SNP (–617C>A) in ARE-Like Loci of the NRF2 Gene: A New Biomarker for Prognosis of Lung Adenocarcinoma in Japanese Non-Smoking Women

Yasuko Okano1,2, Uru Nezu1,3, Yasuaki Enokida1,4, Ming Ta Michael Lee5,6, Hiroko Kinoshita1, Alexander Lezhava1, Yoshihide Hayashizaki1, Satoshi Morita6, Masataka Taguri7, Yasushi Ichikawa2, Takeshi Kaneko1,8, Yutaka Natsumeda9, Tomoyuki Yokose10, Haruhiko Nakayama11, Yohei Miyagi12, Toshihisa Ishikawa1*

1 Omics Science Center, RIKEN Yokohama Institute, Yokohama, Japan, 2 Department of Clinical Oncology, Yokohama City University Graduate School of Medicine, Yokohama, Japan, 3 Department of Clinical Pharmacology and Therapeutics, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan, 4 Division of Thoracic and Visceral Organ Surgery, Gunma University Graduate School of Medicine, Maehashi, Japan, 5 Laboratory for International Alliance on Genomic Research, RIKEN Center for Integrative Medical Sciences, Yokohama, Japan, 6 Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, 7 Department of Biostatistics and Epidemiology, Yokohama City University Medical Center, Yokohama, Japan, 8 Respiratory Disease Center, Yokohama City University Medical Center, Yokohama, Japan, 9 Department of Clinical Research, Yokohama City University Graduate School of Medicine, Yokohama, Japan, 10 Department of Pathology, Kanagawa Cancer Center Research Institute, Yokohama, Japan, 11 Department of Thoracic Surgery, Kanagawa Cancer Center Research Institute, Yokohama, Japan, 12 Kanagawa Cancer Center Research Institute, Yokohama, Japan

Abstract

Purpose: The transcription factor NRF2 plays a pivotal role in protecting normal cells from external toxic challenges and oxidative stress, whereas it can also endow cancer cells resistance to anticancer drugs. At present little information is available about the genetic polymorphisms of the NRF2 gene and their clinical relevance. We aimed to investigate the single nucleotide polymorphisms in the NRF2 gene as a prognostic biomarker in lung cancer.

Experimental Design: We prepared genomic DNA samples from 387 Japanese patients with primary lung cancer and detected SNP (c.–617C>A; rs6721961) in the ARE-like loci of the human NRF2 gene by the rapid genetic testing method we developed in this study. Then we analyzed the association between the SNP in the NRF2 gene and patients’ overall survival.

Results: Patients harboring wild-type (WT) homozygous (c.–617C/C), SNP heterozygous (c.–617C/A), and SNP homozygous (c.–617A/A) alleles numbered 216 (55.8%), 147 (38.0%), and 24 (6.2%), respectively. Multivariate logistic regression models revealed that SNP homozygote (c.–617A/A) was significantly related to gender. Its frequency was four-fold higher in female patients than in males (10.8% female vs 2.7% male) and was associated with female non-smokers with adenocarcinoma. Interestingly, lung cancer patients carrying NRF2 SNP homozygous alleles (c.–617A/A) and the 309T (WT) allele in the MDM2 gene exhibited remarkable survival over 1,700 days after surgical operation (log-rank p = 0.021).

Conclusion: SNP homozygous (c.–617A/A) alleles in the NRF2 gene are associated with female non-smokers with adenocarcinoma and regarded as a prognostic biomarker for assessing overall survival of patients with lung adenocarcinoma.

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* E-mail: toshihisa.ishikawa.r@gmail.com

Introduction

Lung cancer is the leading cause of cancer-related death in many industrial countries. It is classified into two major types, namely, small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC). While long-term exposure to cigarette smoke is the most common cause of lung cancer (80–90% of lung cancers), non-smokers account for 10–15% of lung cancer cases, which are often attributed to a combination of genetic and environmental factors [1–3]. The transcription factor NF-E2-related factor 2 (NRF2) is known to control cellular adaptation/protection to reactive oxygen species and electrophiles by inducing antioxidation and detoxification genes [4–6] as well as mediate cancer cell proliferation and drug resistance [7–12]. We have undertaken the present study to examine the clinical impact of the
**NRF2 Gene in Lung Adenocarcinoma**

**Results**

**Clinicopathological Characterization**

The clinicopathological characterization data for the 387 primary lung cancer patients are summarized in Table 1. The patient population comprised 221 men and 166 women, with an overall mean age of 66 years (range 35 to 87 years). The histological type of lung cancer was determined according to the protocol of the third World Health Organization/International Association for the Study of Lung Cancer Classifications [29]. Among the lung cancer patients, 298 were classified as having adenocarcinomas and 89 non-adenocarcinomas. The p-stage was determined by pathological examination of surgical specimens for 376 patients, and their tumours were staged according to the tumor nodes metastasis (TNM) classification of malignant tumours: 292, 46, 35, and 3 patients were respectively classified into stages I, II, III, and IV. For the remaining 11 patients, the p-stage could not be determined. The smoking history was obtained from each lung cancer patient at Kanagawa Cancer Center Research Institute: 154 patients had no smoking history, whereas 233 patients were smokers.

