Ethnicity affects *EGFR* and *KRAS* gene alterations of lung adenocarcinoma

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Abstract. Mutations or copy number gains (CNGs) of the *EGFR* and *KRAS* genes are representative alterations in lung adenocarcinomas that are individually associated with patient characteristics such as ethnicity, smoking status and gender. However, the effects of combinations of these genetic alterations have not been statistically examined. The present study analyzed previously examined lung adenocarcinoma cases in Asian (n=166) and non-Asian (n=136) individuals in whom all four *EGFR* and *KRAS* alterations had been studied. The polynomial logistic regression models were used following adjustment for gender and smoking status, and using patients without any type of *EGFR*/*KRAS* alterations as a reference. Between the two ethnic groups, *EGFR* CNGs (g*EGFR*) occurred more frequently than *EGFR* mutations (m*EGFR*) (46 vs. 38% in Asians; 21 vs. 10% in non-Asians), whereas *KRAS* mutations (m*KRAS*) were more frequent than *KRAS* CNGs (g*KRAS*) (13 vs. 7% and 35 vs. 4%, respectively). Additionally, g*EGFR* and g*KRAS* occurred significantly more frequently in respective mutant cases, and all *EGFR* alterations were almost exclusive of all *KRAS* alterations. The polynomial logistic regression models confirmed that all types of *EGFR* alterations were significantly more frequent among Asian individuals than among non-Asian individuals, independent of gender and smoking status (odds ratios, 2.36-6.67). *KRAS* alterations occurred less frequently among Asian individuals than among non-Asian individuals, although a significant difference was not detected. The present study results indicated that the *EGFR* and *KRAS* profiles, including mutations and CNGs, differ between Asian and non-Asian individuals with lung adenocarcinoma, suggesting that ethnicity strongly affects the molecular characteristics of lung adenocarcinoma.

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Introduction

Activating mutations of *EGFR* and *KRAS* genes are characteristic mutations, or so-called 'driver mutations', of lung adenocarcinomas (1-3). Approximately 80% of patients with *EGFR* mutations (m*EGFR*) respond efficiently to treatment with EGFR-tyrosine kinase inhibitors (TKIs) (1,2), but *KRAS* mutations (m*KRAS*) are considered to predict resistance to EGFR-TKI therapy (4). Over the past decade, other 'driver mutations' in *ALK* (5), *HER2* (6), and *BRAF* (7) have been found in lung adenocarcinomas, although m*EGFR* and m*KRAS* remain the most frequent 'driver mutations' in lung adenocarcinomas (8). Significantly, m*EGFR* and m*KRAS* are mutually exclusive and exhibit a characteristic association with clinical factors, particularly ethnicity; m*EGFR* are frequently observed in Asian individuals, women and never-smokers, but m*KRAS* are frequently observed in Caucasian individuals, men and smokers (9,10).

A copy number gain (CNG) is another mechanism of oncogenic activation (11). A large-scale project to characterize copy number alterations in primary lung adenocarcinomas confirmed that *EGFR* and *KRAS* loci were significantly recurrent events when using a high-resolution genome-wide

approach (12). A recent systematic review and meta-analysis revealed that *EGFR* CNGs (g*EGFR*) were associated with responsiveness and improved survival outcomes in patients with non-small cell lung cancer (NSCLC) who were treated with EGFR-TKIs (13,14). Although g*EGFR* are reportedly frequent among never-smokers with NSCLC whose samples are collected in Western countries (15,16), ethnic differences in the frequency of g*EGFR* have not been intensively investigated. The frequency of *KRAS* CNGs (g*KRAS*) is reportedly low (7-11%) in lung adenocarcinoma (17-19) and the association between g*KRAS* and clinical factors has been controversial. Of particular note is the fact that g*EGFR* and g*KRAS* occur significantly frequently in m*EGFR* and m*KRAS* cases, respectively (15,17-20).

