *EGFR* mutation inversely correlated with the methylation of at least one of the four genes ($p = 0.010$), at least two of four genes ($p = 0.004$), or three genes ($p = 0.006$) (data not shown). On other hand, *EGFR* methylation did not correlate with *BRCA1*, *MGMT*, *MLH1*, or *RASSF1A* methylation (Table 4). However, *BRCA1* methylation was indicated to correlate with EGFR expression ($p = 0.0193$) (Table 2).

4. Discussion

The clinicopathologic and genomic features of lung cancer are extensively variables depending on age, gender, smoking history and ethnicity. Understanding the molecular and cellular biology of lung cancer can provide a great prognostic molecular signature to develop various diagnostic and adjuvant treatment methods. *EGFR* mutations have been attracting a great interest because *EGFR* mutations in the tyrosine kinase domain are considered to be predictive of response to the tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib [14,15], and enable to isolate cluster of patients that are sensitive to drugs targeted *EGFR*. However, few molecular alterations, which were correlated with smoking history as well as clinicopathologic features toward poor prognosis, have been indicated in wild-type *EGFR* of lung adenocarcinomas. In this study, we analyzed 139 lung adenocarcinomas in Vietnamese patients regrading *EGFR* genetic and epigenetic alterations as well as aberrant promoter methylations of *EGFR*, *BRCA1*, *MGMT*, *MLH1*, and *RASSF1A*, and obtained major findings: (a) Genetic or epigenetic alterations were associated with different clinicopathologic backgrounds; *EGFR* mutation was associated with younger patients, non-smokers, female genders while *MGMT* and *RASSF1A* hypermethylations, but not *EGFR* mutation, had an correlation with smokers (case of *RASSF1A*) and metastases; (b) EGFR overexpression was found to be associated with *EGFR* and *BRCA1* promoter methylations but not *EGFR* mutation in lung adenocarcinomas; and (c) Distribution of *BRCA1*, *MGMT*, and *RASSF1A* methylations was significantly exclusive against that of *EGFR* mutations.

Table 6
Association between *EGFR* mutations, *EGFR* methylation and EGFR overexpression in Vietnamese patients with lung adenocarcinoma.

Variables	N	EGFR overexpression		*P-value*
		Negative (%)	Positive (%)	
Group 4				*0.223*
None	**72**	24 (33.3)	48 (66.7)	
Mutation	**49**	20 (40.8)	29 (59.2)	*0.973*
Methylation	**33**	21 (63.6)	12 (36.4)	*0.002*
Both	**15**	8 (53.3)	7 (46.7)	*0.308*
Group 3				*0.319*
None	**72**	24 (33.3)	48 (66.7)	
Mutation or Methylation	**67**	33 (49.3)	34 (50.7)	*0.227*
Both	**15**	8 (53.3)	7 (46.7)	*0.308*

ANOVA and comparison by Duncan's multiple range test.

EGFR is a 170 kDa transmembrane protein with intrinsic tyrosine kinase activity that triggers multiple downstream signaling pathways, including RAS/RAF/mitogen-activated protein kinase (MAPK), Janus-activated kinase/signal transducers and activators of transcription, and phosphatidylinositol 3′-kinase (PI3K)/ Akt pathways. These pathways regulate cell proliferation, survival, migration, metastasis, and angiogenesis [16,17]. In addition, epigenetic modifications of tumor suppressor genes and/or oncogenic genes are thought to be important molecular mechanisms in the initiation and progression of various cancers including lung cancer, and increased knowledge about the aberrant methylation in tumor may contribute to clarifying biological events with potential clinical applications. *BRCA1* plays an important role in a number of cellular processes such as maintaining cell stability, DNA repair, cell-cycle checkpoint control, ubiquitination, and transcriptional regulation of multiple factors such as p53 and c-Myc [18]. Moreover, *BRCA1* has demonstrated to involve in the regulation of angiogenic process in tumors, which is associated with tumor growth and metastasis. *BRCA1* exerts a transcriptional suppression on angioprotein-1 (ANG1) that enhances tumor growth and extensive vasculature, and *BRCA1*-deleted mouse mammary tumors exhibit ANG1 overexpression, prominent vascularization as well as elevated growth [19]. MGMT protein is a DNA repair enzyme, which specifically removes the methyl group and other alkyl groups at the O^6 position of guanine [20], while MLH1 is responsible for replacement of the mismatched nucleotides during DNA replication to maintain genome stability [21]. As a protection against mutagenic DNA adducts, it is possible that loss of *MGMT* is a pre-tumorigenic mechanism and is related to mutations in other genes. *MGMT* silencing by methylation has been demonstrated to link to *p53* mutation in NSCLC and astrocytic cancer,

