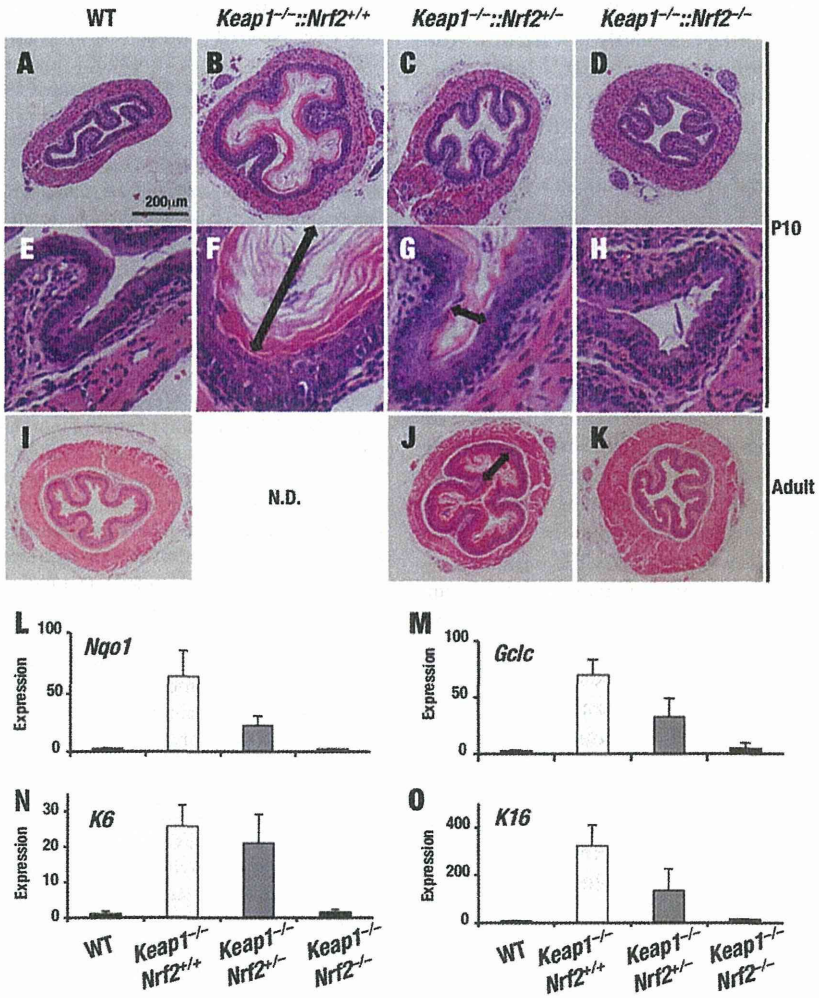

FIG 1 Heterozygous deletion of the *Nrf2* gene alleviates lethal phenotypes of Keap1-deficient mice. (A to K) H&E staining of transverse sections of the esophagus from P10 (A to H) and adult (I to K) mice. Lower (A to D) and higher (E to H) magnifications of the pictures are shown. Arrows indicate the thickened cornified layer. (L to O) Relative expression levels of the *Nqo1*, *Gclc*, *K6*, and *K16* genes compared with the level of 18S rRNA gene expression in the forestomachs of P10 mice. Data are the means ± SDs ($n = 3$). WT, wild type; N.D., no data.

of the *Nrf2* gene is sufficient to rescue the lethality caused by the Keap1 deficiency. We found that the average body weight of the *Keap1*$^{-/-}$::*Nrf2*$^{+/-}$ mice was less than that of the *Keap1*$^{-/-}$::*Nrf2*$^{-/-}$ mice in both males and females (see Fig. S1A and B in the supplemental material). Consistent with our previous observations (25), the *Keap1*$^{-/-}$::*Nrf2*$^{+/+}$ mice showed severe thickening of the cornified layers in the esophagus at 10 days after birth (Fig. 1B and F). On the contrary, the *Keap1*$^{-/-}$::*Nrf2*$^{+/-}$ mice showed clear improvement in the cornification and thickening (Fig. 1C and G). In adult *Keap1*$^{-/-}$::*Nrf2*$^{+/-}$ mouse esophagi, however, the thickening of the cornified layer became apparent (Fig. 1J). These results thus demonstrate that while the Nrf2 level synthesized from a single allele contributes to the esophageal phenotype to a certain extent in the *Keap1* knockout background (*Keap1*$^{-/-}$::*Nrf2*$^{+/-}$), it gives rise to a phenotype much milder than that resulting from the Nrf2 level synthesized from two alleles in the *Nrf2* wild-type background (*Keap1*$^{-/-}$::*Nrf2*$^{+/+}$).

Consistent with the esophageal phenotypes, the levels of expression of Nrf2 target genes, such as *Nqo1* [NAD(P)H:quinone oxidoreductase 1] and *Gclc* (glutamate-cysteine ligase catalytic subunit), and keratin-related genes, including *K6* (keratin 6) and *K16* (keratin 16), were lower in the forestomachs of *Keap1*$^{-/-}$::*Nrf2*$^{+/-}$ mice than in those of *Keap1*$^{-/-}$::*Nrf2*$^{+/+}$ mice but were higher than those in the forestomachs of *Keap1*$^{-/-}$::*Nrf2*$^{-/-}$ mice (Fig. 1L to O). Specifically, the levels of expression in the forestomachs of *Keap1*$^{-/-}$::*Nrf2*$^{+/-}$ mice were approximately half of the levels in *Keap1*$^{-/-}$::*Nrf2*$^{+/+}$ mice, indicating the presence of haploinsufficiency in *Nrf2* gene expression. These results thus indicate that the *Nrf2* gene dosage has an impact on Nrf2 activity *in vivo*.

