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Research Paper

Association of *EGFR* mutations and *HMGB1* genetic polymorphisms in lung adenocarcinoma patients

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Abstract

High-mobility group protein box 1 (HMGB1) is overexpressed and reported to be a prognostic factor in patients with non-small-cell lung cancer (NSCLC). Epidermal growth factor receptor (EGFR) mutants play an important role in NSCLC progression. The aim of this study was to explore potential associations between genetic polymorphisms of *HMGB1* and *EGFR* mutations in a cohort that included 280 patients with NSCLC, some of whom were smokers and others who never smoked. Four tagged single-nucleotide polymorphisms (SNPs) of *HMGB1* were detected by a TaqMan-based real-time polymerase chain reaction (PCR) in patients. We found that after adjusting for other covariates, NSCLC patients who smoked and who respectively had CG, CT, and TC heterozygotes of *HMGB1* rs2249825, rs1045411, and rs1360485, were at lower risk of developing mutant *EGFR*, compared to those patients with wild-type homozygotes. Moreover, significant inverse associations between the CG and CG + GG genotypes of *HMGB1* rs2249825 and the *EGFR* hotspot mutation, an exon 19 in-frame deletion, were also observed among NSCLC patients. Within patients harboring mutant *EGFR*, *HMGB1* rs1360485 C (TC + CC) allele carriers were at higher risk of developing poorly differentiated cancer types (odds ratio=5.493, 95% confidence interval: 1.130~26.696, p=0.019), compared to patients with TT homozygotes. Furthermore, we found that *HMGB1* rs1360485 polymorphisms seemed to be related to susceptibility to developing poorly differentiated cancer linked to tobacco consumption in *EGFR* mutant patients. In conclusion, our results suggested that *HMGB1* variants are significantly inversely associated with *EGFR* mutations among NSCLC patients who smoked. *HMGB1* variants and tobacco consumption might contribute to the pathological development of NSCLC.

Key words: High-mobility group protein box 1, Polymorphism, Susceptibility, Epidermal growth factor receptor, Non-small-cell lung cancer

Introduction

Lung cancer is one of the most prevalent cancers globally and is by far the leading cause of death among males and females worldwide [1]. Small-cell lung cancer (SCLC) and non-SCLC (NSCLC) are two

principal groups of lung cancer; the latter type comprises of 85%~90% of lung cancer diagnoses. NSCLC mainly includes the histologic types of adenocarcinoma (ADC) and squamous cell carcinoma (SCC) [2]. During the last decade, there have been major breakthroughs in many aspects of diagnosing and treating NSCLC, including surgery, chemotherapy, radiation therapy, and targeted drug therapy. However, understanding of the mechanisms underlying NSCLC development and progression remains limited, and effective indicators of an early diagnosis of NSCLC are still lacking. Among the many risk factors known to be associated with a higher lung cancer risk, tobacco smoking appears to have the strongest association [3]. Only about 10%~15% of lung cancer cases occur in people who have never smoked, which indicates that other factors such as genetic polymorphisms may play a role in determining disease risk and prognosis in lung cancer [4]. About 8% of patients have lung cancer solely due to inherited factors, and the contribution of hereditary factors in determining disease risk is also supported by statistics indicating a 2.4-fold increased lung cancer risk in people who are direct relatives of lung cancer patients [5].

The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor encoded by a gene located on the short arm of chromosome 7. Autophosphorylation of EGFR tyrosine kinase domains leads to activation of downstream proteins that mediate cell proliferation and cell survival [6] and was reported to play an important role in tumorigenesis and progression of lung adenocarcinomas [7]. The *EGFR* gene was reported to harbor mutations and/or polymorphisms that increase susceptibility to lung cancer [8]. Most of these mutations/polymorphisms exist within the catalytic kinase domain, which increases EGFR activity. The most common somatic hotspot mutations in the tyrosine kinase domain of the *EGFR* gene are the in-frame deletion in exon 19 and a substitution mutation of lysine for arginine at amino acid position 858 (L858R) in exon 21. Mutations of L858R and the in-frame deletion in exon 19 were found to be more frequent in adenocarcinoma than other NSCLC and were suggested to promote cell viability [7]. Among adenocarcinoma patients, these *EGFR* mutations are more common in Asian patients, female patients, and patients who have never smoked [9]. Recently, genetic polymorphisms associated with susceptibility to various somatic mutations were described, including *EGFR* mutations [10-12], implying that *EGFR* mutations are linked to an individual's genetic background. In addition, genomic DNA is continuously being damaged by mutagens from external agents, such as tobacco smoke in the environment [13]. In the human body, carcinogen-associated DNA damage must be repaired to maintain correct genetic information. If damaged DNA cannot be repaired, DNA lesions will form, such as *EGFR* mutations.