These lines of evidence suggest that a significant mutually exclusive association between *EGFR* and *KRAS* alterations is present in lung adenocarcinomas. In addition, there is already a great deal of information about ethnicity and m*EGFR* and m*KRAS*, but much less about the CNGs of these genes. Only a modest number of studies have analyzed mutations and CNGs in the same study and linked it with ethnicity. To the best of our knowledge, the concordant association between all four genetic alterations and ethnicity has not been extensively investigated using an adequate statistical method. The present study evaluated the impact of ethnic differences on the frequencies of mutations and CNGs of the *EGFR* and *KRAS* genes in lung adenocarcinomas, while considering gender and the smoking status, using a polynomial logistic regression model.

Materials and methods

Tumor samples. We have previously determined the mutational status and copy number of the *EGFR* and *KRAS* genes in resected NSCLC samples (17). Among these samples, the present study restudied 302 surgically resected lung adenocarcinomas with complete information on mutational status and copy number of the *EGFR* and *KRAS* genes, and clinical information such as gender, smoking status and ethnicity. Genomic DNA extracted from frozen tissues was obtained from four countries: Japan [n=148; Okayama University, Okayama, Japan (n=73) and Chiba University, Chiba, Japan (n=75)], the United States (n=87), Australia (n=22) or Canada (n=45)]. All Japanese cases were of Asian individuals; the 87 cases from the United States consisted of 2 Asian, 4 African-American, 4 Hispanic and 77 Caucasian individuals; the 22 Australian cases consisted of 1 Asian and 21 Caucasian individuals; and the 45 Canadian cases consisted of 15 Asian and 30 Caucasian individuals. For this study, the definition of non-Asian individuals (n=136) consisted of Caucasian (n=128), African-American (n=4) and Mexican-American (n=4) individuals. The characteristics of the 302 cases are presented in Table I. Females and never-smokers occurred significantly more frequently in the Asian group than in the non-Asian group. Study permission was granted by the Institutional Review Board of Okayama University (permission ref. Genome 173) and written informed consent was obtained from all patients at each collection site.

Detection of gene mutations by direct sequencing. The mutational status of exons 18 to 21 of the *EGFR* gene and exon 2 of the *KRAS* gene was determined by direct sequencing, as previously described (17,21,22). Briefly, genomic DNA was amplified by conventional PCR using the conditions stated in Table II. The PCR products were incubated with exonuclease I and shrimp alkaline phosphatase (GE Healthcare Life Sciences, Piscataway, NJ, USA), and sequenced using the ABI PRISM® BigDye™ Terminator Cycle Sequencing kit (PerkinElmer, Inc., Foster City, CA, USA). All sequence variants were confirmed by sequencing the products of independent polymerase chain reaction (PCR) in each direction.

Validation of gene copy number alteration by quantitative (q)PCR assay. g*EGFR* and g*KRAS* were determined by qPCR assay using Power SYBR® Green PCR Master Mix (Applied Biosystems Life Technologies, Foster City, CA, USA), as previously reported (17,22). *LINE-1* was used as a reference gene for all copy number analyses. The PCR conditions of each gene are provided in Table II, and gene dosages of *EGFR*, *KRAS* and *LINE-1* were calculated using the standard curve method. The relative copy number of each sample was determined to compare the ratio of the target gene and *LINE-1* in each sample with the ratio in human genomic DNA (EMD Millipore, Billerica, MA, USA) as a diploid control. Based on our previous studies (17,22), CNG was defined as values >3.

Statistical analysis. The primary endpoint of the present cross-sectional study was to examine the ethnic differences (Asian vs. non-Asian) in *EGFR* and *KRAS* alterations (mutations and CNGs) in lung adenocarcinoma. To assess this, polynomial logistic regression models adjusted for gender (female vs. male) and smoking status (never vs. ever) were applied without any type of *EGFR*/*KRAS* alteration as a reference group. Cross-sectional odds ratios (ORs) and 95% confidence intervals (CIs) were applied as a measure of association. Each OR indicates how many times cases of Asian ethnicity are more likely to harbor the specified pattern of alteration of *EGFR*/*KRAS* than cases of non-Asian ethnicity. Fisher's exact test was used for comparing the baseline characteristics of the Asian and non-Asian groups. Exact 95% CIs were estimated with prevalence of each combination of alteration. $P<0.05$ was defined as a threshold of statistical significance. All the statistical analyses were executed by STATA version 11 (StataCorp LP, College Station, TX, USA).