Nrf2 synthesis is a critical determinant of cytoprotection capacity. Our next question was whether the Nrf2 transcription level affects the cellular capacity of cytoprotection even in the presence of Keap1-mediated Nrf2 degradation. To this end, we adopted thioglycolate-elicited mouse peritoneal macrophages as an experimental system, as this peritoneal macrophage system is well established as a system for testing the roles played by Nrf2 in the stress response (5). We first confirmed that in Nrf2 heterozygous

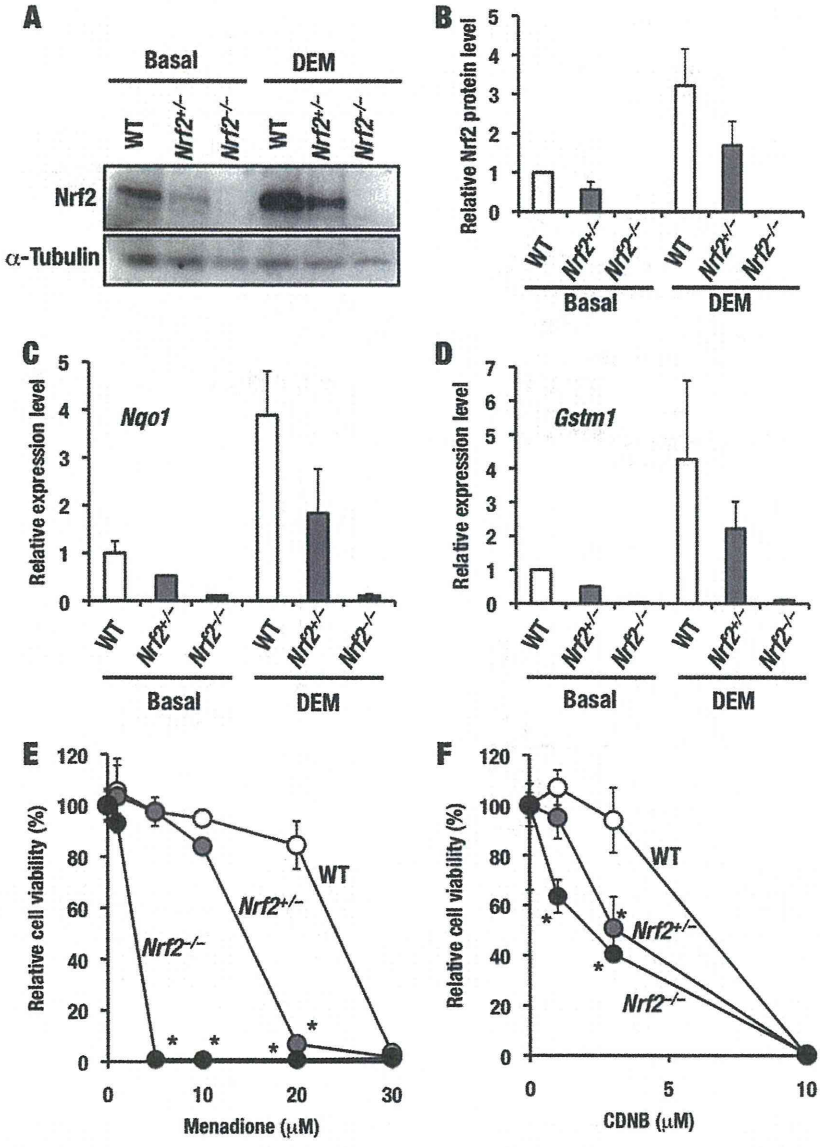

FIG 2 Heterozygous deletion of the *Nrf2* gene attenuates the ultimate activity of Nrf2 and impairs the oxidative stress response. (A) The Nrf2 protein level in macrophages from wild-type, $Nrf2^{+/-}$, and $Nrf2^{-/-}$ mice during the basal and DEM-induced states. (B) A graphical representation of the results in panel A is shown. Data are the means ± SDs ($n = 3$). (C and D) Relative *Nqo1* (C) and *Gstm1* (D) expression levels compared with the level of 18S rRNA gene expression of macrophages from wild-type, $Nrf2^{+/-}$, and $Nrf2^{-/-}$ mice under basal and DEM-induced conditions. Data are the means ± SDs ($n = 3$). (E and F) Relative viability of macrophages from wild-type, $Nrf2^{+/-}$, and $Nrf2^{-/-}$ mice after 12 h of treatment with menadione (E) or CDNB (F). *, statistical significance compared with the result for wild-type cells ($P < 0.05$). Data are the means ± SDs ($n = 3$).