High-mobility group box 1 (HMGB1) is a highly conserved nuclear protein widely expressed in mammalian cells, and it plays a critical role in transcriptional regulation and chromatin construction. Loss of HMGB1 can increase DNA damage caused by potent carcinogens in cigarette smoke (such as benzo[a]pyrene diol epoxide) [14] and chemotherapeutic drugs (such as cisplatin) [15]. The binding of HMGB1 to DNA lesions can facilitate DNA repair, the response to DNA damage, and damage-induced chromatin remodeling; thus, it might prevent a carcinogenic or mutagenic outcome after exposure to DNA-damaging agents [16]. In addition to the nuclear function of HMGB1, HMGB1 was reported to be released into the extracellular matrix, where it exerts crucial functions in immunity, inflammation, and carcinogenesis through its diverse receptors such as the receptor for advanced glycation end-products (RAGE), the toll-like receptor (TLR) 2, and TLR 4 [17]. HMGB1 plays a pivotal role in the diagnosis and prognosis of NSCLC, especially with adenocarcinomas [18]. HMGB1 was upregulated in the serum of progressive NSCLC patients and associated with shorter overall survival and disease-free survival times. Overexpression of HMGB1 correlates with the proliferation, metastasis, and chemotherapy resistance of lung adenocarcinomas. HMGB1 may serve as an important risk factor for the development of lung cancer [19].

Investigation of polymorphisms of *HMGB1* genes may contribute to our understanding of how cigarette smoke-derived carcinogen metabolism and DNA repair mechanisms affect the development of *EGFR* mutations in NSCLC. In this study, four polymorphisms of *HMGB1* genes, including rs1412125, rs2249825, rs1045411, and rs1360485, were examined to study their associations with susceptibility to *EGFR* mutations in patients with lung adenocarcinoma.

Material and Methods

Patient characteristics and consent

Between 2012 and 2015, 280 lung adenocarcinoma patients with wild-type EGFR (67 men and 44 women; mean age = 65.36 ± 13.42 years) or a mutant EGFR (60 men and 109 women; mean age = 65.76 ± 13.57 years) were consecutively recruited from Taichung Cheng-Ching General Hospital

(Taichung, Taiwan). Written ethical consent was obtained from all patients. The study was approved by the Institutional Review Board of Cheng-Ching General Hospital. Data obtained from medical records of each patient included demographics (age and sex), lifestyle variables (tobacco smoking status), and tumor stage and differentiation. Clinical information of patients was staged at the time of diagnosis following the tumor/node/metastasis staging system of the American Joint Committee on Cancer (AJCC).

Selection of single nucleotide polymorphisms (SNPs) of the *HMGB1* gene

We genotyped four *HMGB1* SNPs including rs1412125 (−1615T/C; promoter region), rs2249825 (3814C/G; intron 1), rs1360485 (T/C; 3′ untranslated region (3′ UTR)) and rs1045411 (2262C/T; 3′ UTR) selected according to Chinese HapMap data. Minor allelic frequencies of these SNPs were all >5%. These four polymorphisms are well defined and have been widely evaluated the association with a broad range of cancers such as oral squamous cell carcinoma, hepatocellular carcinoma, cervical cancer, colorectal cancer, breast cancer and lung cancer, especially in Han Chinese population [20]. In addition, the minor allele frequencies of these SNPs were all ≥5 %.

