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Nrf2 gene as a double-edged sword: Clinical relevance of its genetic polymorphisms

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Abstract

In all aerobic organisms on Earth, molecular oxygen is essential for cellular respiration, and our body consisting of those cells is continuously exposed to "oxidative stress". In response to oxidative stress, NF-E2-related transcription factor (Nrf2) encoded by the *Nrf2* gene is considered to play a pivotal role in cellular defense *via* transcriptional up-regulation of many downstream genes, including those for metabolizing enzymes and transporters essential for cellular function. However, it is also true that the *Nrf2* gene is regarded as a double-edged sword. While it plays a role in protecting normal cells from external toxic challenges and oxidative stress, the *Nrf2* gene can also endow cancer cells resistance to anticancer drugs. The Nrf2 protein interacts with the antioxidant responsive element (ARE) located in the promoter region of the *Nrf2* gene as well as its downstream target genes. Genetic polymorphisms and/or mutations in the *Nrf2* gene have hitherto been identified in human samples, one single nucleotide polymorphism (SNP; -617C>A; rs6721961) in the ARE-like loci of the human *Nrf2* gene reportedly affects the positive feedback loop of transcriptional activation of the *Nrf2* gene. Ethnic group-dependent difference is observed for that SNP, where the frequency of the -617A allele is high in Japanese, Taiwanese, and Chinese populations. These allele frequency differences in the *Nrf2* gene may reflect genetic alterations and selection that took place during inter-continental migrations of *Homo sapiens*.

Introduction

The history of the universe including time, space, and matters began shortly after the "Big Bang", 13.7 billion years ago. About 9 billion years later, our solar system was formed in the Milky Way Galaxy. Oxygen was essentially absent from the atmosphere of Earth when this planet was first created about 4.54 billion years ago [1]. Carbon dioxide (CO_2) was the major gas present in the atmosphere of early Earth. After the chemical evolution from the RNA-protein world to the DNA/RNA-protein world, anaerobic microbes were created as original life at least from 3.8 billion to 4.1 billion years ago [2-4]. It is speculated that molecular oxygen (O_2) was first produced somewhere in oceans of Earth around 2.7 billion to 2.8 billion years ago, and then it emerged in atmosphere around 2.45 billion years ago (Figure 1). The origin of molecular oxygen in Earth's atmosphere derived from the biological activity of tiny organisms, namely cyanobacteria that appeared on earth about 3.5 billion years ago [5]. Cyanobacteria were capable to produce carbohydrates from water and carbon dioxide molecules by using the photon energy of sunshine. During the process of photosynthesis, molecular oxygen was produced as exhaust gas. Cyanobacteria are considered to have given rise to plant chloroplasts. As the plants world prospered on Earth and photosynthesis became more common, the rate of O_2 production increased until the present concentration of oxygen in the atmosphere reached about 580 million years ago.

Today, molecular oxygen is essential for cellular respiration in all aerobic organisms, where mitochondria generate ATP *via* oxidative phosphorylation. In contrast, to anaerobic organisms, molecular oxygen is toxic and harmful. It was one of the evolutionally critical points that anaerobes incorporated purple bacteria to acquire high efficiencies of ATP production *via* oxidative phosphorylation. Purple bacteria are considered to have introduced the ancestor responsible for eukaryotic mitochondria. Mitochondria have their own DNA to proliferate in host eukaryotic cells. Since mitochondria-hosting eukaryotic cells (*i.e.*, aerobic cells) had originated anaerobic microbes, those cells started to encounter the risk of oxygen toxicity. Indeed, reactive oxygen species (ROS) generated from molecular oxygen are causal factors in the production of cell damage as well as the mutation of genomic DNA. ROS which consist of superoxide anion radical ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2) play important roles in many essential cellular physiological functions, including growth, differentiation, apoptosis, and aging [6]. Under normal physiological conditions, there is a balance between oxidants and antioxidants, or reduction-oxidation (redox) homeostasis.