**Preparation of Genotyping Primers for Detection SNP (c.–617 C>A) in NRF2 Gene**

The NRF2 gene is located on the negative strand of genomic DNA at q31.2 of chromosome 2. To create the template for preparation of genotyping primers, we synthesized double stranded DNA encoding a 424-bp region of nt.178129735 to nt.178130158, including the SNP (c.–617 C>A) in the NRF2 gene by means of PCR with human genomic DNA. PCR primers used for the DNA synthesis were GACCACTCTCCGACCTAAAGG (forward) and CGAGATAAAAGAGTTGTTTTGCAGGA (reverse).

| Table 1. Clinicopathological characterization of primary lung cancer patients. |
|---------------------------------|---------|----------------|
| Variable                        | No. of patients | (%)       |
| Gender                          | Male    | 221 (57.1)    |
|                                 | Female  | 166 (42.9)    |
| Age (years old)                 | ≤50     | 25 (6.4)      |
|                                 | >50     | 362 (93.5)    |
| Histopathology                  | Adeno   | 298 (77.0)    |
|                                 | Non-adeno | 89 (23.0)    |
| Smoking                         | Non-smoker | 154 (39.8)  |
|                                 | Smoker  | 233 (60.2)    |
| p-Stage                         | I       | 292 (75.4)    |
|                                 | II      | 46 (11.9)     |
|                                 | III     | 35 (9.0)      |
|                                 | IV      | 3 (0.8)       |
|                                 | Undetermined | 11 (2.8)     |

Ages of all patients, 66.4±9.9 (mean±S.D.). Abbreviation: Adeno, adenocarcinoma. doi:10.1371/journal.pone.0073794.t001

**NRF2 gene and its genetic polymorphisms on the risk and prognosis of lung cancer.**

NRF2 is a “cap’n collar” basic region-leucine zipper (CNC-bZip) transcription factor and plays a pivotal role in the induction of antioxidant response element (ARE)-regulated genes [4–13]. Under non-stressed conditions, NRF2 protein is associated with Kelch-like ECH associating protein 1 (KEAP1) [14]. KEAP1 is known to be a negative regulator of NRF2 by retrieving it in the cytoplasm. Oxidative stress and/or electrophilic attack lead to the dissociation of NRF2 from KEAP1 and thereby the NRF2 protein is translocated into the nucleus. NRF2 together with multiple alignment format (MAF) sequences binds to ARE sequences [15]. Many genes encoding detoxifying and antioxidant enzymes have been found to be regulated by NRF2 [4–6,15–18]. It has recently been reported that NRF2 contributes to malignant phenotypes in cancer cells in vivo, including aggressive cell proliferation, drug resistance, and metabolic re-programming [7–11,19,20]. In this context, the NRF2 gene is considered to play split roles, for example, in the protection of normal cells and progression of cancer malignancy.

In 2004, Yamamoto and colleagues first reported the structure of the NRF2 gene and found three SNPs (–653A>G, –651G>A, and –617C>A) and one triplet repeat polymorphism in its regulatory region [21]. Three years later, Marzec et al. examined the impact of those SNPs on the regulation of NRF2 gene expression [22]. In transient transfection assays, they found that the –617C>A SNP significantly affects basal NRF2 protein levels and its function in vitro [22]. SNP –617C>A was found to be associated with a higher risk of oxidant-induced acute lung injury in humans [22]. It has been reported that a SNP (c.–617C>A) in the ARE-like loci of the human NRF2 gene is important for self induction of the NRF2 gene. NRF2 regulates the transcription of numerous phase II drug-metabolizing enzymes and phase III drug-transporters (e.g., ABCG1, ABCG2, ABCC4, and ABCG2) in response to oxidative stress via direct binding to the ARE sequences in those target genes [23–26]. At present, however, little information is available as to the clinical impact of genetic polymorphisms of the NRF2 gene and the prognosis of lung cancer.

To gain insight into the genetic polymorphisms of the NRF2 gene, we have developed rapid genotyping primer sets by utilizing the SmartAmp method, an isothermal DNA amplification process [27,28]. Among a total of 387 lung cancer patients, we found that SNP (c.–617C>A) in the NRF2 gene is a prognostic biomarker for assessing the gender (female)-related risk of lung adenocarcinomas in the Japanese non-smoking sub-population of lung cancer patients. The epidermal growth factor receptor (EGFR) gene was frequently mutated in those female patients harboring the SNP homozygous SNP allele (–617A/A), suggesting a potential link between the SNP homozygote (–617A/A) and EGFR gene mutations. Furthermore, NRF2 reportedly regulates expression of the MDM2 gene that encodes a negative regulator of p53, and this study shows that lung cancer patients with homozygous SNP alleles (–617A/A) in the NRF2 gene and the 309T (WT) allele in the MDM2 gene had markedly better overall survival. This is the first report providing clinical evidence that homozygous SNP (–617A/A), as one of the intrinsic genetic polymorphisms in the NRF2 gene, is associated with the overall survival of lung cancer patients. Our clinical research data strongly suggest that the SNP homozygous allele (–617A/A) is a useful biomarker for clinical diagnosis.
The resulting PCR product was then inserted into the TA-cloning site of the pGEM®-T Easy Vector (Promega, Madison, WI, USA). The vector was amplified in JM109 High Efficiency Competent Cells (Promega), and vector DNA was purified by using the GeneJET™ Plasmid Miniprep Kit (Fermentas, Thermo Fisher Scientific Inc., Waltham, MA, USA). The sequence of the inserted DNA was analyzed with a laser-based automated DNA sequencer (ABI PRISM 3100 DNA Analyzer, Applied Biosystems Ltd., Tokyo, Japan). We designed a number of SNP-typing primer candidates and repeatedly tested them by using the vector-inserted DNA as a template until we obtained the best primer set.