Genomic DNA extraction from blood and *HMGB1* genotyping

Genomic DNA was extracted from preserved whole blood in EDTA anti-coagulant tubes using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol as previously described [21-23]. The TaqMan SNP Genotyping Assay with an ABI StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to analyze allelic discrimination of *HMGB1* SNPs. Allelic frequencies were determined using ABI SDS vers. 3.0 software.

Genomic DNA extraction from tumor tissues and *EGFR* gene sequencing

To acquire genomic DNA from tumor-frozen specimens and paraffin-embedded tissues, a QIAamp DNA Tissue kit (Qiagen) was used to extract genomic DNA according to the manufacturer's protocol. DNA sequencing of the *EGFR* gene in exon 18-21 was amplified by a polymerase chain reaction (PCR), and finally subjected to a DNA sequencing reaction as described previously [9].

Statistical analysis

The distributions of genotype frequencies and demographic characteristics were compared between EGFR wild-type (WT) and mutation type in lung adenocarcinoma patients by using Mann–Whitney *U* test. Odds ratio (ORs) and their 95% confidence intervals (CIs) were assessed using logistic regression models. $p < 0.05$ indicated statistically significant differences. Data were analyzed using SAS (version 9.1, 2005; SAS Institute Inc., Cary, NC).

Results

Demographic and clinical characteristics of patients with lung adenocarcinoma

Our study recruited 280 lung adenocarcinoma patients who were further divided into *EGFR* wild-type (WT) (n = 111; 39.6%) and mutation type (n = 169; 60.4%) groups. With regard to the *EGFR* mutation status, 79 subjects had L858R mutations, 81 had exon19 in-frame deletions, and nine had other mutations. The demographic, lifestyle, and clinical characteristics of both recruited groups with the WT and mutant *EGFR* are shown in Table 1. Between these two groups, there was no significant difference in the distribution of age, clinical stage, or TNM stage. Compared to the group with the WT *EGFR*, subjects with the mutant *EGFR* were significantly observed in females (64.5% vs. 39.6%) and those who had never smoked (77.5% vs. 45.0%), and those who had more well-differentiated (12.4% vs. 7.2%) and moderately differentiated tumors (81.7% vs. 72.1%).

Table 1. Demographics and clinical characteristics of 280 patients with lung adenocarcinoma and their epidermal growth factor mutation status

Variable	Wild type (N=111) n (%)	EGFR mutation (N=169) n (%)	*p* value
Age			
Mean ± SD	65.36 ± 13.42	65.76 ± 13.57	p=0.810
Gender			
Male	67 (60.4%)	60 (35.5%)	p<0.001
Female	44 (39.6%)	109 (64.5%)	
Cigarette smoking status			
Never-smoker	50 (45.0%)	131 (77.5%)	p<0.001
Ever-smoker	61 (55.0%)	38 (22.5%)	
Stage			
I+II	26 (23.4%)	47 (27.8%)	p=0.413
III+IV	85 (76.6%)	122 (72.2%)	
Tumor T status			
T1+T2	60 (54.1%)	108 (63.9%)	p=0.100
T3+T4	51 (45.9%)	61 (36.1%)	
Lymph node status			
Negative	29 (26.1%)	54 (32.0%)	p=0.296
Positive	82 (73.9%)	115 (68.0%)	
Distant metastasis			
Negative	54 (48.6%)	80 (47.3%)	p=0.830
Positive	57 (51.4%)	89 (52.7%)	
Cell differentiation			
Well	8 (7.2%)	21 (12.4%)	p<0.001
Moderately	80 (72.1%)	138 (81.7%)	
Poorly	23 (20.7%)	10 (5.9%)	

EGFR, epidermal growth factor receptor.

Table 2. Distribution frequency of high-mobility group box 1 (*HMGB1*) genotypes of patients with lung adenocarcinoma and multiple logistic regression analysis of epidermal growth factor receptor mutations associated with the cigarette smoking status.