One of the classical but important mechanisms counteracting ROS is the glutathione (GSH) system [7,8]. GSH is a ubiquitous tripeptide thiol (L-γ-glutamyl-L-cysteinyl-glycine). It is a vital intra- and extra-cellular protectant [9,10] and an effective scavenger of ROS. It is estimated that GSH biosynthesis originated about 3 billion years ago. GSH is found in the vast majority of eukaryotes, whereas in eubacteria, GSH biosynthesis is limited to only two groups, namely cyanobacteria and purple bacteria that are thought to be the ancestors of chloroplast and mitochondria, respectively [11].

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Oxidative stress in pathophysiology

Our body consisting of eukaryotic cells is continuously exposed to "oxidative stress". The term "oxidative stress" is defined as a disturbed balance between the production of ROS and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants and enzymatic reactions [12]. This biochemical phenomenon is inevitable as long as we live on Earth. Oxidative stress leads to many pathophysiological conditions in our body. Some of these include cancers, chronic obstructive pulmonary disease (COPD), atherosclerosis, hypertension, diabetes mellitus, and neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease.

The lung is the organ that is continuously exposed to atmosphere's molecular oxygen and has a risk of oxidative damage. Indeed, accumulating evidence strongly suggests that oxidative stress and oxidative damage are involved in the pathogenesis of COPD, where oxidative stress is considered to cause direct injury and inflammation in the respiratory tract, lung, and other organs and cell types. The sources of the increased oxidative stress in the respiratory compartment of COPD patients derive from the increased burden of oxidants from environmental exposures, such as cigarette smoke. Furthermore, ROS and reactive nitric oxide (NO) are also released from leukocytes and macrophages involved in the inflammatory process in the lungs of COPD patients. ROS can induce lipid peroxidation and yield products such as 4-hydroxynonenal and malondialdehyde to stimulate pulmonary inflammation. Increased levels of lipid peroxidation products were detected in the breath condensate and plasma of smokers and patients with stable COPD [13]. Antioxidant defenses, including GSH, vitamin E, glutathione peroxidase, superoxide dismutase, and heme oxygenase-1 (HO-1), are of importance to reduce oxidative damage in the lung.

Key role of heme oxygenase-1 to suppress pulmonary inflammation

HO-1 is a rate-limiting enzyme in the metabolic pathway of heme catabolism to generate biliverdin that is successively metabolized to bilirubin by the enzymatic action of biliverdin reductase. Bilirubin, thus formed, exhibits anti-oxidative, anti-apoptotic, and anti-inflammatory activities. Decrease in the HO-1 level is reportedly associated with the pathogenesis of some age-dependent disorders, including COPD and cancer. It is hypothesized that enhanced expression of HO-1 provides an anti-inflammatory effect and confers cytoprotection. To examine this hypothesis, Shinohara *et al.* [14] examined whether HO-1 overexpression would prevent pulmonary emphysemia induced by porcine pancreatic elastase (PPE). They inoculated mice with an adenovirus encoding HO-1 in the lung [14]. In fact, adenovirus-mediated overexpression of HO-1 in the lung significantly attenuated PPE-induced increase of neutrophils in bronchoalveolar lavage fluid and enlargement of alveoli. In addition, HO-1 overexpression reduced PPE-induced TNFα, IL-6, and keratinocyte-derived chemokine in bronchoalveolar lavage fluid. Furthermore, Sato *et al.* [15] have confirmed the anti-inflammatory effect of HO-1 using a mouse silicosis model where HO-1 expression was up-regulated by treatment with hemin [15]. Hemin is a strong inducer for *HO-1* gene expression, and NF-E2-related transcription factor (Nrf2) induces *HO-1* gene expression, as described below. Other relevant studies have also proven that HO-1 is a key player in the suppression of pulmonary inflammation. HO-1 inhibits IL-13-induced goblet cell hyperplasia associated with CLCA1 suppression in normal human bronchial epithelial cells, as well [16].