and *KRAS* mutation in gastric and colorectal cancer [22]. *MLH1*-deficient mice exhibited an increased spontaneous tumor incidence [23]. On other hand, methylation profiling of selected DNA repair genes has potential for a better identification of clusters of patients who are likely to respond to DNA-damage agents [11,24]. The *RASSF1A* is a member of Ras family genes and is associated with vital signaling pathways, namely Ras/PI3K/Akt, Ras/RAF/MEK/ERK, and Hippo pathways [25]. *RASSF1A* signaling cascades produce various cellular responses such as cell proliferation, differentiation, and apoptosis [26]. In addition, *RASSF1A* functions as a cell-cycle checkpoint molecule at the G1-S transition and has been believed to be a potential tumor suppressor [27]. Ectopic expression of RASSF1A by vector transfections into NSCLC cell line resulted in a decrease in anchorage-dependent and anchorage-independent colony formation, and tumor growth in nude mice [28,29]. Silencing of *RASSF1A* by promoter hypermethylation has been demonstrated to involve in pathogenesis of many kinds of cancer such as breast cancer, gastrointestinal cancer, and lung cancer [25]. These evidences support the hypothesis that *BRCA1*, *MGMT*, *MLH1*, and *RASSF1A* are tumor suppressor genes, and alterations in these genes play an important role in lung tumorigenesis. However, it remains unknown whether or not the methylations of these genes affect signaling pathways caused by *EGFR* mutation. In the present study, we found that *EGFR* mutation and aberrant *BRCA1*, *MGMT*, or *RASSF1A* methylation were mutually exclusive, and associated with different clinicopathologic parameters. This means the probability of having *BRCA1*, *MGMT*, or *RASSF1A* methylation was significantly lower in mutated *EGFR* cases than those in wild-type *EGFR* genotype. Furthermore, we found that *EGFR* mutation did not simultaneously occurred with promoter hypermethylation of at least one of these genes, suggesting that genetic and epigenetic alterations are two independent oncogenic pathways in lung adenocarcinomas. The relationship between *EGFR* mutation and *RASSF1A* methylation was similar to that of previous studies [30,31], which demonstrated that *EGFR* mutation and *RASSF1A* hypermethylation were independent events in lung adenocarcinomas. However, this is the first study to report that distribution of *BRCA1* or *MGMT* methylations was mutually exclusive against that for *EGFR* mutation in lung adenocarcinomas. Thus, further study should be performed to investigate the interaction of *EGFR* mutation and *BRCA1* or *MGMT* methylation. Especially, *BRCA1* hypermethylation was an independent event with *EGFR* mutation but was associated with EGFR overexpression in lung adenocarcinomas. Although the precise mechanisms have not indicated, these findings suggest that genetic and epigenetic modifications do not merely occur randomly but interact systematically in cancer progression [32]. Additionally, it has been demonstrated that EGFR overexpression was associated with gefitinib sensitivity and the patients positive for EGFR IHC had much better outcomes than those negative for EGFR IHC [33]. In contrast, NSCLC cells with *EGFR* methylation were more resistant to gefitinib and the suppression of *EGFR* promoter methylation enhanced the antitumor effects of TKIs in NSCLCs [34,35]. This suggests that promoter hypermethylation of *EGFR* reduces the EGFR expression resulting in the decreased target of TKIs and demethylation adjuvant therapy may improve the sensitivity of cancer to EGFR-based targeted therapy.