($Nrf2^{+/-}$) macrophages the *Nrf2* transcript level was almost half of that in wild-type cells (see Fig. S2A in the supplemental material), corresponding to the allele number difference in the *Nrf2* gene. Since Nrf2 is constitutively degraded in the basal state, we expected that in Nrf2 heterozygous cells the Nrf2 protein level would not change significantly from the wild-type level under normal conditions. To our surprise, however, the Nrf2 protein level in $Nrf2^{+/-}$ macrophages was clearly decreased compared with that in wild-type cells under basal conditions (Fig. 2A and B). When cells were stimulated with DEM, an electrophilic Nrf2 inducer, the Nrf2 protein level in $Nrf2^{+/-}$ cells became almost half of that in wild-type cells. Consistent with the Nrf2 protein level, the *Nqo1* and *Gstm1* (glutathione *S*-transferase, mu 1) mRNA level was significantly reduced under both basal and induced conditions (Fig. 2C and D). These results suggest that gene dosage does influence cellular Nrf2 activity and cytoprotection under both basal and induced conditions.

To test whether the decrease in the *Nrf2* allele reduces cytoprotective functions, we examined whether $Nrf2^{+/-}$ macrophages are more susceptible to the cytotoxic effect of xenobiotics than wild-type cells. We employed menadione, CDNB, and BITC, which are well-established stressors for testing the roles played by Nrf2 in the stress response (30). Consistent with the reduced expression of *Nqo1* and *Gstm1* in Nrf2 heterozy-

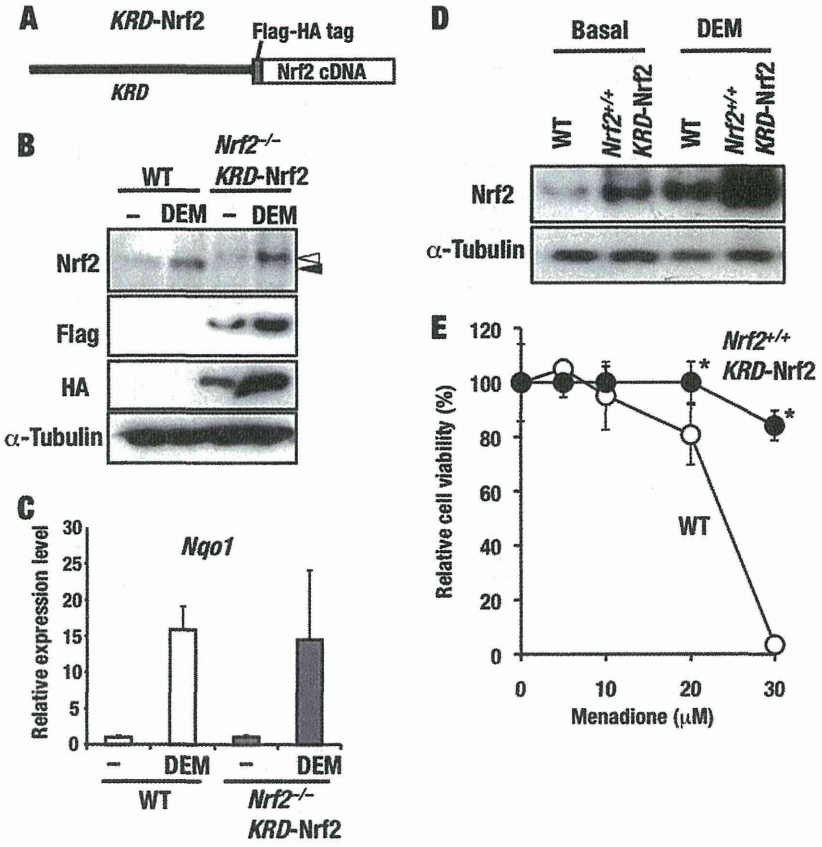

FIG 3 Increase in Nrf2 synthesis enhances Nrf2 activity and makes cells resistant to oxidative stress. (A) Structure of the transgene *KRD*-Nrf2. The Flag-HA double tag was fused to the N terminus of mouse Nrf2, and the fusion protein was expressed under the regulation of the Keap1 regulatory domain (*KRD*). (B) Level of transgene-derived Nrf2 expression in macrophages from $Nrf2^{-/-}$::*KRD*-Nrf2 mice. Closed and open arrowheads, endogenous Nrf2 and transgene-derived Flag-HA tagged Nrf2, respectively. (C) Relative levels of *Nqo1* expression compared with 18S rRNA gene expression in macrophages in the basal and DEM-induced states. Data are the means ± SDs ($n = 3$). (D) Nrf2 protein level in macrophages from wild-type and $Nrf2^{+/+}$::*KRD*-Nrf2 mice in the basal and DEM-induced states. Note the increase in the Nrf2 protein level in basal and DEM-treated $Nrf2^{+/+}$::*KRD*-Nrf2 macrophages. (E) Relative viability of macrophages from wild-type and $Nrf2^{+/+}$::*KRD*-Nrf2 mice after 12 h of treatment with menadione. Data are the means ± SDs ($n = 3$). *, statistical significance compared with the result for wild-type cells ($P < 0.05$).

gous macrophages, the cells appeared to be more susceptible to menadione, CDNB, and BITC than the wild-type cells but less susceptible to the insult than Nrf2-null macrophages (Fig. 2E and F; see Fig. S3 in the supplemental material), showing the haploinsufficiency of the *Nrf2* gene. These results thus demonstrate that the *Nrf2* mRNA expression level is critical for protecting cells from a wide range of xenobiotics.