Results

Mutations and CNGs of EGFR or KRAS and clinical factors. m*EGFR* (*EGFR* mutation independent of *EGFR* CNG), g*EGFR* (*EGFR* CNG independent of *EGFR* mutation), m*KRAS* and g*KRAS* were present in 26% (n=77), 34% (n=104), 23% (n=69) and 6% (n=17) of the 302 cases, respectively. m*EGFR*, g*EGFR*, m*KRAS* and g*KRAS* were present in 38, 46, 13 and 7% of Asian individuals (n=166) and 10, 21, 35 and 4% of non-Asian individuals (n=136), respectively, indicating that CNGs were more frequently present than mutations in *EGFR* but not in *KRAS* between the two ethnic groups (Table I; Fig. 1). m*EGFR* ($P<0.0001$), g*EGFR* ($P<0.0001$) or any *EGFR* alteration (m*EGFR* or g*EGFR*; $P<0.0001$) were significantly more frequent in Asian compared with non-Asian individuals. By contrast, m*KRAS* ($P<0.0001$) and any *KRAS* alteration (m*KRAS*

Table I. Patient characteristics and genetic alterations in Asian and non-Asian groups.

		Asian (n=166)		Non-Asian (n=136)		
Subsets	Total, n	n	%	n	%	P-value
Gender						
Female	143	70	42.2	73	53.7	0.049
Male	159	96	57.8	63	46.3	
Smoking status						
Never	115	75	45.2	40	29.4	0.006
Ever	187	91	54.8	96	70.6	
Stage						
I	188	109	65.7	79	58.1	NS*
II	35	15	9.0	20	14.7	
III	61	35	21.1	26	19.1	
IV	13	3	1.8	10	7.4	
No data	5	4	2.4	1	0.7	
EGFR mutation						
Mutation	77	63	38.0	14	10.3	<0.0001
Wild	225	103	62.0	122	89.7	
EGFR CNG						
CNG	104	76	45.8	28	20.6	<0.0001
No gain	198	90	54.2	108	79.4	
Any *EGFR* alterations						
Mutation or CNG	143	107	64.5	36	26.5	<0.0001
None of *EGFR*	159	59	35.5	100	73.5	
KRAS mutation						
Mutation	69	22	13.3	47	34.6	<0.0001
Wild	233	144	86.7	89	65.4	
KRAS CNG						
CNG	17	11	6.6	6	4.4	NS
No gain	285	155	93.4	130	95.6	
Any *KRAS* alterations						
Mutation or CNG	78	28	16.9	50	36.8	0.0001
None of *KRAS*	224	138	83.1	86	63.2	

*Comparison of stage I vs. stages II-IV; Any genetic alteration consisted of either mutation or CNG. CNG, copy number gain; NS, not significant.

or *gKRAS*; P=0.0001) were significantly more frequent in non-Asian compared with Asian individuals (Table I; Fig. 1). With regard to other clinical factors, the never smoking status was significantly associated with m*EGFR* (P<0.0001) and g*EGFR* (P=0.046), whereas the presence of a smoking history was significantly associated with m*KRAS* (P<0.0001; Table III). The female gender was a significant factor that was associated with m*EGFR* (P=0.0005).