The schematic illustration in Figure 1A shows the annealing sites of the best primer set, which comprises four different primers, i.e., TP, OP, FP, and BP. The TP primer was designed such that its 3'-end of the primer (see Figure 1A). Furthermore, two SNPs, c.–651G>A and c.653A>G, were not included in the annealing sites of the four primers. Figure 1B depicts the sequences of primers in the WT (–617C)-typing and SNP (–617T)-typing sets. Exciton dye was linked to thymine in the FP primer as symbolized by “Z” in the lower panel of Figure 1B.

Figure 1C shows the time courses of genotyping reactions as functions of fluorescence intensity. By using the genotyping primer sets, we could detect the WT homozygote (–617C/C), WT/SNP heterozygote (–617C/A), and SNP homozygote (–617T/A) in genomic DNA samples. These results were verified by DNA sequence analysis. Namely, we performed sequencing analysis for determination of the WT homozygote (–617C/C), WT/SNP heterozygote (–617C/A), and SNP homozygote (–617A/A) in those genomic DNA samples. We tested 24 samples for each group (i.e., C/C, C/A, and A/A) and confirmed the 100% accordance between the SmartAmp method and the DNA sequencing analysis method.

Detection of SNP (c.–617 C>A) in the NRF2 Gene in Lung Cancer Patients

Using the genomic DNA samples prepared from a total of 387 lung cancer patients, we have detected WT and SNP alleles in the NRF2 gene by the rapid genotyping method described above. Table 2 summarizes these results, showing that 216, 147, and 24 patients could be typed as WT homozygote (–617C/C), WT/SNP heterozygote (–617C/A), and SNP homozygote (–617T/A), respectively. Accordingly, the allele frequency was calculated to be 74.8% and 25.2% for WT (–617C) and SNP (–617A), respectively, when both male and female patients were grouped together. It is of interest to note, however, that the allele frequency of SNP (c.–617A) was 28.6% for female patients, compared with 22.6% for male patients. Indeed, 18 female patients carried the SNP homozygote (–617A/A), a number three-fold higher than that of male patients. The ratio of homozygous SNP (–617A/A) was 10.8% for female patients, about four-fold higher than the 2.7% found in males (Table 2; \( P=0.004 \)). In contrast, the ratios of WT homozygote (–617C/C) and heterozygote (–617 C/A) were moderately higher in male patients than in female patients (Table 2). On the other hand, with respect to smoking experience, the ratio of homozygous SNP (–617A/A) was 10.4% in the non-smoker sub-population, being about three times (\( P=0.021 \)) higher than the ratio (4.5%) observed in the smokers (Table 2).

By multivariate analysis, the SNP homozygote (–617T/A) in the NRF2 gene was found to be independently associated with gender (Table 3). Among a total of 20 adenocarcinoma patients (females+males) carrying the SNP homozygote (–617T/A), 16 patients were females who had no cigarette-smoking experience (Table 4). In contrast, there were no male adenocarcinoma patients in the sub-group of non-smokers carrying the SNP homozygote (–617A/A) (Table 4). These results demonstrate a marked gender difference in terms of non-smoking patients carrying the SNP homozygote (–617A/A).

To gain more insight into the gender difference among 24 patients homozygous for the SNP (c.–617A/A), we have analyzed the genetic polymorphisms of human CYP2A6*4 (whole gene deletion) and the numbers of (GT)\(_n\) repeats in the HO-1 gene 5′-flanking region. These data are summarized in Table 5. CYP2A6*4/*4 (whole gene deletion) was found in only one female patient in this subgroup (Table 5), whereas among 387 lung cancer patients, CYP2A6*4/*4 was detected in seven patients (1.8%) with lung adenocarcinoma. The number of (GT)\(_n\) repeats in the HO-1 gene 5′-flanking region varied greatly (14 to 34 repeats) among these 24 patients.

Interestingly, as shown in Table 5, either exon 19 or exon 21 of the EGFR gene was frequently mutated in female patients who were non-smokers and had homozygous SNP alleles (–617A/A). Two of those patients were diagnosed as p-stage IIA (cases 1 and 16). They were treated first surgically and then with gefitinib. These patients had no relapse over 1879 days (case 1) and 939 days (case 16).

Association of SNP (c.–617 C>A) in the NRF2 Gene with Overall Survival of Lung Cancer Patients

We investigated a potential association between SNP (c.–617 C>A) in the NRF2 gene and the overall survival of lung cancer patients, since we could obtain follow-up information on 369 patients among the total of 387 lung cancer patients over 1,700 days after surgical operation at Kanagawa Cancer Center. A survival Kaplan-Mayer plot is shown in Figure 2A. Our univariate analysis has revealed that lung cancer patients (p-stages I to IV) carrying homozygous SNP alleles (–617A/A) in the NRF2 gene experienced significantly better overall survival, as compared with patients with heterozygous alleles (c.–617C/A) (log-rank \( P=0.021 \)). In contrast, no association was found between patients with homozygous WT alleles (–617C/C) and those with heterozygous alleles (c.–617C/A) (Figure 2A). It is important to mention that one female patient with the homozygous SNP (–617A/A) (case 5 in Table 5) died due to primary pancreatic cancer during the follow-up study.

We further analyzed the associations between the NRF2 genotypes and patients’ overall survival in the p-stage I of non-small cell lung cancer (NSCLC) including adenocarcinoma, squamous cell cancer, and large cell cancer. In the case of stage I, patients (n = 285) were treated by surgical excision of the tumor, with no follow-up treatment by adjuvant therapy or chemother-apy. Nonetheless, patients harboring homozygous alleles (–617A/A) in the NRF2 gene exhibited the best record of overall survival among the members of these three different allele groups (Figure 2B).