Nrf2-mediated transcriptional regulation

Nrf2 is a master switch for transcriptional up-regulation of a variety of target genes, including those for metabolizing enzymes (*e.g.*, HO-1)

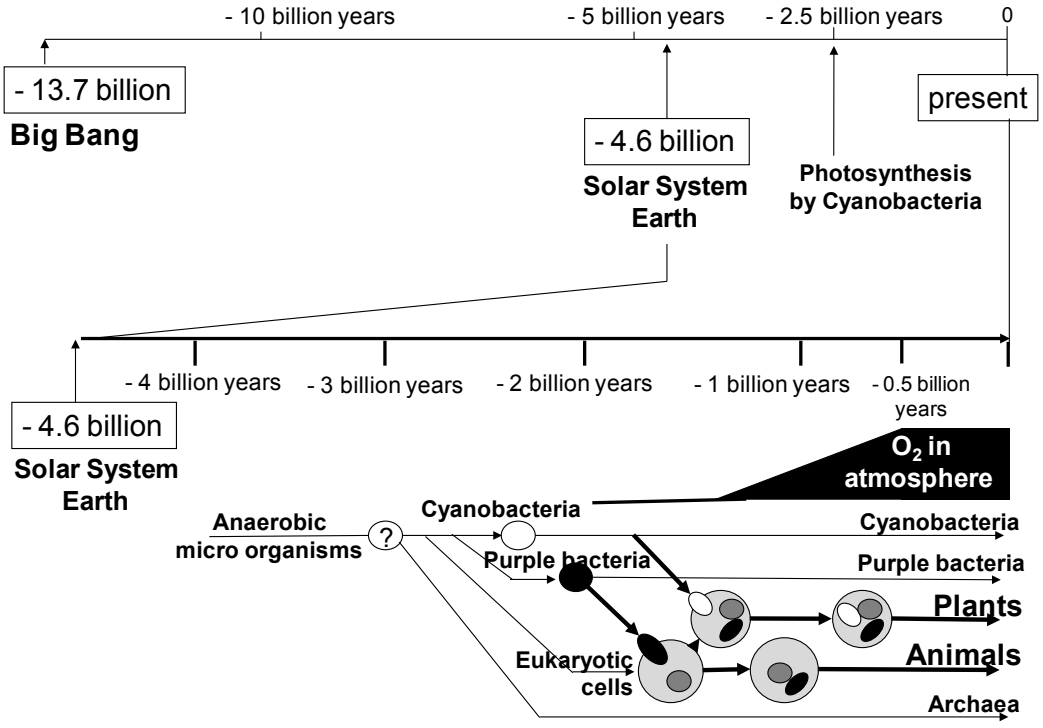

Figure 1. The evolution of organisms on Earth and the emergence of molecular oxygen (O_2) in the history of the universe after Big Bang.

and transporters essential for cellular defense in response to oxidative and/or electrophilic stress [17-19]. The Nrf2 protein is a basic region-leucine zipper (bZip)-type transcription factor, and it targets the anti-oxidant response element (ARE) with the consensus sequence of 5'- A/GTGACNNNGC) [19,20] This Nrf2 system has been evolutionarily conserved among different species [21].

The activation and nuclear translocation processes of Nrf2 seem to be complex. As shown in Figure 2, at least three distinct pathways may be involved in the activation of the Nrf2 protein leading to HO-1 induction: oxidation of critical cysteinyl residues of the Keap1 protein and concomitant inhibition of ubiquitination activity of Keap1 (Pathway A); phosphorylation of the Nrf2 protein via protein kinases, such as p38^{MAPK}, PI3K, PKC, and PERK (Pathway B); direct binding of heme to Bach1 and facilitation of Nrf2/small Maf heterodimer formation (Pathway C).

The HO-1 induction is dependent on transcription and *de novo* protein synthesis, and it is preceded by the nuclear accumulation of the Nrf2 transcription factor. In pathway A, Nrf2 is repressed under quiescent conditions, where Keap1 and Cul3 constitute a unique ubiquitin E3 ligase that leads to the degradation of Nrf2. Upon exposure to oxidants/electrophiles, the enzymatic activity of this ligase complex is inhibited and the complex fails to degrade Nrf2, resulting in the transcriptional activation of Nrf2 target genes [22,23]. Cys151 of Keap1 reportedly plays an important role to facilitate Nrf2 activation in response to oxidants/electrophiles [23].

Regarding pathway B, Martin *et al.* [24] reported that HO-1 expression is regulated through the PI3K/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol. Kocanova *et al.* [25] more recently characterized the signaling pathways and the mechanisms leading to the up-regulation of HO-1 in cancer cells subjected to hypericin-based photodynamic therapy. They have shown that HO-1 induction mechanisms involve the p38MAPK and PI3K signaling cascade. Besides p38MAPK and PI3K, the activation of Nrf2 is mediated by other protein kinases, such as PERK and PKC, being dependent on cell types [26-31].