Inter-association between mutations and CNGs of EGFR and KRAS is retained between the two ethnic groups. The present study evaluated the effect of ethnic difference on the inter-association between mutations and CNGs of the *EGFR* and *KRAS* genes by categorizing 302 cases into three groups according to mutational status: i) m*EGFR* (n=77), ii) m*KRAS* (n=69) and iii) wild-type for *EGFR* and *KRAS* (n=156). g*EGFR* (Asian individuals, P=0.338; non-Asian individuals, P=0.041) and g*KRAS* (Asian individuals, P=0.007; non-Asian individuals, P=0.124) occurred significantly more frequently in their respective mutant cases (Fig. 2). Between the Asian and non-Asian individuals, the frequencies of g*EGFR* and g*KRAS* were lowest in the m*KRAS* and m*EGFR* groups, respectively (Fig. 2). m*EGFR* and m*KRAS* were completely mutually exclusive in the two ethnic groups and any *EGFR* alterations (either m*EGFR* or g*EGFR*) were almost exclusive with any *KRAS* alterations (either m*KRAS* or g*KRAS*) between the two ethnic groups (P=0.016 in Asians and P=0.004 in non-Asians). These findings suggested that the inter-association between the mutation and CNG of an identical gene and between alterations of the *EGFR* and *KRAS* genes were retained in the two ethnic groups.

Table II. Conditions for direct PCR sequencing and quantitative PCR of gene copy number.

Gene	Primer sequence, 5' to 3'	Amplicons, bp	Tm, ˚C	Cycles, n
Direct sequencing				
KRAS, exon 2	F: GTATTAACCTTATGTGTGACA R: GTCCTGCACCAGTAATATGC	222	55	37
EGFR, exon 18	F: AGCATGGTGAGGGCTGAGGTGAC R: ATATACAGCTTGCAAGGACTCTGG	263	65	35
EGFR, exon 19	F: CCAGATCACTGGGCAGCATGTGGCACC R: AGCAGGGTCTAGAGCAGAGCAGCTGCC	265	65	35
EGFR, exon 20	F: GATCGCATTCATGCGTCTTCACC R: TTGCTATCCCAGGAGCGCAGACC	362	65	35
EGFR, exon 21	F: TCAGAG CCTGGCATGAACATGACCCTG R: GGTCCCTGGTGTCAGGAAAATGCTGG	297	65	35
Gene copy number				
KRAS	F: CACCCTAGACAAGCAGCCAATA R: AAGCCCTGCCGCAAAAA	-	60	45
EGFR	F: CAAGGCCATGGAATCTGTCA R: CTGGAATGAGGTGGAGGAACA	-	60	45
LINE-1	F: AAAGCCGCTCAACTACATGG R: TGCTTTGAATGCGTCCCAGAG	-	60	45

PCR, polymerase chain reaction; Tm, tempertaure; F, forward; R, reverse.

Table III. Association between *EGFR* and *KRAS* alterations and characteristics in 302 lung adenocarcinomas.

		m*EGFR* (n=77)			g*EGFR* (n=104)			m*KRAS* (n=69)			g*KRAS* (n=17)		
Subsets	Total, n	n	%	P-value	n	%	P-value	n	%	P-value	n	%	P-value
Gender													
Female	143	50	35.0	0.0005	42	29.4	NS	32	22.4	NS	4	2.8	0.048
Male	159	27	17.0		62	39.0		37	23.3		13	8.2	
Ethnicity													
Asian	166	63	38.0	<0.0001	76	45.8	<0.0001	22	13.3	<0.0001	11	6.6	NS
Non-Asian	136	14	10.3		28	20.6		47	34.6		6	4.4	
Smoking status													
Never	115	57	49.6	<0.0001	48	41.7	0.046	12	10.4	<0.0001	4	3.5	NS
Smoker	187	20	10.2		56	29.9		57	30.5		13	7.0	
Stage													
I	188	50	26.6	NS[a]	65	34.6	NS[a]	48	25.5	NS	11	5.9	NS[a]
II	35	11	31.4		12	34.3		6	17.1		0	0.0	
III	61	13	21.3		20	32.8		8	13.1		4	6.6	
IV	13	2	15.4		4	30.8		4	30.8		1	7.7	
No data	5	1	20.0		3	60.0		3	60.0		1	20.0	

[a]Comparison of stage I vs. stages II-IV. CNG, copy number gain; mut, mutation; NS, not significant; m, with mutation; g, with CNG.