Through the extensive research into lung cancer, we now know that the prevalence of genetic alterations in *EGFR* gene often exhibits ethnic and environmental differences. Somatic mutations of *EGFR* are more common in Asian patients than in Caucasian patients [36]. In the present study, the occurrence of *EGFR* mutations was found to be approximately identical with that of a previous study, which showed 40.7% of tumor mutation rate in a adenocarcinoma population ($n = 332$) of Vietnamese patient [37]. This rate of *EGFR* mutations (35.3%) was found to be a lower frequency than that in Asian-Pacific countries (overall 47%) [38–40], although higher than that in Western countries (15% in Europe, 22% in North America, and 12% in Oceania) [40–42]. However, the prevalence of *EGFR* mutations in published literatures was different in geographic distribution even within a country, the discrepant results might be due to differences in environmental variables, genetic backgrounds of the populations studied, and methodologies. Therefore, further studies with a large number of cases are needed to confirm the frequency of *EGFR* mutations in Vietnamese lung adenocarcinomas. Likely to *EGFR* mutations, the documented frequency of promoter methylation in lung adenocarcinoma varies between studies, namely *BRCA1* (4–30%), *MGMT* (8–50%), *MLH1* (0–68%) [11], and *RASSF1A* (30–50%) [43]. In addition, it has been demonstrated that the prevalence of *MGMT* methylation is more common in Caucasians than in Asians [44]. In the study, the methylation frequencies of *EGFR*, *BRCA1*, *MGMT*, *MLH1*, and *RASSF1A* genes were 23.7%, 29.5%, 33.1%, 20.1%, and 29.5% of tumors in lung adenocarcinomas, respectively. In comparison to *EGFR* mutation, methylation of these genes had relatively lower prevalences in Vietnamese lung adenocarcinomas. Moreover, our results exhibited a contrast in a clinicopathologic feature between *RASSF1A* methylation and *EGFR* mutation. *RASSF1A* methylation was associated with smoking habit, whereas *EGFR* mutation frequently occurred in non-smokers. It has demonstrated that tobacco smoking importantly contribute to pathogenesis of lung cancer, evidenced that smoking is a cause of approximately 90% and 70% of male and female cases of lung cancer [45]. An association of smoking status and abnormalities in oncogenes and/or tumor suppressor genes, including promoter hypermethylation has been observed in many previous studies [46,47]. These results suggest that smoking may trigger *RASSF1A* promoter hypermethylation in lung adenocarcinomas. These evidences support our findings that *EGFR* mutation and *RASSF1A* methylation target different subsets of lung adenocarcinomas based on smoking status, and *RASSF1A* methylation arising in adenocarcinomas is frequently associated with metastatic status of tumors. *RASSF1A* hypermethylation may be one of the important mechanisms for tumorigenesis but is rarely involved in *EGFR*-related pathway. Genes in the same biological pathway tend to modify in a mutually exclusive manner [48]. This hypothesis has been evidenced by the fact that *KRAS* mutations rarely occur in tumors with *EGFR* mutations [49]. These suggest that *EGFR* mutations may activate PI3K/Akt and Ras/RAF/MAPK pathways, and thus *RASSF1A* methylation may not be required to further potentiate these signaling pathways. However, our results were contradictory to those of several studies, which did not find the relationship between *EGFR* mutation and *RASSF1A* methylation [32,50]. These discrepant results may be due to the differences in size of sample, environmental background, frequencies of genetic and epigenetic abnormalities in lung cancer among ethnic populations studied, and methodologies.

Furthermore, aberrant promoter methylations of *MGMT* and *RASSF1A* significantly correlated with the clinicopathologic factor concerning tumor metastasis in adenocarcinomas of the lung. A recent study reported that *RASSF1A* inactivation enhanced tumorigenic and metastatic capacities in NSCLC by the activation of Yes-associated protein [51]. In addition, *RASSF1A* methylation was found to correlate with poor survival [28,52]. Furthermore, *RASSF1A* methylation was found to simultaneously occur with *BRCA1* and *MLH1* methylations. Our results of *MLH1* and *RASSF1A* methylations were in agreement with previous study which reported that the concordant *MLH1* and *RASSF1A* methylation has been indicated to decrease survival rate of patients [8]. These data imply that epigenetic abnormalities may be closely related to the advanced lung cancer toward poor prognosis, however, their mechanisms are needed to elucidate extensively.

In conclusion, our findings indicate that genetic and epigenetic abnormalities in Vietnamese lung adenocarcinomas have different clinicopathologic features such as metastatic status and smoking habit. In addition, epigenetic modifications of *BRCA1*, *MGMT*, or *RASSF1A* were mutually independent events against *EGFR* mutations. Our data contribute to a better understanding of molecular pathogenesis through genetic and epigenetic interactions in lung adenocarcinomas. However, further studies with a large number of samples are essential to confirm significance of genetic and epigenetic abnormalities, and clarify the role

of molecular alterations in development of lung adenocarcinomas.