Elevation of Nrf2 synthesis makes cells resistant to oxidative stress. *Keap1* gene knockout in mice results in an increase in Nrf2 protein levels. However, how the increase in *Nrf2* mRNA levels influences Nrf2 protein levels and cytoprotection is unclear. To assess the influence of *Nrf2* mRNA induction, we generated transgenic mouse lines that expressed Flag-HA-tagged Nrf2 under the control of the *Keap1* gene regulatory domain (*KRD*-Nrf2) (26) (Fig. 3A). Four independent lines were established for the *KRD*-Nrf2 transgene. We first examined whether transgene-derived Nrf2 protein was functional by crossing the transgenic mice with *Nrf2*-null mice ($Nrf2^{-/-}$::*KRD*-Nrf2). The transgenic mouse line expressed the transgene-derived Nrf2 protein at a level comparable to that of endogenous Nrf2 in macrophages under the basal and DEM-induced conditions (Fig. 3B), although the transgene-derived transcript was much more abundant than the endogenous Nrf2 transcript (see Fig. S2B in the supplemental material). This result is most likely due to the limited efficiency of translation from the transgene-derived mRNA, but the reasons for this are unknown. *Nqo1* expression was increased in the presence of DEM in $Nrf2^{-/-}$::*KRD*-Nrf2 macrophages to an extent similar to that in wild-type macrophages (Fig. 3C), indicating that the transgene-derived Nrf2 activated its target gene in response to DEM as efficiently as endogenous Nrf2.

We next analyzed the *KRD*-Nrf2 mice in the wild-type background ($Nrf2^{+/+}$::*KRD*-Nrf2). The Nrf2 protein level was robustly increased in the macrophages of $Nrf2^{+/+}$::*KRD*-Nrf2 mice compared with that in wild-type macrophages, irrespective of the induction status (Fig. 3D). When challenged with menadione, $Nrf2^{+/+}$::*KRD*-Nrf2 macrophages were more resistant to the cytotoxic effect of a high concentration of menadione than the wild-type control (Fig. 3E). Thus, although previous analyses argued that Nrf2 accumulated within the cells due to the derepression of rapid proteasome-dependent degradation, our present results demonstrate that the increase in *Nrf2* synthesis also effectively contributes to the increase in total cellular Nrf2 activity. Collectively, regulation of syn-

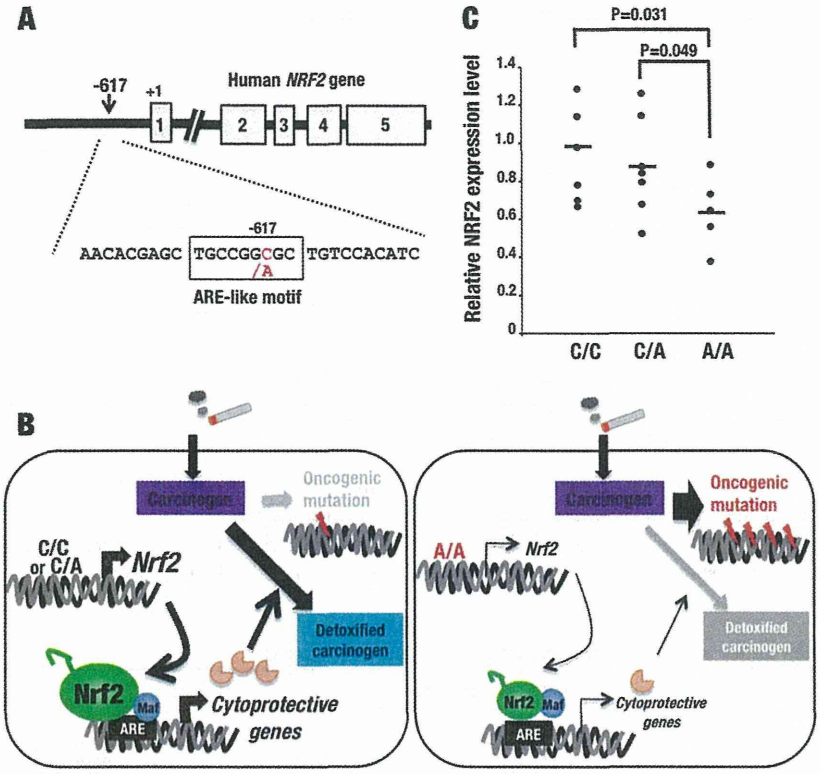

FIG 4 Genotypes of NRF2 rSNP-617 that affect *NRF2* gene expression. (A) Location of NRF2 rSNP-617 in the *NRF2* gene locus. The five exons are indicated by the numbered boxes. NRF2 rSNP-617 is located in the ARE-like motif in the promoter region of the *NRF2* gene. The polymorphic nucleotides are shown in red. (B) Schematic presentation of the putative mechanism for the association of NRF2 rSNP-617 and an increased risk of lung cancer. A/A homozygotes for NRF2 rSNP-617 significantly exhibit decreased expression of the *NRF2* gene and its downstream cytoprotective genes, resulting in the impaired detoxification of tobacco carcinogens and frequent oncogenic events. (C) Relative levels of human *NRF2* gene expression compared with *GAPDH* gene expression in immortalized lymphocytes of three different genotypes of NRF2 rSNP-617.

thesis and degradation in combination determines the cellular Nrf2 levels under basal and induced conditions.