Ethnic differences of EGFR and KRAS alterations with respect to gender and smoking status. As gender and smoking status were other significant factors associated with the frequency of the mutations and CNGs of the *EGFR* and *KRAS* genes (Table III) and as the proportions of females and never-smokers were significantly biased toward the Asian group, a polynomial logistic regression model was performed with adjustments for gender and smoking status; patients without any *EGFR*/*KRAS* alterations were used as the reference group (Table IV). According to the polynomial logistic regression models, it

Table IV. Odds ratios for Asian individuals harboring *EGFR* or *KRAS* alterations.

Genetic alterations (*EGFR*/*KRAS*)	Asian, n (%)	Non-Asian, n (%)	OR (95% CI)	P-value
None/None	43 (25.9)	56 (41.2)	1.00 (Reference)	
Mut alone/None	31 (18.7)	8 (5.9)	4.94 (1.95-12.6)	<0.001
Mut+CNG/None	31 (18.7)	6 (4.4)	6.67 (2.48-17.9)	<0.001
CNG alone/None	33 (19.9)	16 (11.8)	2.43 (1.17-5.07)	0.017
None/Mut alone	16 (9.6)	40 (29.4)	0.56 (0.27-1.16)	0.118
None/Mut+CNG	0 (0.0)	3 (2.2)	NE	NE
None/CNG alone	0 (0.0)	1 (0.7)	NE	NE
Mut+CNG/CNG	1 (0.6)	0 (0.0)	NE	NE
CNG/Mut	1 (0.6)	4 (2.9)	0.34 (0.04-3.25)	0.35
CNG/Mut+CNG	5 (3.0)	0 (0.0)	NE	NE
CNG/CNG	5 (3.0)	2 (1.5)	2.92 (0.53-16.1)	0.219
Mut or CNG/None	95 (57.2)	30 (22.1)	4.24 (2.29-7.86)	<0.001
None/Mut or CNG	16 (9.6)	44 (32.4)	0.51 (0.25-1.03)	0.059
Mut/None	62 (37.4)	14 (10.3)	4.76 (2.30-9.83)	<0.001
CNG/None	64 (38.6)	22 (16.2)	2.36 (1.30-4.28)	0.005
None/Mut	16 (9.6)	43 (31.6)	0.53 (0.26-1.08)	0.080
None/CNG	0 (0.0)	3 (2.9)	NE	NE

Odds ratios (ORs) were adjusted for smoking status (never vs. ever) and gender. Mut, mutation; CNG, copy number gain; CI, confidence interval; NE, not evaluated.