Conflict of interest

The authors received no funding for this project and have no conflicts of interest

Acknowledgements

We thank Prof. Jong Park, Department of Cancer Epidemiology, Moffitt Cancer Center, Tampa, United States for comments that greatly improved this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.prp.2019.01.032.

References

[1] W.D. Travis, E. Brambilla, A.G. Nicholson, Y. Yatabe, J.H.M. Austin, M.B. Beasley, L.R. Chirieac, S. Dacic, E. Duhig, D.B. Flieder, K. Geisinger, F.R. Hirsch, Y. Ishikawa, K.M. Kerr, M. Noguchi, G. Pelosi, C.A. Powell, M.S. Tsao, I. Wistuba, The 2015 World Health Organization classification of lung tumors: impact of genetic, clinical and radiologic advances since the 2004 classification, J. Thorac. Oncol. 10 (2015) 1243–1260.

[2] S.Y. Ha, S.J. Choi, J.H. Cho, H.J. Choi, J. Lee, K. Jung, D. Irwin, X. Liu, M.E. Lira, M. Mao, H.K. Kim, Y.S. Choi, Y.M. Shim, W.Y. Park, Y.-L. Choi, J. Kim, Lung cancer in never-smoker Asian females is driven by oncogenic mutations, most often involving EGFR, Oncotarget 6 (2015) 5465–5474.

[3] T. Mitsudomi, T. Kosaka, Y. Yatabe, Biological and clinical implications of EGFR mutations in lung cancer, Int. J. Clin. Oncol. 11 (2006) 190–198.

[4] T. Kosaka, Y. Yatabe, H. Endoh, H. Kuwano, T. Takahashi, T. Mitsudomi, Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications, Cancer Res. 64 (2004) 8919–8923.

[5] M.G. Kris, R.B. Natale, R.S. Herbst, et al., Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non–small cell lung cancer: a randomized trial, JAMA 290 (2003) 2149–2158.

[6] A. Inoue, K. Yoshida, S. Morita, F. Imamura, T. Seto, I. Okamoto, K. Nakagawa, N. Yamamoto, S. Muto, M. Fukuoka, Characteristics and overall survival of EGFR mutation-positive non-small cell lung cancer treated with EGFR tyrosine kinase inhibitors: a retrospective analysis for 1660 Japanese patients, Jpn. J. Clin. Oncol. 46 (2016) 462–467.

[7] M. Pesek, M. Kopeckova, L. Benesova, A. Meszarosova, P. Mukensnabl, F. Bruha, M. Minarik, Clinical significance of hypermethylation status in NSCLC: evaluation of a 30-gene panel in patients with advanced disease, Anticancer Res. 31 (2011) 4647–4652.

[8] T.J. Seng, N. Currey, W.A. Cooper, C.S. Lee, C. Chan, L. Horvath, R.L. Sutherland, C. Kennedy, B. McCaughan, M.R.J. Kohonen-Corish, DLEC1 and MLH1 promoter methylation are associated with poor prognosis in non-small cell lung carcinoma, Br. J. Cancer 99 (2008) 375–382.

[9] Y.J. Kwon, S.J. Lee, J.S. Koh, S.H. Kim, H.W. Lee, M.C. Kang, J.B. Bae, Y.-J. Kim, J.H. Park, Genome-wide analysis of DNA methylation and the gene expression change in lung Cancer, J. Thorac. Oncol. 7 (2012) 20–33.

[10] J.A. Tsou, J.S. Galler, K.D. Siegmund, P.W. Laird, S. Turla, W. Cozen, J.A. Hagen, M.N. Koss, I.A. Laird-Offringa, Identification of a panel of sensitive and specific DNA methylation markers for lung adenocarcinoma, Mol. Cancer 6 (2007) 70.

[11] H. Do, N.C. Wong, C. Murone, T. John, B. Solomon, P.L. Mitchell, A. Dobrovic, A critical re-assessment of DNA repair gene promoter methylation in non-small cell lung carcinoma, Sci. Rep. 4 (2014) 4186.

[12] T. Shinichi, S. Makoto, T. Toshihide, O. T.K, M. Riichiroh, T. Kazunori, F. Yasuro, I. Toshihiko, F. Takehiko, S. Nobuyoshi, M.J. D, G.A. F, Dose effect of smoking on aberrant methylation in non-small cell lung cancers, Int. J. Cancer 110 (2004) 462–464.