Potential Link between NRF2 and MDM2 Genotypes

NRF2 reportedly regulates expression of the MDM2 gene that encodes a crucial negative regulator of p53. To gain insight into a potential link between MDM2 and NRF2 genotypes, we have analyzed the SNP (c.309T>G) in the MDM2 gene as well as the SNP (–617C>A) in the NRF2 gene using the genomic DNA samples from lung cancer patients. Table 6 summarizes the corresponding results, where the number of patients harboring T/T, T/G, or G/G genotype in the MDM2 gene has been given for each genotype of the NRF2 gene (i.e., –617C/C, C/A, or A/A). In the genotype groups of –617C/C and –617C/A, the 309G (SNP)
A Chromosome 2

NRF2 gene

Exon 1

5' FP 3' 5' BP 3' 5' OP 5'

Exon 1

ARE-like

MZF1

SNP (c.-617C>A)
SNP (c.-651G>A)
SNP (c.-653A>G)

B WT (c.-617C)-typing primer set

5'-GTGGGCCCTGCGTGGAGATGAGGCGAGCGAGCGCAGCTCGGTGGCAGTCAACCTGAAG

Exciton

CCTCCTGTGAGACCTCCACGTGTTCTCCATCTCTCATAAGCTCAGTGTCGTC-3'

SNP (c.-617A)-typing primer set

5'-GTGGGCCCTGCGTGGAGATGAGGCGAGCGAGCGCAGCTCGGTGGCAGTCAACCTGAAG

Exciton

CCTCCTGTGAGACCTCCACGTGTTCTCCATCTCTCATAAGCTCAGTGTCGTC-3'

TP (c.-617C) 5'-CGCCGCCATGGAGACACGTGGGAGTTCAGAGGG-3'
TP (c.-617A) 5'-CTCCGCGCTGGAGACACGTGGGAGTTCAGAGGG-3'
FP (c.-617C) 5'-GATCCCGCTGAGGACACGTGGGAGTTCAGAGGG-3'
FP (c.-617A) 5'-TTTATATATATAAACCTCGCTTAGGGAGATG-3'
Exciton 5'-AGGACGCTGAGATGCTGCTCTTAGGGAAGTGGGA-3'
BP 5'-CGTGGTCGTCAGTCAACC-3'
OP 5'-GACGACCTGAGCTTAGGA-3'

C

Fluorescence (a.u.)

Time (min)

-617C/C

-617C/A

-617A/A

G C C G G G C G T

G C C G G G A G C T

G C C G G A G C T
allele frequency was 0.606 and 0.541, respectively. In contrast, the SNP allele frequency was found to be markedly lower (0.333) in the genotype group of $NRF2$–617A/A. In the case of adenocarcinoma, female patients harboring 309T/T, T/G, and G/G genotype in the $MDM2$ gene were 7, 8, and 1, respectively (Table S1); most patients were harboring the 309T (WT) allele in the $MDM2$ gene.

**Discussion**

**SNP (c.–617C>A) in the $NRF2$ Gene and Female Non-smokers with Adenocarcinoma**

Recent genome-wide association studies (GWAS) have identified several loci associated with lung cancer susceptibility in never-smoking women in Asia; they were, 5p15.33 ($rs2736100$) [30], 6p21.3 ($rs3817963$) [30], 3q28 ($rs10937405$ and $rs4488809$) [31], 1q25.2 ($rs7086803$) [32], 6q22.2 ($rs9387478$) [32], and 6p21.32 ($rs2951815$) [32].

In the present study, differing from those reports, we found that SNP (c.–617C>A) in the $NRF2$ gene located on chromosome 2q31.2 is associated with Japanese non-smoking female patients with adenocarcinoma and their overall survival. While the allele frequency of SNP c.–617C>A in the $NRF2$ gene was estimated to be 25.2%, non-smoking females harboring homozygous alleles (–617A/A) had a markedly higher incidence of the $NRF2$ gene (Table 4), suggesting a potential link between the SNP homozygote (–617A/A) and $EGFR$ gene mutations.

Recent studies have demonstrated that mutations in the tyrosine kinase domain of the $EGFR$ are frequently found among non-smoker patients with NSCLC [33]. Approximately 90% of these mutations are exon 19 deletions or exon 21 L858R point mutations in the tyrosine kinase domain [34]. In the vast majority of cases, $EGFR$ mutations are non-overlapping with other oncogenic mutations (e.g., $KRAS$ mutations, $ALK$ rearrangements) found in NSCLC [34]. A large randomized clinical study named the “iPAS$SA$ Pan-Asian Study (IPASS)” has reported that high rates of mutations in the $EGFR$ gene were observed in female NSCLC patients without smoking experience [35]. A high incidence of $EGFR$ gene mutations was reported in female non-smokers with adenocarcinoma of lung: 30–40% in East Asians, as compared with 15% in Caucasians [36–38]. Both $EGFR$ gene mutations and homozygous SNP alleles (–617A/A) in the $NRF2$ gene were frequently observed in Japanese female adenocarcinoma patients without smoking experience (Table 4). As shown in Table 7, ethnic group-dependent difference was observed in the $NRF2$ genotype, where the frequency of the –617A allele is high in Japanese, Taiwanese, and Chinese populations. Thus, it is of great interest to investigate the link between the SNP homozygote (–617A/A) and $EGFR$ gene mutations and to gain insight into the underlying molecular mechanism.