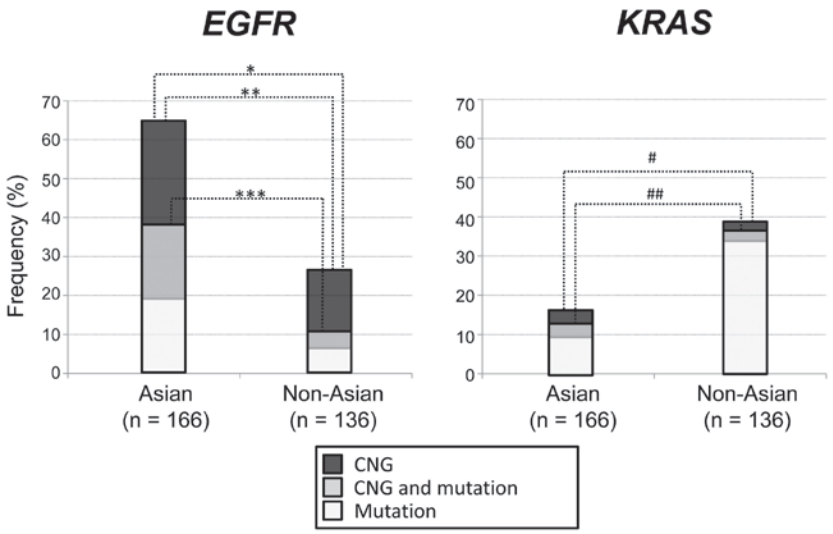

Figure 1. Inter-association between mutations and copy number gains (CNGs) of *EGFR* and *KRAS* among Asian and non-Asian individuals. *EGFR* alterations were more frequent among Asian individals than non-Asian individuals, while *KRAS* alterations were more frequent among non-Asian individuals than Asian individuals. *P<0.0001 (any *EGFR* alteration); **P<0.0001 (g*EGFR*); ***P<0.0001 (m*EGFR*); $^{\#}$P=0.0001 (any *KRAS* alteration); $^{\#\#}$P<0.0001 (m*KRAS*); there is no statistical significance between the two ethnic groups with regard to the frequency of g*KRAS*. m, with mutation; g, with CNG.

was confirmed that all types of *EGFR* alterations [mEGFR (P<0.001), g*EGFR* (P=0.005), mg*EGFR* (mutation and CNG of *EGFR* gene; P<0.001), any EGFR (P<0.001), m*EGFR* alone (m*EGFR* without *EGFR* CNG; P<0.001) and g*EGFR* alone (g*EGFR* without *EGFR* mutation; P=0.017)] were significantly more frequent among Asian individuals compared with among non-Asian individuals (ORs, 2.36-6.67). *KRAS* alterations occurred less frequently among Asian individuals than among non-Asian individuals, although a statistical significance was not detected using the polynomial model.

Additionally, the 302 cases were subcategorized into 8 groups according to gender, smoking status and ethnicity: i) Asian never-smoker, female (A-NS-F; n=58), ii) Asian never-smoker, male (A-NS-M; n=17), iii) Asian smoker, female (A-SM-F; n=12), iv) Asian smoker, male (A-SM-M; n=79), v) non-Asian never-smoker, female (NA-NS-F; n=26), vi) non-Asian never-smoker, female (NA-NS-M; n=14), vii) non-Asian smoker, female (NA-SM-F; n=47), and viii) non-Asian smoker, male (NA-SM-M; n=49). Each group was compared, noting the effect of ethnicity on the

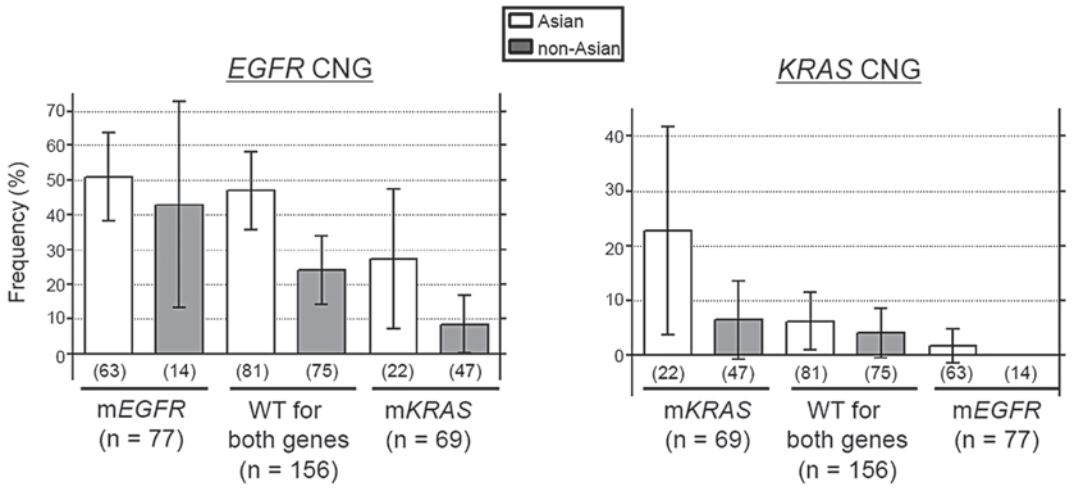

Figure 2. Ethnic difference of the inter-association between mutations and copy number gains (CNGs) of *EGFR* and *KRAS*. The frequencies of the cases with CNGs of each gene are shown. CNGs of the two genes occurred more frequently in the respective mutant cases, independent of ethnicity. m, with mutation; g, with CNG; WT, wild-type.