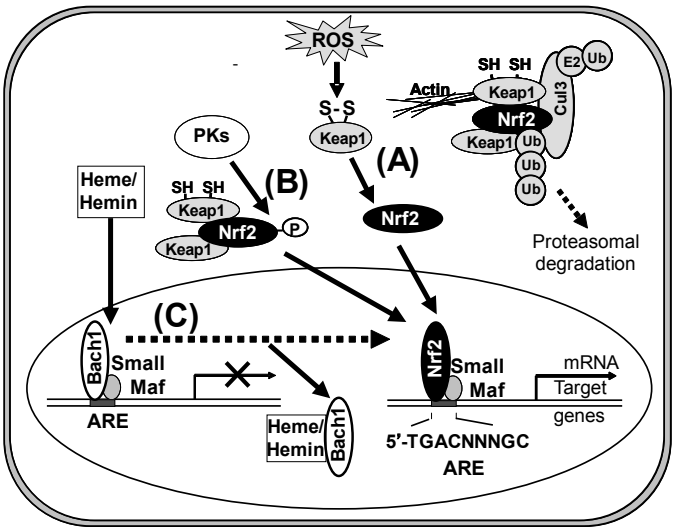

Figure 2. Activation of Nrf2 via three different pathways (A, B, and C). Pathway A, under homeostatic conditions, Nrf2 is sequestered in the cytoplasm by the Keap1-Cul3 complex and rapidly degraded in a ubiquitin-proteasome-dependent manner. After an oxidative challenge, oxidation of two reactive cysteine residues of Keap1 inhibits the ubiquitination reaction of Nrf2 mediated by the Keap1-Cul3 complex, which results in both cytoplasmic accumulation and nuclear translocation of Nrf2. Pathway B, activation of Nrf2 is mediated by protein kinases (PKs), such as p38^{MAPK}, PI3K, PERK, and PKC. Pathway C, under normal conditions, the chromatin structure of many target genes is in a pre-activation state, but transcription is repressed by Bach1. Heme or hemin binds to Bach1, inhibiting its DNA binding activity and inducing its nuclear export. In the nuclei, the activated Nrf2 dimerizes with small Maf nuclear protein (MafK) for effective binding to the ARE consensus sequence in the promoter region of Nrf2-target genes, such as HO-1 and γ-glutamyl cysteine ligase.

In pathway C, heme or hemin regulates the dynamic exchange of Bach1 and Nrf2 in the Maf transcription factor network. Igarashi and his colleagues proposed this direct interaction model [32-35]. The transcription repressor Bach1 is a sensor and effector of heme/hemin that regulates the expression of *HO-1* and *globin* genes [36-38]. Under normal conditions, the chromatin structure of HO-1 is in a preactivation state, but transcription is repressed by Bach1. Heme/hemin binds to Bach1, inhibiting its DNA binding activity [39] and inducing its nuclear export [40]. Furthermore, heme/hemin induce ubiquitination and degradation of the transcription factor Bach1 [41]. As a consequence, heme/hemin induces the switching of Nrf2/small Maf hetero-dimers, resulting in HO-1 expression [42].

SNP (-617C>A; rs6721961) in the *Nrf2* gene

Yamamoto *et al.* 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 [43] (Figure 3). Three years later, Marzec *et al.* examined the impact of those SNPs on the regulation of *Nrf2* gene expression [44]. In transient transfection assays, they found that the -617C>A SNP significantly affects basal Nrf2 protein levels and its function *in vitro* [44]. SNP -617C>A was found to be associated with a higher risk of oxidant-induced acute lung injury in humans [44]. It is likely that the SNP (c.-617C>A) in the ARE-like loci of the human *Nrf2* gene is important for self-induction of the *Nrf2* gene (Figure 4). To our knowledge, the report of Marzec *et al.* [44] is the first one demonstrating that functional polymorphisms in the transcription factor *NRF2* in humans increase the risk of acute lung injury.