**SNP (c.–617C>A) in the $NRF2$ Gene as a Biomarker for Prognosis of Lung Cancer**

The $NRF2$ gene is regarded as a double-edged sword. It plays an important role in protecting normal cells from external toxic challenges and oxidative stress, whereas it can also endow cancer cells resistance to anticancer drugs. Recently it has been reported that $NRF2$ contributes to the malignant phenotypes of cancer cells in vitro, including aggressive cell proliferation, drug resistance, and metabolic re-programming [8,20]. Indeed, $NRF2$ activation is involved in the emergence of cancer resistance to various anticancer drugs by transcriptionally activating a battery of self-defense genes, such as those encoding antioxidant enzymes, phase

**Table 2. Classification of primary lung cancer patients with respect to $NRF2$ genotypes, gender, and histopathology.**

| $NRF2$ gene SNP (c.–617C>A) | C/C | C/A | A/A | P-value* |
|-----------------------------|-----|-----|-----|---------|
| Patients                    | 216 | 147 | 24  | 0.62    |
| Gender*                     |     |     |     |         |
| Male                        | 127 | 88  | 6   | 0.27    |
| Female                      | 89  | 59  | 18  | 0.004   |
| Histopathology              |     |     |     |         |
| Adeno                       | 164 | 114 | 20  | 0.67    |
| Non-adeno                   | 52  | 33  | 4   | 0.68    |
| Smoking behavior*           |     |     |     |         |
| Smoker                      | 133 | 92  | 8   | 0.45    |
| Non-smoker                  | 83  | 55  | 16  | 0.021   |
| p-Stage                     |     |     |     |         |
| I                           | 156 | 114 | 22  | 0.75    |
| II                          | 28  | 16  | 2   | 0.43    |
| III                         | 23  | 12  | 0   | 0.00    |
| IV                          | 1   | 2   | 0   | 0.46    |

*p-value calculated by Fisher’s exact test. Abbreviation: Adeno, adenocarcinoma.

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**Table 3. Logistic regression analysis for evaluation of the association among homozygous SNP alleles (–617A/A) in the $NRF2$ gene and gender/smoking experience of lung cancer patients.**

| Variable   | P     | Odds Ratio | 95% CI       |
|------------|-------|------------|--------------|
| Gender     | 0.041 | 3.48       | 1.05 to 11.51|
| Smoking    | 0.463 | 0.66       | 0.22 to 2.00 |

Abbreviation: CI, confidence interval. Gender code: 1 = female; 0 = male. Smoking experience code: 1 = smoker; 0 = non-smoker. The multivariate logistic regression analysis was performed under two categories, i.e., the gender (female and male) and the smoking experience (smoker and non-smoker).

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**Figure 1. SmartAmp-based detection of SNP (c.–617C>A) in the $NRF2$ gene.** SNP (c.–617C>A) resides in the promoter region of the $NRF2$ gene on chromosome 2q31.2. Panel A presents a schematic illustration of annealing sites of the TP, FP, and OP primers. Panel B shows cDNA encoding a partial sequence of the $NRF2$ gene and primer annealing sites. Panel C depicts the results of SNP detection. a.u. = arbitrary unit.
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II detoxifying enzymes, and ABC transporters [23–25]. ABCG2 is known to mediate the efflux of gefitinib (Iressa®) from cancer cells [39], and its expression is regulated by NRF2 [26] and the EGFR-tyrosine kinase cascade [40,41].

As revealed in the Kaplan-Meier plot (Figure 2), lung cancer patients (both females and males) with homozygous SNP alleles (–617A/A) in the NRF2 gene had markedly high overall survival. Univariate analysis showed a significant difference between the –617 A/A and –617 C/A groups in terms of overall survival (log-rank P=0.021). It is important to note that, except for one patient (case 5 in Table 5) who died because of primary pancreatic cancer, all of the adenocarcinoma patients with homozygous SNP alleles (–617A/A) in the NRF2 gene survived over 1,000 days after surgical excision of the tumor that was followed up with neither adjuvant therapy nor chemotherapy, even when p-stage I patients were considered alone (Figure 2B). To our knowledge, this is the first report providing clinical evidence that homozygous SNP (–617A/A), as one of the intrinsic genetic polymorphisms in the NRF2 gene, is associated with overall survival of lung cancer patients.

### Table 4. Classification of primary lung cancer patients with respect to NRF2 genotypes, smoking behavior, adenocarcinoma, and gender.

| NRF2 gene SNP (–617) | C/C | C/A | A/A | P-value* |
|----------------------|-----|-----|-----|----------|
| Patients (M+F)       | 216 | 147 | 24  |
| Smoking behavior     |     |     |     |          |
| Smoker (M)           | 114 | 75  | 6   |
| Smoker (F)           | 19  | 17  | 2   |
| Non-smoker (M)       | 13  | 13  | 0   |
| Non-smoker (F)       | 70  | 42  | 16  | 0.014    |
| Adenocarcinoma       |     |     |     |          |
| Smoker (M)           | 75  | 48  | 4   |
| Smoker (F)           | 15  | 16  | 0   |
| Non-smoker (M)       | 11  | 13  | 0   |
| Non-smoker (F)       | 63  | 37  | 16  | 0.003    |

*P-values were calculated by Fisher’s exact test. Abbreviation: M, male; F, female. doi:10.1371/journal.pone.0073794.t004