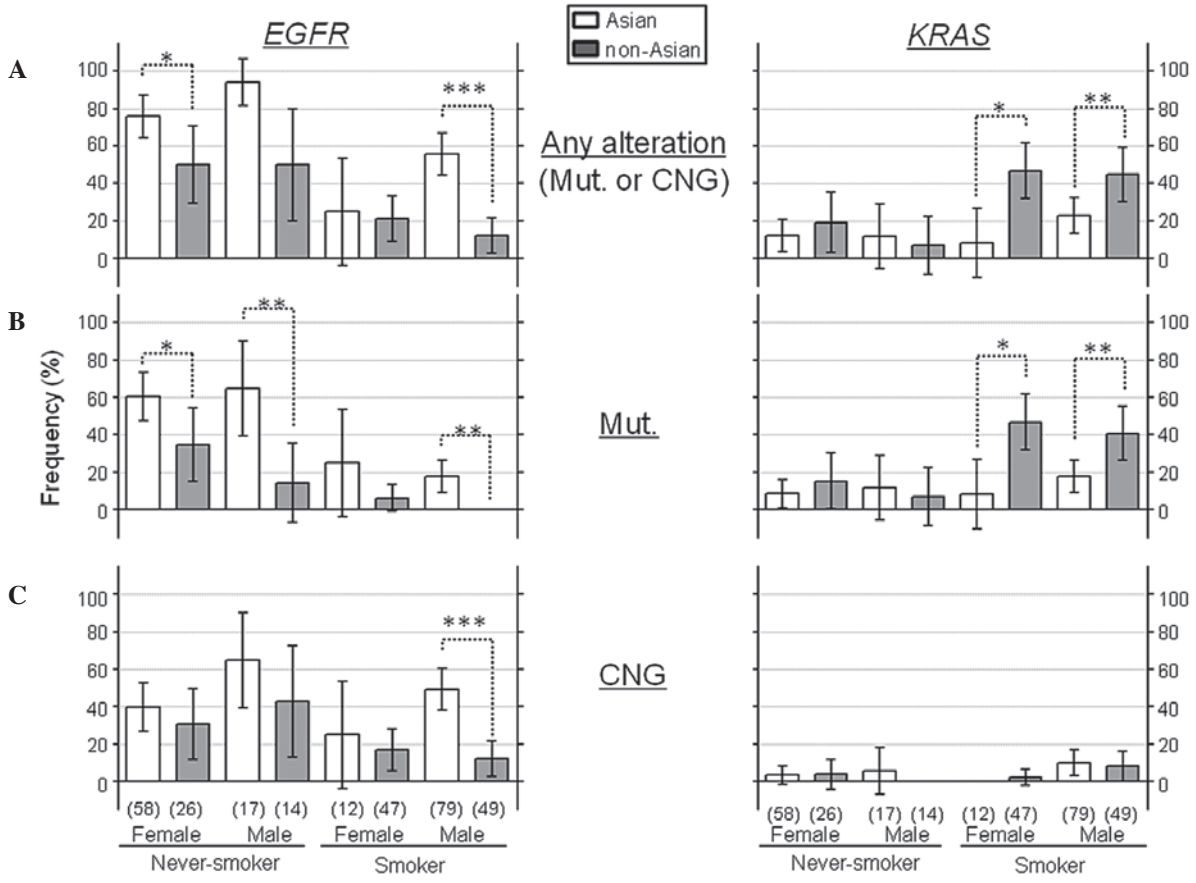

Figure 3. Impact of ethnic difference on *EGFR* and *KRAS* alterations. The frequencies of the cases with (A) any alteration (mut. and/or CNG of each gene), (B) mut., including mut. and CNG, and (C) CNG, including mut. and CNG are shown. *P<0.05; **P<0.01; ***P<0.0001. mut., mutation; CNG, copy number gain.

frequencies of these genetic alterations (Fig. 3). m*EGFR* and g*EGFR* occurred more frequently in every Asian group than in their corresponding non-Asian group, independent of gender and smoking status, whereas m*KRAS* occurred more frequently in non-Asians than in Asians among the smoker groups, but this ethnic difference was not observed among the never-smoker groups (Fig. 3).