Interestingly, 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 (Table 1). The ethnic group-dependent difference may reflect genetic alterations and selection that took place in the *Nrf2* gene during inter-continental migrations of *Homo sapiens* (Figure 5). With this respect, the authors have recently reported that inter-continental migration of *Homo sapiens* is well associated with ethnic-dependent differences in one SNP 538G>A (rs17822931: Gly180Arg) in the human *ABCG11* gene [45].

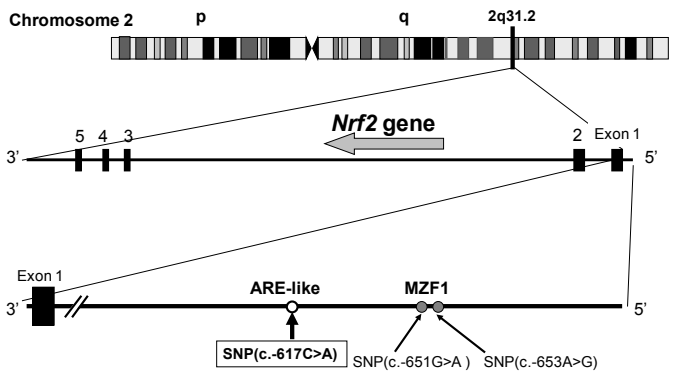

Figure 3. SNP (- 617C>A) in the promoter region of the human *Nrf2* gene located on chromosome 2q31.2.

Table 1. Frequencies of wild type (-617C) and SNP (-617A) alleles in the *Nrf2* gene among different ethnic groups.

	Allele frequency			Nrf2 (-617)			
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	[64]
African-American	0.893	0.107	0.787	0.213	0.000	61	[64]
European	0.883	0.117	0.778	0.208	0.013	379	[64]
American in Utah	0.888	0.112	0.788	0.200	0.012	85	[64]
American mixed	0.862	0.138	0.757	0.210	0.033	181	[64]
Mexican in Los Angels	0.803	0.197	0.667	0.273	0.061	66	[64]
Japanese	0.775	0.225	0.618	0.315	0.067	89	[64]
Japanese (lung cancer)	0.748	0.252	0.558	0.380	0.062	387	[50]
Taiwanese	0.726	0.274	0.524	0.405	0.071	168	[50]
Chinese in Beijing	0.722	0.278	0.515	0.412	0.072	97	[64]
Southern Han Chinese	0.710	0.290	0.500	0.420	0.080	100	[64]

N, the number of subjects

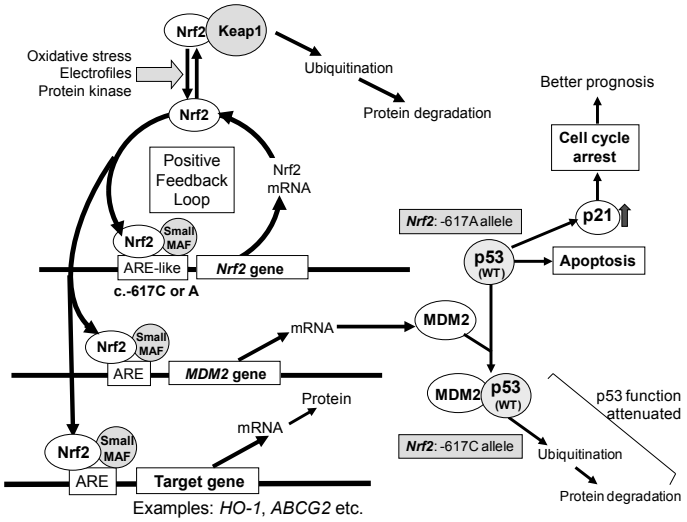

Figure 4. Schematic illustrations showing the effect of *NRF2* SNP -617C>A and *MDM2* gene on the p53-mediated suppression of cancer cell proliferation and ABCG2-mediated drug resistance to gefitinib [50].

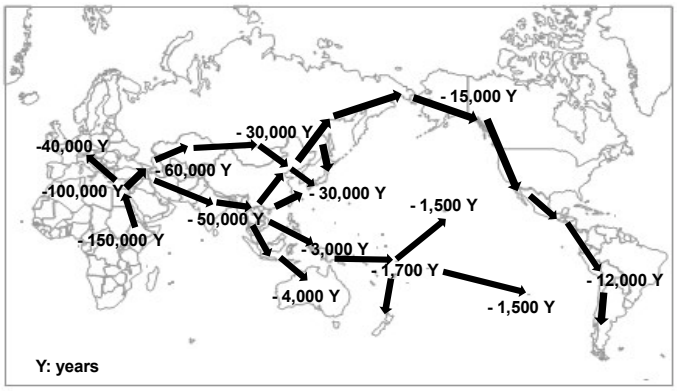

Figure 5. Schematic illustration of inter-continental migration of *Homo sapiens* [45].