	All cases (*N*=280)			Non-smokers (*N*=181)			Smokers (*N*=99)		
SNP genotype	**Wild type (*N*=111)**	**Mutation type (*N*=169)**	**AOR (95% CI)**	**Wild type (*N*=50)**	**Mutation type (*N*=131)**	**AOR (95% CI)**	**Wild type (*N*=61)**	**Mutation type (*N*=38)**	**AOR (95% CI)**
rs1412125									
TT	59 (53.2%)	89 (52.7%)	1.00	28 (56.0%)	67 (51.1%)	1.00	31 (50.8%)	22 (57.9%)	1.00
TC	43 (38.7%)	66 (39.1%)	0.82 (0.44~1.54)	20 (40.0%)	53 (40.5%)	0.76 (0.29~1.95)	23 (37.7%)	13 (34.2%)	0.82 (0.21~3.18)
CC	9 (8.1%)	14 (8.2%)	0.95 (0.27~3.32)	2 (4.0%)	11 (8.4%)	3.55 (0.28~45.12)	7 (11.5%)	3 (7.9%)	0.23 (0.02~3.00)
TC+CC	52 (46.8%)	80 (47.3%)	0.84 (0.46~1.54)	22 (44.0%)	64 (48.9%)	0.89 (0.35~2.22)	30 (49.2%)	16 (42.1%)	0.64 (0.18~2.29)
rs2249825									
CC	74 (66.7%)	127 (75.1%)	1.00	39 (78.0%)	95 (72.5%)	1.00	35 (57.4%)	32 (84.2%)	1.00
CG	34 (30.6%)	37 (21.9%)	0.55 (0.27~1.10)	10 (20.0%)	33 (25.2%)	1.19 (0.41~3.50)	24 (39.3%)	4 (10.5%)	**0.07 (0.01~0.74)***
GG	3 (2.7%)	5 (3.0%)	2.06 (0.36~11.87)	1 (2.0%)	3 (2.3%)	1.52 (0.09~27.00)	2 (3.3%)	2 (5.3%)	2.21 (0.09~57.15)
CG+GG	37 (33.3%)	42 (24.9%)	0.63 (0.32~1.23)	11 (22.0%)	36 (27.5%)	1.22 (0.43~3.45)	26 (42.6%)	6 (15.8%)	0.16 (0.03~1.02)
rs1045411									
CC	62 (55.9%)	103 (60.9%)	1.00	33 (66.0%)	76 (58.0%)	1.00	29 (47.5%)	27 (71.1%)	1.00
CT	41 (36.9%)	56 (33.1%)	0.80 (0.42~1.52)	14 (28.0%)	48 (36.6%)	2.23 (0.84~5.92)	27 (44.3%)	8 (21.1%)	**0.12 (0.02~0.69)***
TT	8 (7.2%)	10 (6.0%)	1.12 (0.33~3.81)	3 (6.0%)	7 (5.4%)	0.76 (0.13~4.48)	5 (8.2%)	3 (7.9%)	0.71 (0.06-8.89)
CT+TT	49 (44.1%)	66 (39.1%)	0.84 (0.46~1.55)	17 (34.0%)	55 (42.0%)	1.83 (0.75~4.47)	32 (52.5%)	11 (28.9%)	**0.38 (0.15~0.96)***
rs1360485									
TT	60 (54.1%)	94 (55.6%)	1.00	32 (64.0%)	69 (52.7%)	1.00	28 (45.9%)	25 (65.8%)	1.00
TC	40 (36.0%)	64 (37.9%)	1.05 (0.55~1.97)	15 (30.0%)	55 (42.0%)	**3.07 (1.12~8.38*)**	25 (41.0%)	9 (23.7%)	**0.12 (0.02~0.72)***
CC	11 (9.9%)	11 (6.5%)	0.96 (0.29~3.16)	3 (6.0%)	7 (5.3%)	0.89 (0.15~5.34)	8 (13.1%)	4 (10.5%)	0.51 (0.05~5.11)
TC+CC	51 (45.9%)	75 (44.4%)	1.03 (0.56~1.89)	18 (36.0%)	62 (47.3%)	2.47 (0.98~6.20)	33 (54.1%)	13 (34.2%)	**0.18 (0.04~0.86)***

The adjusted odds ratios (AORs) with 95% confidence intervals (CIs) were estimated by multiple logistic regression models after controlling for age and gender.
Note: Bold text indicates a significant association with p value of <0.05.
Abbreviations: SNP, single-nucleotide polymorphism.