### Table 5. Clinicopathological profiling of 24 patients harboring homozygous SNP alleles (–617A/A) in the NRF2 gene.

| Case | Histology | p stage | Age | Gender | smoker | (GT)n repeats | CYP2A6 | EGFR mutation | Gefitinib therapy |
|------|-----------|---------|-----|--------|--------|---------------|--------|---------------|-------------------|
| 1    | Ad        | IIA     | 74  | F      | non-smoker | 19,30          | Wt     | Exon 21       | Yes               |
| 2    | Ad        | IA      | 53  | F      | non-smoker | 23             | *4/*4  | Exon 19       |                   |
| 3    | Ad        | IB      | 70  | F      | non-smoker | 24             | Wt     | Exon 21       |                   |
| 4    | Mix       | IB      | 63  | F      | non-smoker | 34             | Wt     | Exon 21       |                   |
| 5    | Ad        | IB      | 61  | F      | non-smoker | 23             | Wt     | Exon 19       |                   |
| 6    | Ad        | IB      | 72  | F      | non-smoker | 22             | Wt     | Exon 21       |                   |
| 7    | Ad        | IA      | 40  | F      | non-smoker | 19             | Wt     | Exon 19       |                   |
| 8    | Ad        | IA      | 71  | F      | non-smoker | 17             | Wt     | Exon 21       |                   |
| 9    | Ad        | IA      | 45  | F      | non-smoker | 30             | Wt     | Exon 21       |                   |
| 10   | Ad        | IA      | 74  | F      | non-smoker | 30             | Wt     | Exon 21       |                   |
| 11   | Ad        | IA      | 73  | F      | non-smoker | 22             | Wt     | Exon 19       |                   |
| 12   | Ad        | IA      | 69  | F      | non-smoker | 22             | Wt     | Exon 21       |                   |
| 13   | Ad        | IA      | 62  | F      | non-smoker | 28             | Wt     | None          |                   |
| 14   | Ad        | IA      | 73  | F      | non-smoker | 14             | Wt     | Exon 19       |                   |
| 15   | Ad        | IA      | 63  | F      | non-smoker | 15             | Wt     | Exon 19       |                   |
| 16   | Ad        | IIA     | 73  | F      | non-smoker | 30             | Wt     | None          | Yes               |
| 17   | Sq        | IA      | 72  | F      | smoker     | 20             | Wt     | None          |                   |
| 18   | Ple       | IA      | 75  | F      | smoker     | 29             | Wt     | None          |                   |
| 19   | Ad        | IA      | 74  | M      | smoker     | 23,27          | Wt     | None          |                   |
| 20   | Sq        | IA      | 78  | M      | smoker     | 23             | Wt     | None          |                   |
| 21   | Ad        | IA      | 65  | M      | smoker     | 30             | Wt     | Exon 21       |                   |
| 22   | Sq        | IB      | 75  | M      | smoker     | 20             | Wt     | None          |                   |
| 23   | Ad        | IA      | 77  | M      | smoker     | 28,31          | Wt     | Exon 21       |                   |
| 24   | Ad        | IA      | 80  | M      | smoker     | 29,30          | Wt     | Exon 19       |                   |

Abbreviation: Ad, adenocarcinoma; Mix, adenocarcinoma and squamous cell carcinoma; Ple, pleomorphic carcinoma; Sq, squamous cell carcinoma; F, female; M, male; Wt, wild type.

1Patient (case 5) died because of primary pancreatic cancer. doi:10.1371/journal.pone.0073794.t005
MAF [22], it is anticipated that the homozygote –617A/A significantly attenuates the positive feedback loop of transcriptional activation of the NRF2 gene. It has recently been reported that NRF2 regulates the basal expression of the murine double minute-2 (Mdm2) gene [42]. Since human MDM2 is an oncoprotein that binds to p53 protein and inactivates the tumor suppressor activity of p53 [43], NRF2 can indirectly contribute to p53-mediated cell cycle control and/or apoptosis [44]. One SNP in the MDM2 promoter region, a T-to-G change at nucleotide c.309 (rs2279744) in the first intron, increases the binding affinity toward stimulatory protein 1 (Sp1) and results in higher expression levels of MDM2 protein [45]. This, in turn, attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans [45]. Asians, including Japanese, have higher frequencies of the 309G allele as compared with African-Americans and Caucasians [46]. It has been reported that this polymorphism in the MDM2 gene is associated with the prognosis for several types of tumors, including lung cancer [47].

As demonstrated in Table 6, the 309G (SNP) allele frequency of the MDM2 gene was markedly lower (0.333) in the genotype group of NRF2 –617A/A, as compared with those observed in the genotype groups of NRF2 –617C/C and –617C/A. It is suggested that lung cancer patients harboring both the 309T (WT) allele in the MDM2 gene and the –617A allele in the NRF2 gene have better prognosis owing to well-controlled tumor suppression via

**Table 6. Classification of primary lung cancer patients with respect to genotypes of NRF2 and MDM2 genes.**

| NRF2 (–617) | C/C | C/A | A/A |
|-------------|-----|-----|-----|
| Patients (N) | 216 | 147 | 24  |
| MDM2 (c.309) | N (%) | N (%) | N (%) |
| T/T | 35 (16.2) | 36 (24.5) | 11 (45.8) |
| T/G | 100 (46.3) | 63 (42.9) | 10 (41.7) |
| G/G | 81 (37.5) | 48 (32.6) | 3 (12.5) |

N, the number of patients; % in parentheses.