Discussion

The present study investigated the impact of ethnic differences on the genetic alterations of *EGFR* and/or *KRAS* genes, and found that ethnic differences were associated with the frequencies of these genetic alterations, particularly *EGFR* alterations, even when gender and smoking status were taken into consideration.

Ethnic differences in the frequencies of molecular alterations have been described in several studies. We previously reported that the frequencies of the aberrant methylation of CpG islands in certain tumor-suppressor genes, such as *MGMT* and *GSTP1*, were different between non-Asian populations (American and Australian cases) and Asian populations (Japanese and Taiwanese cases) (23). A recent study that evaluated CNGs in lung adenocarcinomas using a common high-resolution single nucleotide polymorphism microarray, also reported that discrete differences in copy number aberrations was present between East-Asian and Western European individuals (chromosome 16p CNGs in East-Asian individuals, and chromosome 19p losses in Western European individuals) (24).

Oncogenes can be activated by mutations, CNGs and/or translocations (11). Any one of these genetic alterations is able to activate oncogenes, but interactions among these alterations can occur. In fact, the *EGFR* and *KRAS* genes are known to be activated by activating mutations and CNGs, and the inter-association between these genetic alterations have been investigated in previous studies (15,17,18,25). Although these studies have not revealed ethnic differences for these genetic alterations, it has been reported that i) the *EGFR* gene is more dominantly activated by CNGs than by mutations, while the *KRAS* gene is more activated by mutations than by CNGs; ii) m*EGFR* and m*KRAS* are mutually exclusive; and iii) g*EGFR* and g*KRAS* occur significantly more frequently among their respective mutant cases. As a result of these findings, *EGFR* alterations (m*EGFR* and/or g*EGFR*) may be almost exclusive of *KRAS* alterations, as confirmed in the present study. This study added novel insights into the inter-association between these genetic alterations and ethnicity. The inter-association between mutations and CNGs of the same gene, and between alterations of the *EGFR* and *KRAS* genes, were similar in the Asian and non-Asian groups: i.e., in each ethnic group, g*EGFR* and g*KRAS* were significantly frequent among the respective mutant cases, and *EGFR* alterations (m*EGFR* and/or g*EGFR*) were exclusive of *KRAS* alterations. This fact strongly suggests that the inter-association between CNG and mutations in each gene is retained in Asian and non-Asian ethnicities.

In the present study, DNA samples were collected from four countries with mixed populations of different ethnicities, such as Japanese, other Asian (non-Japanese), Caucasian, African-American and Mexican-American. A total of 4 African-Americans were included in the non-Asian group, as African-Americans have been reported to show similar frequencies of m*EGFR* and m*KRAS* to Caucasians (26-28), and it was confirmed that none of the 4 African-Americans harbored m*EGFR* and that 2 harbored m*KRAS*. The mutations and CNGs in 12 Asian patients with lung adenocarcinomas whose DNA samples were obtained from Western countries were also determined. It was confirmed that *EGFR* alterations were more frequent than *KRAS* alterations among these Asians samples (data not shown), as previously reported (29). In addition, Asian patients with NSCLC who immigrated to Canada from Asian countries reportedly showed a preferential response to EGFR-TKI treatment (30). These lines of evidence suggest that ethnic differences in the molecular spectra of *EGFR* and *KRAS* are not affected by environmental factors, and that ethnicity is an important factor determining the molecular spectrum of lung adenocarcinoma.

In conclusion, the *EGFR* and *KRAS* profiles in lung adenocarcinoma differ between Asian and non-Asian populations, suggesting that ethnicity affects the molecular characteristics of lung adenocarcinoma.

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