Associations between *HMGB1* genotypes and *EGFR* mutations in adenocarcinoma patients who did or did not smoke

Regardless of sex, cigarette smoking is a major risk factor for lung cancer, and cigarette smoking was reported to affect the *EGFR* status in lung adenocarcinoma patients [24]. In this study, we divided our recruited lung adenocarcinoma patients into smoking and non-smoking groups and further investigated the difference between *HMGB1* polymorphisms and *EGFR* status in these two groups. Distributions of *HMGB1* genetic polymorphisms in the promoter region (rs1412125, −1615T/C), intron 1 (rs2249825, 3814C/G), and the 3′ UTR (rs1045411, 2262C/T and rs1360485, T/C) in patients with the WT or mutant *EGFR* are shown in Table 2. Alleles with the highest distribution frequency of rs1412125, rs2249825, rs1045411, and rs1360485 in our recruited adenocarcinoma patients with the WT or mutant *EGFR* were homozygous for TT, CC, CC, and TT, respectively. For rs1412125 polymorphisms, there was no significant association demonstrated between variant types (TC, CC, and TC+CC) and the *EGFR* mutation type. In the smoking group, subjects with the *HMGB1* polymorphic rs2249825 CG, rs1045411 CT, and rs1360485 TC genotypes exhibited significantly (p<0.05) lower frequencies of 0.07- (95% CI: 0.01~0.74), 0.12- (95% CI: 0.02~0.69), and 0.12-fold (95% CI: 0.02~0.72) respectively, of having an *EGFR* mutation compared to their corresponding WT homozygotes (Table 2). In the non-smoking and overall cohorts we recruited, we observed that there was no significant difference in having an *EGFR* mutation in individuals with rs2249825 and rs1045411 polymorphisms of the *HMGB1* gene compared to WT individuals (Table 2).

Associations between *HMGB1* SNPs and *EGFR* hotspot mutations among lung adenocarcinoma patients

Two most common *EGFR* hotspot mutations, L858R and an in-frame deletion, were also observed in most (95%) of our recruited patients with EGFR mutations. We further assessed associations of the L858R mutation or in-frame deletion of the *EGFR* gene with *HMGB1* genotypes. The CG and CG+GG genotypes of *HMGB1* rs2249825 were significantly associated with a decreased proportion of in-frame deletions (adjusted odds ratio (AOR)=0.473, 95% CI: 0.228~0.979, p=0.044; AOR=0.463, 95% CI: 0.227~0.941, p=0.033), but not with the L858R mutation (Table 3). In contrast, no significant association was observed of *HMGB1* rs1412125, rs1045411, or rs1360485 polymorphisms with either hotspot mutations of the *EGFR* (Table 3).

Correlations between polymorphic genotypes of *HMGB1* and the clinical status of lung adenocarcinoma patients with *EGFR* mutations

Impacts of the variant *HMGB1* genotypes on the clinicopathological development of NSCLC harboring *EGFR* mutations, such as clinical cancer stage, primary tumor size, lymph node, distant metastasis,

and degree of tumor cell differentiation, were further evaluated, and results are shown in Table 4. We classified patients into two subgroups of patients who had the homozygous WT alleles or who had at least one polymorphic allele. Among 169 lung adenocarcinoma patients with an *EGFR* mutation who carried at least one polymorphic C allele of *HMGB1* rs1360485, they showed a significantly higher risk of having a poorly differentiated carcinoma (5.493-fold, 95% CI: 1.130~26.696, p=0.019) than those carrying the WT gene (Table 4). Next, we analyzed the synergistic effects of cigarette smoking combined with rs1360485 SNPs in lung adenocarcinoma patients with an *EGFR* mutation. Sixty patients who smoked cigarettes and harbored at least one polymorphic C allele of *HMGB1* rs1360485 had an enhanced risk (9.9-fold, 95% CI: 1.109~88.339, p=0.016) for developing poorly differentiated tumors compared to patients with TT homozygotes (Table 4). However, no significant associations between rs1360485 gene polymorphisms and the clinicopathologic status were observed in 109 non-smoking patients with *EGFR* mutations.