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**Table 7. Frequencies of wild type (–617C) and SNP (–617A) alleles in the NRF2 gene among different ethnic groups.**

| Ethnic group | C | A | C/C | C/A | A/A | N | Data source |
|-------------|---|---|-----|-----|-----|---|-------------|
| African     | 0.925 | 0.075 | 0.850 | 0.150 | 0.000 | 246 * | 1000 Genomes. http://browser.1000genomes.org/Homo_sapiens/Variation/Population?db = core;r = 2:178129537–178130537;v = rs6721961;vdb = variation;vf = 4574214. doi:10.1371/journal.pone.0073794.t007 |
| African-American | 0.893 | 0.107 | 0.787 | 0.213 | 0.000 | 61 * |
| European    | 0.883 | 0.117 | 0.778 | 0.208 | 0.013 | 379 * |
| American in Utah | 0.888 | 0.112 | 0.788 | 0.200 | 0.012 | 85 * |
| American mixed | 0.862 | 0.138 | 0.757 | 0.210 | 0.033 | 181 * |
| Mexican in Los Angeles | 0.803 | 0.197 | 0.667 | 0.273 | 0.061 | 66 * |
| Japanese    | 0.775 | 0.225 | 0.618 | 0.315 | 0.067 | 89 * |
| Japanese (lung cancer) | 0.748 | 0.252 | 0.558 | 0.380 | 0.062 | 387 This study |
| Taiwanese   | 0.726 | 0.274 | 0.524 | 0.405 | 0.071 | 168 This study |
| Chinese in Beijing | 0.722 | 0.278 | 0.515 | 0.412 | 0.072 | 97 * |
| Southern Han Chinese | 0.710 | 0.290 | 0.500 | 0.420 | 0.080 | 100 * |
| Chinese     | 0.710 | 0.290 | 0.500 | 0.420 | 0.080 | 100 * |

N, the number of subjects.

doi:10.1371/journal.pone.0073794.t007

Figure 2. Kaplan-Meier plots showing the overall survival of patients harboring the WT homozygote (–617C/C), WT/SNP heterozygote (–617C/A), or SNP homozygote (–617A/A) in the NRF2 gene. Patients with p-stages I to IV (A) and p-stage I only NSCLC (B). The number of patients at times 0, 500, 1000, or 1500 days after surgical operation is described along with genotypes of the NRF2 gene.

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|-------------|-----|-----|-----|
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|-------------|---|---|-----|-----|-----|---|-------------|
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| African-American | 0.893 | 0.107 | 0.787 | 0.213 | 0.000 | 61 * |
| European    | 0.883 | 0.117 | 0.778 | 0.208 | 0.013 | 379 * |
| American in Utah | 0.888 | 0.112 | 0.788 | 0.200 | 0.012 | 85 * |
| American mixed | 0.862 | 0.138 | 0.757 | 0.210 | 0.033 | 181 * |
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| Southern Han Chinese | 0.710 | 0.290 | 0.500 | 0.420 | 0.080 | 100 * |
| Chinese     | 0.710 | 0.290 | 0.500 | 0.420 | 0.080 | 100 * |
cell cycle arrest and/or apoptosis mediated by p53 (WT); refer to Figure 3. This may explain, in part, our finding that the lung cancer patients with homozygous SNP alleles (–617A/A) in the NRF2 gene had markedly high overall survival rates (Figure 2).

In cancer tissues, somatic mutations take place frequently. In addition to the above-mentioned genetic polymorphisms as the “intrinsic” mechanism, mutations in the KEAP1 and/or NRF2 genes are the “acquired” mechanisms that lead to constitutive activation of NRF2. In fact, mutations in the NRF2 and KEAP1 genes have been found in carcinomas of the lung [12], breast [48], liver [49], and stomach [49]. Abnormalities in NRF2 activity were correlated with poor prognosis, when measured either as recurrence-free or overall 5-year survival. A recent immunohistochemical study has revealed that increased expression of NRF2 protein and decreased expression of KEAP1 protein are common abnormalities in NSCLC and are associated with poor prognosis [50], suggesting the involvement of other mechanisms such as intrinsic genetic polymorphisms of those genes. To bridge the gap between the homozygous SNP alleles (–617A/A) in the NRF2 gene and the high overall survival of lung cancer patients shown in this study, we need to carry out further clinical follow-up studies with lung cancer patients (p-stages III and IV) who have been subjected to chemotherapeutic treatments. As exemplified in the present study, genetic polymorphisms/mutations and fine balances among NRF2, KEAP1, MDM2, p53, p21WAF1/cip1 and other genes are likely to contribute to the progression of cancer and, consequently, the prognosis of cancer patients.

Development of a Rapid Genotyping Method for Personalized Cancer Therapy

One of the challenges in lung cancer management is to identify biomarkers for personalized cancer therapy. To effectively advance personalized medicine, cost-effective methods should be developed for genotyping. It would be desirable to include such

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**Figure 3. Schematic illustration showing the effect of NRF2 SNP–617C>A and MDM2 SNP c.309 T>G on the p53-mediated suppression of cancer cell proliferation and drug resistance.** In response to oxidative stress, electrophiles challenge, or protein kinase-mediated phosphorylation (e.g., via the PI3K-Akt pathway), the NRF2 protein is released from KEAP1 and then translocated into the nuclei. The SNP–617C>A in the ARE-like motif is considered to play a role in the positive feedback loop of transcriptional activation of the NRF2 gene. The SNP homozygote (–617 A/A) significantly attenuates the positive feedback loop and also expression of NRF2-target genes, such as MDM2 and ABCG2. In the case of MDM2 gene expression, the SNP (c.309 T>G) in the first intron of the MDM2 gene increases the binding affinity toward Sp1 and results in higher expression levels of MDM2 protein. MDM2 protein, thus highly expressed, binds to p53 (wild type; WT) protein and leads to ubiquitination and proteasomal degradation of p53 (WT) protein. Combination of the 309G (SNP) allele of the MDM2 gene and the –617C (WT) allele of the NRF2 gene may have negative impacts on p53 (WT)-mediated tumor suppression. On the other hand, lung cancer patients harboring both the 309T (WT) allele of the MDM2 gene and the –617A (SNP) allele of the NRF2 gene may have better prognosis due to the tumor suppressor function of p53 (WT), such as apoptosis and p21WAF1/cip1-mediated cell cycle arrest. Expression of the ABCG2 gene is known to be up-regulated by NRF2. Gefitinib, an inhibitor of EGFR tyrosine kinase, is extruded by ABCG2 out of cancer cells. Thus, NRF2-mediated induction of ABCG2 expression can confer cancer cells with acquired resistance to gefitinib and other anticancer drugs.