Association of *NRF2* SNP and lung cancer susceptibility. Analyses of mouse models revealed that weakened transcription of the *Nrf2* gene results in the reduction of Nrf2 activity. We surmise that this reduction of NRF2 activity due to an *NRF2* SNP and reduced *NRF2* mRNA expression underlies various disease susceptibilities and/or pathophysiologies in humans. Therefore, we decided to examine the association of the *NRF2* SNP and lung cancer susceptibility.

A few SNPs within the *NRF2* gene have been described (20, 21). Of these SNPs, we focused on SNP rs6721961, located 617 bp upstream from the transcription start site of the gene (Fig. 4A), in this study. This SNP has been reported to be associated with the risk of acute lung injury (21), and its minor allele frequency varies among populations, as shown by the HapMap and 1,000 Genomes Projects (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=6721961). We refer to rSNP rs6721961 as NRF2 rSNP-617 in this study. We conducted a case-control study consisting of 2,701 lung cancer patients (1,987 patients with ADC, 411 patients with squamous cell carcinoma [SQC], and 303 patients with small-cell lung carcinoma [SCC]) and 1,167 controls who had distributions of age, gender, ethnicity, and smoking status similar to those of the patient population (Table 1). All 3,868 case and control subjects were genotyped for the NRF2 rSNP-617, and the association of the genotypes with the risk for development of lung cancers was examined (Table 2). Notably, the frequency of the minor allele (i.e., the A allele, which causes a low level of expression, as described below) for this SNP was more prevalent in lung cancer patients than in controls (Table 2). Homozygotes for the minor allele (A/A) and the recessive mode (A/A homozygotes versus C/A heterozygotes plus C/C homozygotes) for the minor allele showed significant associations with overall lung cancer risk (odds ratio [OR] = 1.54 [$P = 0.0084$] and OR = 1.53 [$P = 0.0083$], respectively).

We next examined the association of NRF2 rSNP-617 with lung cancer risk according to clinicopathological factors. Minor homozygotes showed similarly increased risks for all histological types of lung cancers, including ADC, SQC, and SCC (Table 2). The homozygotes showed a higher risk in ever smokers than in never smokers (OR = 2.57 [$P = 0.00041$] versus OR = 1.13 [$P = 0.58$]) (Table 2).

We further examined the association of NRF2 rSNP-617 with the risk for developing lung ADC according to oncogenic pathway, i.e., *EGFR* and *KRAS* driver gene mutations (31) (Table 3). Minor homozygotes showed similarly increased risks for both lung ADC-bearing *EGFR* and *KRAS* mutations, with the association of ADC with the *EGFR* mutation being significant due to a large number of subjects (OR = 1.55 [$P = 0.0497$] and OR = 1.39 [$P = 0.47$], respectively). In contrast, an increase in the OR was

TABLE 1 Profiles of lung cancer cases and control cases that were analyzed in this study

Variable	Controls	Cases[a]						
			ADC					
				Mutation status				
		All	All	*EGFR*	*KRAS*	None	SQC	SCC
Total no. of cases	1,167	2,701	1,987	600	114	489	411	303
Mean ± SD age (yr)	49 ± 11	59 ± 11	58 ± 11	59 ± 10	59 ± 8	58 ± 10	61 ± 8	62 ± 11
No. (%) of subjects of the following sex:								
Male	725 (62)	1,746 (65)	1,137 (57)	243 (41)	81 (71)	315 (64)	366 (89)	243 (80)
Female	442 (38)	955 (35)	850 (43)	357 (59)	33 (29)	174 (36)	45 (11)	60 (20)
No. (%) of subjects with the following smoking status:								
Never smoker	760 (65)	884 (33)	853 (43)	361 (60)	30 (26)	192 (39)	17 (4)	14 (5)
Ever smoker	407 (35)	1,817 (67)	1,134 (57)	239 (40)	84 (74)	297 (61)	394 (96)	289 (95)

[a] ADC, adenocarcinoma; SQC, squamous cell carcinoma; SCC, small-cell carcinoma.

not evident for lung ADC without *EGFR* and *KRAS* mutations (OR = 1.14, $P = 0.61$). These results therefore indicate that minor homozygotes (A/A) of the NRF2 rSNP-617 are associated with the risk for lung cancers, especially ever smokers (Fig. 4B). Notably, in lung ADC cases, the association was evident in cancers harboring *EGFR* mutations.

NRF2 rSNP-617 affects gene expression in lymphocytes. The NRF2 rSNP-617 coincides with the ARE motif, which is important

TABLE 2 Genotype distribution for the rs6721961 SNP between controls and cancer cases