Table 3. Associations between the polymorphisms of high-mobility group box 1 (*HMGB1*) and the epidermal growth factor receptor hotspot mutations in lung adenocarcinoma patients.

Variable	Wild type	L858R		Exon 19 in-frame deletion	
	(*N*=111) (%)	(*N*=79) (%)	AOR (95% CI)	(*N*=81) (%)	AOR (95% CI)
rs1412125					
TT	59 (53.2%)	39 (49.4%)	1.00	46 (56.8%)	1.00
TC	43 (38.7%)	34 (43.0%)	1.033 (0.522~2.044)	28 (34.6%)	0.640 (0.328~1.250)
CC	9 (8.1%)	6 (7.6%)	1.109 (0.319~3.852)	7 (8.6%)	0.870 (0.290~2.608)
TC+CC	52 (46.8%)	40 (50.6%)	1.045 (0.545~2.003)	35 (43.2%)	0.683 (0.366~1.272)
rs2249825					
CC	74 (66.7%)	55 (69.6%)	1.00	65 (80.2%)	1.00
CG	34 (30.6%)	21 (26.6%)	0.767 (0.370~1.591)	15 (18.5%)	**0.473 (0.228~0.979)**[*,a]
GG	3 (2.7%)	3 (3.8%)	1.234 (0.196~7.757)	1 (1.2%)	0.349 (0.032~3.811)
CG+GG	37 (33.3%)	24 (30.4%)	0.804 (0.397~1.626)	16 (19.8%)	**0.463 (0.227~0.941)**[*,b]
rs1045411					
CC	62 (55.9%)	44 (55.7%)	1.00	52 (64.2%)	1.00
CT	41 (36.9%)	29 (36.7%)	0.913 (0.457~1.823)	26 (32.1%)	0.766 (0.402~1.459)
TT	8 (7.2%)	6 (7.6%)	0.963 (0.275~3.370)	3 (3.7%)	0.448 (0.106~1.884)
CT+TT	49 (44.1%)	35 (44.3%)	0.921 (0.477~1.779)	29 (35.8%)	0.715 (0.385~1.330)
rs1360485					
TT	60 (54.1%)	42 (53.2%)	1.00	46 (56.8%)	1.00
TC	40 (36.0%)	31 (39.2%)	0.989 (0.493~1.986)	31 (38.3%)	1.042 (0.549~1.977)
CC	11 (9.9%)	6 (7.6%)	0.843 (0.252~2.820)	4 (4.9%)	0.498 (0.140~1.769)
TC+CC	51 (45.9%)	37 (46.8%)	0.961 (0.496~1.864)	35 (43.2%)	0.929 (0.504~1.714)

The adjusted odds ratios (AORs) with 95% confidence intervals (CIs) were estimated by multiple logistic regression models after controlling for age and gender.
Note: Bold text indicates a significant association with a p value of <0.05.
Abbreviations: SNP, single-nucleotide polymorphism.
[a] p value=0.044; [b] p value=0.033.

Table 4. Associations between polymorphic genotypes of high-mobility group box 1 (*HMGB1*) (rs1360485) and clinicopathologic characteristics of lung cancer with epidermal growth factor receptor (EGFR) mutations.