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information in each patient’s record as guidance for medical doctors to provide individualized treatment. In the present study, we have developed a rapid genetic testing method to elucidate the impact of genetic polymorphisms in the NRP2 gene on the risk and survival of patients with primary lung cancer. The method enables the detection of genetic polymorphisms in target genes within 30 to 45 minutes under isothermal conditions that do not require DNA isolation and PCR amplification. Thus, this genotyping method would provide a simple and practical tool for personalized cancer therapy and assessment of prognosis.

Materials and Methods

Patients and Sample Collection

This clinical research was conducted according to the Declaration of Helsinki Principles. Under written informed consent, we collected blood samples from patients with primary lung cancer who received surgical operation at the Kanagawa Cancer Center. Protocols for sample collection, anonymity, storage, and transportation to RIKEN Yokohama Institute required for the present study were approved by both the Institutional Review Board of the Kanagawa Cancer Center Research Institute and the Research Ethics Committee at RIKEN Yokohama Institute. Procedures for analyzing the HO-1 gene 5′-flanking region sequence as well as for genotyping the NRP2, CYP2A6, and MDM2 genes in the genomic DNA samples were approved by the Research Ethics Committee at RIKEN Yokohama Institute.

The Taiwanese samples (N = 168) used in this study were randomly selected from the Han-Chinese Cell and Genome Bank in Taiwan described previously [51], in which more than 3,300 healthy controls were collected and randomly selected through registry.

Preparation of Genomic DNA

Peripheral venous blood samples from lung cancer patients were collected into tubes containing Na2EDTA. Genomic DNA was extracted by the use of the QIAamp blood kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer’s instructions.

Genotyping

Based on the SmartAmp method, rapid genotyping primers were developed for detecting both the SNP (c.–617C/A) in the ARE-like loci of the human NRP2 gene (Figure 1) and the CYP2A6*4 genotype (whole gene deletion) [51]. We used excision-controlled hybridization-sensitive fluorescent primers for optical detection of genotyping reactions (Figure 1). After genomic DNA was denatured at 98°C for 3 min, the genotyping reactions were allowed to proceed isothermally at 60°C for 60 min in a Mx3000P PCR system (Agilent Technologies, Santa Clara, CA, USA). The SNP c.309T>G in of the MDM2 gene were detected by the Duplex SmartAmp method, as described previously [46].

Analysis of Length Variability of (GT)ₙ Repeats in the HO-1 Gene Promoter

The partial genomic DNA including (GT)ₙ repeats located in the 5′-flanking region of the HO-1 gene was amplified by the polymerase chain reaction (PCR) [33,34] with a fluorescence probe-labeled sense primer p1-s (5′-AGAGGCGTGCAAGGTTCCAG-TACAGA-3′) and an unlabeled anti-sense primer p1-as (5′-ACAAAGTCTGCCATAGGAC-3′). These primers were designed according to the previously reported sequences [55]. The PCR cycle program of 95°C for 30 seconds, 63°C for 30 seconds, and 60°C for 30 seconds was carried out for a total of 30 cycles. The resulting PCR products were visualized by electrophoresis in 3% agarose gels containing ethidium bromide. The electrophoresis revealed two differently-sized PCR products attributable to two alleles with different (GT)ₙ repeat sequences in the HO-1 gene. The (GT)ₙ repeats in the PCR products were analyzed with a laser-based automated DNA sequencer (ABI PRISM 3100 DNA Analyzer, Applied Biosystems Ltd., Tokyo, Japan).

Analysis of Mutation Status in the EGFR Gene

DNA samples were isolated from frozen tissues or formalin-fixed and paraffin-embedded tissue sections that had been surgically excised from lung cancer loci. Epidermal growth factor receptor (EGFR) gene exons 19 and 21 were analyzed for their mutational status by the loop-hybrid mobility shift assay, a PCR-based heteroduplex analysis method, as described previously [56,57].

Statistical Analysis

The association of lung cancer with the allele frequency of the gene of interest was assessed by considering the confounding effects derived from known risk factors, such as age, gender, smoking history, and histopathology. After preliminary bivariate analysis using Fisher’s exact test or χ² test, the multivariate logistic regression method was performed to estimate independent variables associated with the SNP homozygote (–617A/A) in the NRP2 gene. Furthermore, the Kaplan-Meier method was used to estimate survival curves for overall survival. Log-rank tests were used to compare the survival curves of patients in different NRP2 subgroups. The statistical significance of all the data was tested by the analysis of variance. We performed statistical analysis with the SPSS statistics program (v.19.0; SPSS Inc., Chicago, USA). Values of P<0.05 were considered to indicate statistical significance.

Supporting Information

Table S1 Classification of female adenocarcinoma patients with respect to genotypes of NRP2 and MDM2 genes.

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Author Contributions

Conceived and designed the experiments: YO UN AL YI YN TK YH. Performed the experiments: YO UN YI AL YN TK YH. Analyzed the data: YO TI AL HK. Wrote the paper: YO TI. Contributed reagents/materials/analysis tools: YO UN YE AL HK. Contributed to the sample collection: YM HN MT ML TY. Contributed to the clinical study: YM HN MT ML TY.

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