Category[a]	Genotype	No. (%) of subjects		Adjusted OR (95% CI)	*P*
		Controls	Cancer cases		
All	C/C	627 (53.7)	1,466 (54.3)	Reference	
	C/A	477 (40.9)	1,026 (38.0)	1.02 (0.87–1.19)	0.85[b]
	A/A	63 (5.4)	209 (7.7)	1.54 (1.12–2.16)	0.0084[b]
	Dominant			1.08 (0.92–1.26)	0.34[b]
	Recessive			1.53 (1.11–2.12)	0.0083[b]
ADC	C/C		1,071 (53.9)	Reference	
	C/A		761 (38.3)	1.02 (0.87–1.21)	0.77[b]
	A/A		155 (7.8)	1.55 (1.12–2.18)	0.0088[b]
	Dominant			1.09 (0.93–1.27)	0.30[b]
	Recessive			1.53 (1.11–2.13)	0.0092[b]
SQC	C/C		230 (56.0)	Reference	
	C/A		149 (36.3)	0.89 (0.66–1.21)	0.46[b]
	A/A		32 (7.8)	2.05 (1.11–3.85)	0.023[b]
	Dominant			1.00 (0.75–1.33)	0.99[b]
	Recessive			2.19 (1.18–4.12)	0.013[b]
SCC	C/C		165 (54.5)	Reference	
	C/A		116 (38.3)	1.00 (0.72–1.39)	0.99[b]
	A/A		22 (7.3)	1.82 (0.91–3.68)	0.092[b]
	Dominant			1.08 (0.79–1.48)	0.63[b]
	Recessive			1.83 (0.93–3.61)	0.082[b]
Never smoker	C/C	408 (53.7)	476 (53.8)	Reference	
	C/A	302 (40.0)	344 (39.0)	1.12 (0.89–1.41)	0.33[c]
	A/A	50 (6.6)	64 (7.2)	1.19 (0.77–1.83)	0.44[c]
	Dominant			1.13 (0.91–1.41)	0.26[c]
	Recessive			1.13 (0.74–1.73)	0.58[c]
Ever smoker	C/C	219 (53.8)	990 (54.5)	Reference	
	C/A	175 (43.0)	682 (37.5)	0.94 (0.75–1.18)	0.58[c]
	A/A	13 (3.2)	145 (8.0)	2.48 (1.42–4.70)	8.9×10^{-4}[c]
	Dominant			1.05 (0.84–1.31)	0.68[c]
	Recessive			2.57 (1.49–4.86)	4.1×10^{-4}[c]

[a] ADC, adenocarcinoma; SQC, squamous cell carcinoma; SCC, small-cell carcinoma.
[b] Adjusted for sex, age, and smoking status.
[c] Adjusted for sex and age.

TABLE 3 Association of the rs6721961 SNP and risk for development of lung ADC with or without the *EGFR* or *KRAS* mutation

Category	Genotype	No. (%) of subjects		Adjusted OR[a] (95% CI)	*P*
		Controls	Cancer cases		
EGFR mutation	C/C	627 (53.7)	327 (54.5)	Reference	
	C/A	477 (40.9)	224 (37.3)	1.00 (0.80–1.25)	1.00
	A/A	63 (5.4)	49 (8.2)	1.55 (1.00–2.38)	0.0497
	Dominant			1.07 (0.86–1.32)	0.56
	Recessive			1.55 (1.01–2.36)	0.044
KRAS mutation	C/C		63 (55.3)	Reference	
	C/A		44 (38.6)	0.95 (0.62–1.44)	0.80
	A/A		7 (6.1)	1.39 (0.54–3.10)	0.47
	Dominant			0.99 (0.66–1.48)	0.97
	Recessive			1.55 (0.61–3.44)	0.33
None	C/C		273 (55.8)	Reference	
	C/A		190 (38.9)	0.99 (0.78–1.25)	0.95
	A/A		26 (5.3)	1.14 (0.68–1.90)	0.61
	Dominant			1.00 (0.80–1.26)	0.95
	Recessive			1.14 (0.68–1.87)	0.61

[a] Adjusted for sex, age, and smoking status.

for *NRF2* expression in a feed-forward activation mechanism. Because the A allele-containing ARE is mutated, the transcription level of the *NRF2* gene is expected to be lower in the A allele case than in the C allele case, but no conclusive answer to whether the NRF2 rSNP-617 affects transcription of the *NRF2* gene *in vivo* has been obtained. To address this issue, we quantified the *NRF2* mRNA in immortalized human lymphocytes with distinct NRF2 rSNP-617 genotypes (Fig. 4C). We found that the *NRF2* mRNA levels were significantly lower in A/A homozygotes than in C/A heterozygotes and C/C homozygotes by approximately 40% ($P = 0.031$ and 0.049, respectively). No significant difference was observed between C/C homozygotes and C/A heterozygotes ($P = 0.47$), indicating that a homozygous nucleotide change from C to A at NRF2 rSNP-617 significantly decreased *NRF2* mRNA expression. Consistent with the NRF2 mRNA level, the levels of expression of *tert*-butylhydroquinone-induced NRF2 protein (see Fig. S4 in the supplemental material) and *NQO1* mRNA (see Fig. S5 in the supplemental material) were lower in A/A homozygote than in C/C genotype lymphocytes. These results strongly support the notion that the level of *NRF2* gene transcription is important for the role of NRF2 in cytoprotection, including cancer prevention (Fig. 4B).

DISCUSSION

In this study, we showed clinical and experimental lines of evidence that the final Nrf2 protein level in cells is under dual regulation at the protein degradation level and gene transcription level. The characteristic phenotypes of *Keap1*-null mice, i.e., hyperkeratosis and growth retardation, which are attributable to high Nrf2 activity, are significantly improved by deletion of a single allele of the *Nrf2* gene. Under physiological conditions (in the presence of Keap1-dependent Nrf2 degradation), a decrease in the *Nrf2* mRNA level markedly attenuates the final Nrf2 protein level, which in turn increases the susceptibility of mice to a wide range of xenobiotics. Conversely, when the Nrf2 mRNA level is increased, the Nrf2 protein level is enhanced and the cellular defense against oxidative stress is augmented. In excellent agreement with these results, we found that minor A/A homozygotes of NRF2 rSNP-617 exhibit significantly decreased *NRF2* gene expression and, consequently, increased the risk of lung cancers, especially in ever smokers. Thus, as summarized in Fig. 5, coordinated synthesis and degradation of Nrf2 are critically important for the maintenance of cellular redox homeostasis. Of note, we verified in this study that the transcription level of the *NRF2* gene is indeed important for the roles that NRF2 plays in cytoprotection.