[13] M.V. Brock, C.M. Hooker, E. Ota-Machida, Y. Han, M. Guo, S. Ames, S. Glöckner, S. Piantadosi, E. Gabrielson, G. Pridham, K. Pelosky, S.A. Belinsky, S.C. Yang, S.B. Baylin, J.G. Herman, DNA methylation markers and early recurrence in stage I lung cancer, N. Engl. J. Med. 358 (2008) 1118–1128.

[14] J.G. Paez, P.A. Jänne, J.C. Lee, S. Tracy, H. Greulich, S. Gabriel, P. Herman, F.J. Kaye, N. Lindeman, T.J. Boggon, K. Naoki, H. Sasaki, Y. Fujii, M.J. Eck, W.R. Sellers, B.E. Johnson, M. Meyerson, *EGFR* mutations in lung cancer: correlation with clinical response to gefitinib therapy, Science 304 (2004) 1497–1500.

[15] M. Ladanyi, W. Pao, Lung adenocarcinoma: guiding EGFR-targeted therapy and beyond, Mod. Pathol. 21 (2008) 16–22.

[16] H. Shigematsu, L. Lin, T. Takahashi, M. Nomura, M. Suzuki, I.I. Wistuba, K.M. Fong, H. Lee, S. Toyooka, N. Shimizu, T. Fujisawa, Z. Feng, J.A. Roth, J. Herz, J.D. Minna, A.F. Gazdar, Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers, J. Natl. Cancer Inst. 97 (2005) 339–346.

[17] R. Roskoski, The ErbB/HER receptor protein-tyrosine kinases and cancer, Biochem. Biophys. Res. Commun. 319 (2004) 1–11.

[18] N. Reguart, A.F. Cardona, E. Carrasco, P. Gomez, M. Taron, R. Rosell, BRCA1: a new genomic marker for non–small-cell lung cancer, Clin. Lung Cancer 9 (2008) 331–339.

[19] S. Furuta, J.M. Wang, S. Wei, Y.M. Jeng, X. Jiang, B. Gu, P.L. Chen, E.Y.H.P. Lee, W.H. Lee, Removal of BRCA1/CtIP/ZBRK1 repressor complex on ANG1 promoter leads to accelerated mammary tumor growth contributed by prominent vasculature, Cancer Cell 10 (2006) 13–24.

[20] A.E. Pegg, Repair of O6-alkylguanine by alkyltransferases, Mutat. Res. Rev. Mutat. Res. 462 (2000) 83–100.

[21] P. Hsieh, K. Yamane, DNA mismatch repair: molecular mechanism, cancer, and ageing, Mech. Ageing Dev. 129 (2008) 391–407.

[22] S. Sharma, F. Salehi, B.W. Scheithauer, F. Rotondo, L.V. Syro, K. Kovacs, Role of MGMT in tumor development, progression, diagnosis, treatment and prognosis, Anticancer Res. 29 (2009) 3759–3768.

[23] Z. Fu, K. Regan, L. Zhang, M.H. Muders, S.N. Thibodeau, A. French, Y. Wu, S.H. Kaufmann, W.L. Lingle, J. Chen, D.J. Tindall, Deficiencies in Chfr and Mlh1 synergistically enhance tumor susceptibility in mice, J. Clin. Invest. 119 (2009) 2714–2724.

[24] M.R. Middleton, G.P. Margison, Improvement of chemotherapy efficacy by inactivation of a DNA-repair pathway, Lancet Oncol. 4 (2003) 37–44.

[25] A.M. Grawenda, E. O'Neill, Clinical utility of RASSF1A methylation in human malignancies, Br. J. Cancer 113 (2015) 372–382.

[26] N. Yanagawa, G. Tamura, H. Oizumi, N. Kanauchi, M. Endoh, M. Sadahiro, T. Motoyama, Promoter hypermethylation of RASSF1A and RUNX3 genes as an independent prognostic prediction marker in surgically resected non-small cell lung cancers, Lung Cancer 58 (2007) 131–138.

[27] M.S. Song, S.J. Song, N.G. Ayad, J.S. Chang, J.H. Lee, H.K. Hong, H. Lee, N. Choi, J. Kim, H. Kim, J.W. Kim, E.J. Choi, M.W. Kirschner, D.-S. Lim, The tumour suppressor RASSF1A regulates mitosis by inhibiting the APC–Cdc20 complex, Nat. Cell Biol. 6 (2004) 129.