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 a key role in protecting normal cells from oxidative stress as well as external toxic challenges, whereas it can also endow cancer cells resistance to anticancer drugs. Recently it has been reported that Nrf2 contributes to malignant phenotypes of cancer cells *in vitro*, including aggressive cell proliferation, drug resistance, and metabolic re-programming [46,47]. Indeed, Nrf2 activation and/or up-regulated expression of the *Nrf2* gene are involved in the emergence of cancer resistance to various anticancer drugs by transcriptionally activating a battery of cell-protection genes [48,49].

The SNP -617C>A [43] is considered to regulate the positive feedback loop of transcriptional activation of the *Nrf2* gene and thereby it can modulate the Nrf2 protein expression level (Figure 4). Since the SNP (-617A) in the ARE-like loci of the human *Nrf2* gene decreases the binding affinity to the transcription factors of Nrf2/small Maf (MafK) [44], it is anticipated that the homozygous -617 A/A allele significantly attenuate the positive feedback loop of transcriptional activation of the *Nrf2* gene.

The authors have recently found that lung cancer patients with homozygous SNP alleles (-617A/A) in the *Nrf2* gene had markedly high overall survival [50]. Univariate analysis showed a significant difference between the -617 A/A and -617 C/A groups in terms of overall survival (log-rank $P = 0.021$). As one of the intrinsic genetic polymorphisms in the *Nrf2* gene, homozygous SNP (-617A/A) is considered to be associated with overall survival of lung cancer patients [50].

Nrf2 is known to regulate the basal expression of the murine double minute-2 (*MDM2*) gene [51]. Since human MDM2 is an oncoprotein that binds to p53 protein and inactivates the tumor suppressor activity of p53 [52], Nrf2 can indirectly contribute to p53-mediated cell cycle control and/or apoptosis [53]. This, in turn, attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans [54]. In addition, Nrf2 activation may be involved in the emergence of cancer resistance to various anticancer drugs by transcriptionally activating a battery of self-defense genes. For example, ABCG2 is one of the Nrf2 target genes and it is known to mediate the efflux of gefitinib from cancer cells [55]. Its expression is regulated by Nrf2 [56] and the EGFR-tyrosine kinase cascade [57,58]. In this context, it is suggested that the genetic polymorphism in the *Nrf2* gene could be a clinically useful biomarker for prognosis of lung cancer patients.

Concluding remarks

Recently, Figarska *et al.* genotyped SNPs, *i.e.*, rs4243387, rs2364723, rs13001694, rs1806649, and rs6726395) located in intron 1 between exons 1 an2 of the human *Nrf2* gene in 1,390 subjects from the Vlagtwedde-Vlaardingen cohort in the Netherlands [59]. Their study suggested that Nrf2 is indeed associated with reduced risk of cardiovascular and COPD mortality. At present, however, information is still limited as to the clinical impact of genetic polymorphisms of the

Nrf2 gene. Insight into molecular mechanisms underlying the impact of genetic polymorphisms as well as epidemiological studies with genotyping should be necessary. 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 *Kea1* genes have been found in carcinomas of the lung [60], breast [61], liver [62], and stomach [62]. 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 non-small cell lung cancer (NSCLC) and are associated with poor prognosis [50]. Importantly, abnormal expression of Nrf2 and Keap1 proteins was more common than that of the corresponding gene mutaions [63], suggesting the involvement of other mechanisms such as intrinsic genetic polymorphisms of those genes. Genetic polymorphisms/mutations and fine balances among Nrf2, Keap1, MDM2, p53, $p21^{WAF1/cip1}$ and other genes are likely to contribute to the progression of cancer and, consequently, the prognosis of cancer patients.

Conflict of interest/disclosure

Hereby the authors declare that there is no conflict of interest for the present study.

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