Variable	EGFR mutation (*N*=169)			EGFR mutation with non-smoking (*N*=109)			EGFR mutation with smoking (*N*=60)		
	TT (*N*=94)	TC+CC (*N*=75)	p value	TT (*N*=60)	TC+CC (*N*=49)	p value	TT (*N*=34)	TC+CC (*N*=26)	p value
Stage									
I+II	30 (31.9%)	17 (22.7%)	p=0.182	20 (33.3%)	12 (24.5%)	p=0.313	10 (29.4%)	5 (19.2%)	p=0.367
III+IV	64 (68.1%)	58 (77.3%)		40 (66.7%)	37 (75.5%)		24 (70.6%)	21 (80.8%)	
Tumor T status									
T1+T2	62 (66.0%)	46 (61.3%)	p=0.534	40 (66.7%)	33 (67.3%)	p=0.940	22 (64.7%)	13 (50.0%)	p=0.252
T3+T4	32 (34.0%)	29 (38.7%)		20 (33.3%)	16 (32.7%)		12 (35.3%)	13 (50.0%)	
Lymph node status									
Negative	34 (36.2%)	20 (26.7%)	p=0.188	24 (40.0%)	14 (28.6%)	p=0.213	10 (29.4%)	6 (23.1%)	p=0.582
Positive	60 (63.8%)	55 (73.3%)		36 (60.0%)	35 (71.4%)		24 (70.6%)	20 (76.9%)	
Distant metastasis									
Negative	47 (50.0%)	33 (44.0%)	p=0.438	32 (53.3%)	22 (44.9%)	p=0.381	15 (44.1%)	11 (42.3%)	p=0.889
Positive	47 (50.0%)	42 (56.0%)		28 (46.7%)	27 (55.1%)		19 (55.9%)	15 (57.7%)	
Cell differentiation									
Well + Moderately	92 (97.9%)	67 (89.3%)	**p=0.019***[,a]	59 (98.3%)	47 (95.9%)	p=0.443	33 (97.1%)	20 (76.9%)	**p=0.016***[,b]
Poorly	2 (2.1%)	8 (10.7%)		1 (1.7%)	2 (4.1%)		1 (2.9%)	6 (23.1%)	

* p value of <0.05 as statistically significant.
[a] Odds ratio (OR) (95% confidence interval (CI)) : 5.493 (1.130~26.696); [b] OR (95% CI): 9.900 (1.109~88.339).

Discussion

Increasing evidence has shown that HMGB1 plays a key role in various cancer types including NSCLC [18], and HMGB1 dysfunction affects tumor development and therapy through regulating several hallmarks of cancer, such as metastasis, angiogenesis, resisting cell death, genome instability, mutations, and so on [15]. HMGB1 was characterized as having both oncogenic and tumor-suppressive roles in promoting both cell survival and death by regulating multiple signaling pathways, including genome stability, inflammation, immunity, proliferation, apoptosis, autophagy, metastasis, and metabolism [15, 17]. Moreover, the HMGB1 expression level was reported to be inversely correlated with tumor differentiation [25]. In the past few years, several studies evaluated the association of *HMGB1* polymorphisms with cancer susceptibility. In total, four frequently used SNPs within the human *HMGB1* gene, rs1412125, rs2249825, rs1045411, and rs1360485, were studied for their associations with various cancer types including oral [26], liver [27], cervical [28], colorectal [29], and lung [30] cancers, especially in in Chinese Han population [20]. These four *HMGB1* SNPs with minor allele frequencies were of 0.05 (5%) or greater in the Chinese Han Beijing population. Moreover, previous GWAS study indicated that an association might exist between rs1360485 and rs1412125 SNPs with lung cancer risk [31] and rs1360485 showed a strong linkage disequilibrium with rs1045411 and rs2249825 [30, 32]. In lung cancer, knockdown of HMGB1 can increase the chemosensitivity of cells [33]. Moreover, the rs1412125 and rs2249825 polymorphisms were recently reported to be associated with the response to platinum-based chemotherapy in Chinese population [34]. High frequency of *EGFR* mutations in NSCLC was found in Asian population and the *EGFR* mutation of NSCLC was demonstrated to be a predictor of responsiveness to targeted therapy. Overexpression of HMGB1 was reported to promote cell proliferation and invasion in EGFR-mutant lung adenocarcinoma cells [35]. Based on the above annotations, we first investigated the association of *EGFR* mutation statuses in lung adenocarcinoma with variations (rs1412125, rs2249825, rs1045411, and rs1360485) of *HMGB1* and their interactions with subjects who smoke in a Taiwanese population. In this study, our recruited cohort revealed a high prevalence of *EGFR* mutations in females and subjects who had never smoked and who harbored a more-differentiated tumor type. Characteristics of our recruited cohort were similar to Asian NSCLC patients as previously reported [36].