[28] D.G. Burbee, E. Forgacs, S. Zöchbauer-Müller, L. Shivakumar, K. Fong, B. Gao, D. Randle, M. Kondo, A. Virmani, S. Bader, Y. Sekido, F. Latif, S. Milchgrub, S. Toyooka, A.F. Gazdar, M.I. Lerman, E. Zabarovsky, M. White, J.D. Minna, Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression, J. Natl. Cancer Inst. 93 (2001) 691–699.

[29] R. Dammann, C. Li, J.H. Yoon, P.L. Chin, S. Bates, G.P. Pfeifer, Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3, . Nature Genet. 25 (2000) 315–319.

[30] N. Yanagawa, G. Tamura, H. Oizumi, M. Endoh, M. Sadahiro, T. Motoyama, Inverse correlation between EGFR mutation and FHIT, RASSF1A and RUNX3 methylation in lung adenocarcinoma: relation with smoking status, Anticancer Res. 31 (2011) 1211–1214.

[31] M.O. Hoque, M. Brait, E. Rosenbaum, M.L. Poeta, P. Pal, S. Begum, S. Dasgupta, A.L. Carvalho, S.A. Ahrendt, W.H. Westra, D. Sidransky, Genetic and epigenetic analysis of erbB signaling pathway genes in lung cancer, J. Thorac. Oncol. 5 (2010) 1887–1893.

[32] S. Toyooka, M. Tokumo, H. Shigematsu, K. Matsuo, H. Asano, K. Tomii, S. Ichihara, M. Suzuki, M. Aoe, H. Date, A.F. Gazdar, N. Shimizu, Mutational and epigenetic evidence for independent pathways for lung adenocarcinomas arising in smokers and never smokers, Cancer Res. 66 (2006) 1371–1375.

[33] F. Cappuzzo, F.R. Hirsch, E. Rossi, S. Bartolini, G.L. Ceresoli, L. Bemis, J. Haney, S. Witta, K. Danenberg, I. Domenichini, V. Ludovini, E. Magrini, V. Gregorc, C. Doglioni, A. Sidoni, M. Tonato, W.A. Franklin, L. Crino, J.P.A. Bunn, M. Varella-Garcia, Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non–small-cell lung cancer, J. Natl. Cancer Inst. 97 (2005) 643–655.

[34] X.Y. Li, J.Z. Wu, H.X. Cao, R. Ma, J.Q. Wu, Y.J. Zhong, J.F. Feng, Blockade of DNA methylation enhances the therapeutic effect of gefitinib in non-small cell lung cancer cells, Oncol. Rep. 29 (2013) 1975–1982.

[35] Z.Y. Pan, Z.S. Jiang, H.Q. Ouyang, Study of the methylation patterns of the EGFR gene promoter in non-small cell lung cancer, Genet. Mol. Res. 14 (2015) 9813–9820.

[36] S. Hisayuki, F. G.A, Somatic mutations of epidermal growth factor receptor signaling pathway in lung cancers, Int. J. Cancer 118 (2006) 257–262.

[37] V.H. Anh, X.P. Thi, H.H.T. Ngoc, H.N.T. Tuyet, B.N. Duc, T.D.T. Phuong, D.N. Quoc, T.N. Sao, Spectrum of EGFR gene mutations in Vietnamese patients with non–small cell lung cancer, Asia Pac. J. Clin. Oncol. 12 (2016) 86–90.

[38] K. Sugio, H. Uramoto, K. Ono, T. Oyama, T. Hanagiri, M. Sugaya, Y. Ichiki, T. So, S. Nakata, M. Morita, K. Yasumoto, Mutations within the tyrosine kinase domain of EGFR gene specifically occur in lung adenocarcinoma patients with a low exposure of tobacco smoking, Br. J. Cancer 94 (2006) 896–903.

[39] C.H. Tseng, C.-J. Chiang, J.S. Tseng, T.Y. Yang, K.H. Hsu, K.C. Chen, C.L. Wang, C.Y. Chen, S.H. Yen, C.M. Tsai, M.S. Huang, C.C. Ho, C.J. Yu, Y.H. Tsai, J.S. Chen, T.Y. Chou, M.H. Tsai, H.Y. Chen, K.Y. Su, J.J.W. Chen, H.W. Chen, S.L. Yu, T.W. Liu, G.C. Chang, EGFR mutation, smoking, and gender in advanced lung adenocarcinoma, Oncotarget 8 (2017) 98384–98393.