The experiments utilizing genetically engineered mice demonstrate that a decrease in the *Nrf2* transcript to approximately half of its level is physiologically critical. This observation supports the contention that minor A/A homozygotes of NRF2 rSNP-617 are susceptible to lung cancers because of the 40% reduction in the *NRF2* transcript. Notably, changes in *Nrf2* transcript level alter the Nrf2 protein level, even in the basal state, in which Keap1 actively degrades Nrf2. This observation has led us to consider the kinetic properties of Keap1-dependent degradation of Nrf2. We surmise two possible models here. One is the threshold model, in which the Keap1-based ubiquitin E3 ligase system degrades Nrf2 efficiently and completely if its abundance is below a certain threshold. The other is the probability model, in which the Keap1-based ubiquitin ligase system degrades a certain ratio of Nrf2 irrespective of its abundance. Our present results support the latter model, as the status of Nrf2 synthesis exquisitely reflects the Nrf2 protein level, especially under basal conditions.

The analysis of lung cancer patient cohort and non-cancer patient populations revealed that NRF2 rSNP-617 has an association with susceptibility to lung cancer, especially for ever smokers. Although smoking is the top-ranked risk factor for lung cancer, little is known about genetic variations that increase the cancer risk related to smoking. Previous large-scale genome-wide association studies revealed the associations between variations in the nicotine receptor gene (32, 33) or the CYP1A1 and CYP2A6 detoxification enzyme genes (34) and susceptibility in smoking-associated lung cancers. Because oxidative stress has been well established to be one of the main factors in smoking-associated carcinogenesis, the result of our clinical study is in very good agreement with the function of Nrf2 as a key regulator of the cellular response against oxidative stresses.

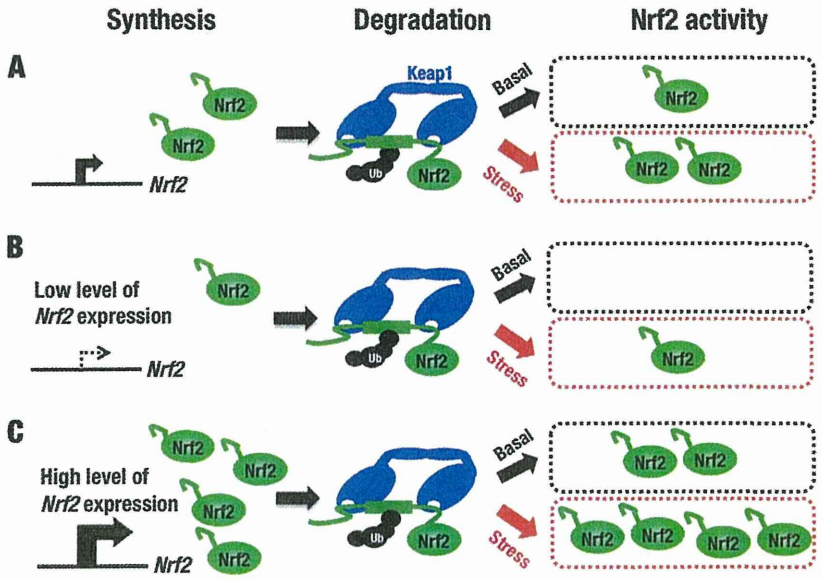

FIG 5 Dual regulation of Nrf2 activity by synthesis and degradation. (A) When an adequate level of the *Nrf2* transcript is supplied, the Nrf2 protein is maintained at low levels by Keap1-mediated degradation under the basal condition, and a relatively high level of the Nrf2 protein is accumulated after the inactivation of Keap1. (B) When the *Nrf2* transcription level is low, the Nrf2 protein is decreased in both the basal and induced states. (C) When a high *Nrf2* transcript level is achieved, a large amount of Nrf2 is produced in both the basal and induced states.

In addition to NRF2 rSNP-617, there appear to be other causes that result in a reduction of Nrf2 synthesis. Indeed, *Nrf2* expression is decreased in aged smokers and patients with chronic obstructive pulmonary diseases (35). We showed that transgenic overexpression of Nrf2 significantly increases the Nrf2 protein level and makes cells resistant to oxidative stress. These observations suggest that induction of the Nrf2 transcript is an effective approach for enhancing the activity of Nrf2. To date, several factors have been found to increase the Nrf2 transcript level, including Nrf2 itself by autoactivation (19), the aryl hydrocarbon receptor (36), and Jun (37).