As to correlations between *HMGB1* SNPs and frequencies of *EGFR* mutations, variant types (CG or CG + GG) of *HMGB1* 3814C/G polymorphism (rs2249825) were significantly negatively correlated with the *EGFR* mutation type of an exon 19 in-frame deletion. Furthermore, we observed that the rs2249825 C/G, rs1045411 C/T, and rs1045411 T/C heterozygous polymorphisms were associated with a significantly lower proportion of developing *EGFR* mutations compared to their respective homozygous C/C, C/C, and T/T polymorphisms in the smoking population. These results suggested that the respective G, T, and C alleles of rs2249825, rs1045411, and rs1360485 might be protective factors against developing *EGFR* mutations in individuals who smoke. Moreover, individuals who smoke and carry at least one C allele of rs1360485 had a higher risk of having a poorly differentiated tumor type. Cigarette smoking, the best-known causative factor of lung cancer, was reported to induce HMGB1 upregulation in the blood and lung tissues of smokers [37]. A carcinogen of cigarette smoke, benzo[a]pyrene, can cause DNA damage, and base excision repair is a critical repair pathway that removes benzo[a]pyrene-induced DNA damages [38]. It was postulated that impaired base excision repair may be associated with increased genomic instability and increased tumor mutation rates such as *EGFR* mutations, and HMGB1 was reported to play an important role in base excision repair [39]. Based on these findings, we suggest that *HMGB1* SNPs might affect cigarette smoke-induced HMGB1 expression to further decrease genomic instability and tumor *EGFR* mutations. Actually, expression of another base excision repair gene, excision repair cross complementing 1, was reported to be inversely correlated with *EGFR* mutations in NSCLC patients [40].

Previous studies demonstrated that polymorphisms of certain genes can influence gene expressions and biological functions. The C-to-G variation of the rs2249825 polymorphism, located in intron 1, might affect the binding site of v-myb and provide transcriptional control to enhance HMGB1 expression [34]. The rs2249825 polymorphism was also correlated with lipopolysaccharide-induced HMGB1 production in neutrophils [41]. In addition to rs2249825, two closely related SNPs, rs1045411 and rs1360485, showed strong linkage, are located in the 3′ UTR of the *HMGB1* gene, and may alter *HMGB1* messenger (m)RNA expression through micro (mi)RNA binding. For example, rs1045411 is in close proximity to the mir-505 binding site, and a significant influence of CT/CC genotypes of rs1045411 was reported to alter *HMGB1* gene

expression by regulating miR-505-binding activity [32]. Until now, the rs2249825 C/G polymorphism was reported to enhance HMGB1 expression, and the functional role of this SNP might have prevented *EGFR* mutations in our study cohort by maintaining genomic stability. In smokers with *EGFR*-mutant adenocarcinoma, not only rs2249825, but also rs1045411 and rs1360485 SNPs were all correlated with lower incidences of *EGFR* mutations. Although the rs1045411 SNP was reported to decrease HMGB1 expression by miR-505 [32], cigarette smoking might influence other miRNAs [42, 43] to regulate HMGB1 expression. Moreover, rs1360485 polymorphisms, TC and CC genotypes, were reported to reduce risk of lung adenocarcinoma, but there was no addictive interaction between rs1360485 polymorphisms and tobacco smoking [31]. Until now, interactions among tobacco consumption, rs1045411 and rs1360485 polymorphisms, and HMGB1 expression are unclear and worthy of future investigations.

In conclusion, in a Taiwanese population, we first identified associations among *HMGB1* genetic polymorphisms, cigarette smoking, and the *EGFR* mutation status. Our results indicated that the exon 19 in-frame deletion mutation of the *EGFR* was associated with polymorphisms of the *HMGB1* gene related to carcinogen detoxification and DNA repair in lung adenocarcinoma patients who smoked. These findings provide novel insights into the genesis of *EGFR* mutations and can be utilized as a predictor of responsiveness to EGFR targeted therapy.

Competing Interests

The authors have declared that no competing interest exists.

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