[40] J.Y. Park, S.H. Jang, Epidemiology of lung Cancer in Korea: recent trends, Tuberc. Respir. Dis. (Seoul) 79 (2016) 58–69.

[41] A. Midha, S. Dearden, R. McCormack, EGFR mutation incidence in non-small-cell lung cancer of adenocarcinoma histology: a systematic review and global map by ethnicity (mutMapII), Am. J. Cancer Res. 5 (2015) 2892–2911.

[42] S. Dearden, J. Stevens, Y.L. Wu, D. Blowers, Mutation incidence and coincidence in non small-cell lung cancer: meta-analyses by ethnicity and histology (mutMap), Ann. Oncol. 24 (2013) 2371–2376.

[43] Y. Liu, W. Gao, J.M. Siegfried, J.L. Weissfeld, J.D. Luketich, P. Keohavong, Promoter methylation of RASSF1A and DAPK and mutations of K-ras, p53, and

EGFR in lung tumors from smokers and never-smokers, BMC Cancer 7 (2007) 074.
[44] T. Shinichi, M. Riichiroh, T.K. O, M. Dale, F. Ziding, F. Yasuro, V.A. K, Z.-M. Sabine, T. Kazunori, S. Kenji, S. Nobuyoshi, S. Kenji, L. Huei, C. Chih-Yi, F.K. M, G. Michael, R.J. A., M.J. D, F. G.A, Smoke exposure, histologic type and geography-related differences in the methylation profiles of non-small cell lung cancer, Int. J. Cancer 103 (2003) 153–160.
[45] D.R. Shopland, Tobacco use and its contribution to early cancer mortality with a special emphasis on cigarette smoking, Environ. Health Perspect. 103 (1995) 131–142.
[46] J. Gu, Y. Wen, S. Zhu, F. Hua, H. Zhao, H. Xu, J. You, L. Sun, W. Wang, J. Chen, Q. Zhou, Association between P16INK4a promoter methylation and non-small cell lung cancer: a meta-analysis, PLoS One 8 (2013) e60107.
[47] Y. Ma, M.D. Li, Establishment of a strong link between smoking and cancer pathogenesis through DNA methylation analysis, Sci. Rep. 7 (2017) 1811.
[48] K. Takaomi, T. Masafumi, Y. Tokujiro, M. Yoshihiko, S. Katsuo, CHFR hypermethylation and EGFR mutation are mutually exclusive and exhibit contrastive clinical backgrounds and outcomes in non-small cell lung cancer, Int. J. Cancer 128 (2011) 1009–1017.
[49] S.M. Lee, J.E. Choi, Y.K. Na, E.J. Lee, W.K. Lee, Y.Y. Choi, G.S. Yoon, H.-S. Jeon, D.S. Kim, J.Y. Park, Genetic and epigenetic alterations of the LKB1 gene and their associations with mutations in TP53 and EGFR pathway genes in Korean non-small cell lung cancers, Lung Cancer 81 (2013) 194–199.
[50] Y. Liu, W. Gao, J.M. Siegfried, J.L. Weissfeld, J.D. Luketich, P. Keohavong, Promoter methylation of RASSF1A and DAPK and mutations of K-ras, p53, and EGFR in lung tumors from smokers and never-smokers, BMC Cancer 7 (2007) 74.
[51] F. Dubois, M. Keller, O. Calvayrac, F. Soncin, L. Hoa, A. Hergovich, M.-C. Parrini, J. Mazières, M. Vaisse-Lesteven, J. Camonis, G. Levallet, G. Zalcman, RASSF1A suppresses the invasion and metastatic potential of human non–small cell lung cancer cells by inhibiting YAP activation through the GEF-H1/RhoB pathway, Cancer Res. 76 (2016) 1627–1640.
[52] Y. Tomizawa, T. Kohno, H. Kondo, A. Otsuka, M. Nishioka, T. Niki, T. Yamada, A. Maeshima, K. Yoshimura, R. Saito, J.D. Minna, J. Yokota, Clinicopathological significance of epigenetic inactivation of *RASSF1A* at 3p21.3 in stage I lung adenocarcinoma, Clin. Cancer Res. 8 (2002) 2362–2368.