Increasing numbers of studies have revealed that NRF2 is also involved in the malignant progression of various human cancers (13–15). Constitutive activation of NRF2 gives a strong advantage to cancer cells by conferring chemo- and radioresistance and accelerating proliferation (38–40). A recent study demonstrated that NRF2 is constitutively activated in lung cancer cells bearing *EGFR* mutations (41). Transcriptional activation of *NRF2* by the RAS oncogenic pathway also substantially contributes to the enhancement of NRF2 activity in cancer (42). Accordingly, we expect that *EGFR* and *KRAS* mutations are associated with NRF2 rSNP-617. We found that there are associations of the minor A/A homozygotes with the risk for developing lung cancers with *EGFR* and *KRAS* mutations (Table 3). One plausible explanation for this association is that *EGFR* and *KRAS* mutations may compensate for the compromised transcription of *NRF2*, allowing the A/A homozygous cancer cells to achieve a sufficiently high level of NRF2 activity.

In summary, Nrf2 has been considered a transcription factor which is mainly regulated by a posttranslational derepression mechanism. In this study, we found that weakened transcription of the *Nrf2* gene provides a basis for the development of lung cancers, possibly through the reduced expression of cytoprotective enzymes. This observation leads to the notion that in addition to control over proteasomal degradation and derepression from degradation/repression, transcriptional regulation of the *Nrf2* gene in response to various signals/insults is an important pathway in determining cellular Nrf2 levels. Collectively, the contribution of NRF2 to these physiological and pathological processes is regulated at various nodes, and the delineation of these mechanisms is a critical step toward a better understanding of our defense machinery.

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Molecular Mechanisms Underlying Oncogenic *RET* Fusion in Lung Adenocarcinoma

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Background: Oncogenic *RET* fusion, caused by an inversion in chromosome 10, was recently identified as a driver mutation for the development of lung adenocarcinoma (LADC). Nevertheless, the molecular mechanism(s) underlying the rearrangement of the *RET* locus during lung carcinogenesis are unknown.

Methods: Genomic segments containing breakpoint junctions for *RET* fusions were cloned and analyzed by genomic polymerase chain reaction and genome capture sequencing using a next-generation sequencer to identify the mechanisms involved in DNA strand breaks and illegitimate joining of DNA ends. Of the 18 cases studied, 16 were identified by screening 671 LADC cases and two were previously published.

Results: Almost all (17 of 18, 94%) of the breakpoints in *RET* were located within a 2.0-kb region spanning exon 11 to intron 11 and no breakpoint occurred within 4 bp of any other. This suggested that as in papillary thyroid carcinoma, DNA strand breaks formed at nonspecific sites within this region trigger *RET* fusion. Just over half of the *RET* fusions in LADC (10 of 18, 56%) were caused by simple reciprocal inversion, and two DNA-repair mechanisms, namely nonhomologous end joining and break-induced replication, were deduced to have contributed to the illegitimate joining of the DNA ends.

Conclusions: Oncogenic *RET* fusion in LADC occurs through multiple pathways and involves the illegitimate repair of DNA strand breaks through mechanisms different from those identified in papillary thyroid carcinoma, where *RET* fusion also functions as a driver mutation.

Key Words: Lung adenocarcinoma, Molecular target therapy, Personalized medicine, *RET*, Gene fusion, DNA strand break.

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Oncogenic fusion of *RET* (rearranged during transfection) tyrosine kinase gene partnered with *KIF5B* (kinesin family member 5B) and *CCDC6* (coiled-coil domain containing 6) was identified as a novel druggable driver mutation in a small subset (1–2%) of patients with lung adenocarcinoma (LADC).[1–4] Vandetanib (ZD6474) and cabozantinib (XL184), two U.S. Food and Drug Administration–approved inhibitors of the *RET* tyrosine kinase showed therapeutic responses in a few patients with *RET* fusion-positive LADC.[5,6] Several clinical trials are currently underway to examine the therapeutic effects of RET tyrosine kinase inhibitors, including these two drugs.[7,8] *RET* fusions are generated by pericentric (includes the centromere, with a breakpoint in each arm) and paracentric (not including the centromere, with both breaks in the same arm) inversions of chromosome 10 (Fig. 1*A*). As the majority of *RET* fusion-positive patients are never-smokers,[3,9,10] cigarette smoking does not cause a predisposition. Therefore, the mechanism(s) responsible for the rearrangement of the *RET* locus are unknown. Elucidation of such a mechanism(s) may help to identify risk factors that can be modified or other preventive methods that can reduce the incidence of LADC; however, no such mechanism has been identified.[8]

Analyzing the breakpoints and structural aberrations in cancer cell genomes is a powerful method of identifying the underlying molecular mechanism(s) responsible, as the breakpoints retain "traces" of the DNA strand breaks and the illegitimate joining of DNA ends.[11–13] In fact, several studies have characterized the structure of the breakpoints responsible for the *ELE1* (also known as *RFG, NCOA4*, and *ARA70*)-*RET* oncogenic fusion in cases of papillary thyroid cancer (PTC), including post-Chernobyl irradiation-induced cases, to elucidate the mechanism underlying chromosome 10 inversion generating this fusion (Fig. 1*A*).[14–17]

Here, we examined the molecular processes underlying chromosome inversions that generate oncogenic *RET* fusions in LADC by cloning genomic segments containing breakpoint junctions and by comparing their structures with those identified in PTC. The results will increase our understanding of how *RET* fusions are generated and will also have implications for diagnosis of *RET* fusion-positive LADCs.

PATIENTS AND METHODS

Patient Samples

Fourteen frozen tissues (13 surgical specimens and a pleural effusion) and two methanol-fixed paraffin-embedded tissues from surgical specimens were obtained from the

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