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Nrf2, A Target for Precision Oncology in Cancer Prognosis and Treatment

Hoang Kieu Chi Ngo[1], Hoang Le[2], Young-Joon Surh[1,3]

[1]Tumor Microenvironment Global Core Research Center, College of Pharmacy, Seoul National University, Seoul, Korea, [2]Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA, USA, [3]Cancer Research Institute, Seoul National University, Seoul, Korea

Activating nuclear factor-erythroid 2-related factor (Nrf2), a master regulator of redox homeostasis, has been shown to suppress initiation of carcinogenesis in normal cells. However, this transcription factor has recently been reported to promote proliferation of some transformed or cancerous cells. In tumor cells, Nrf2 is prone to mutations that result in stabilization and concurrent accumulation of its protein product. A hyperactivated mutant form of Nrf2 could support the cancer cells for enhanced proliferation, invasiveness, and resistance to chemotherapeutic agents and radiotherapy, which are associated with a poor clinical outcome. Hence understanding mutations in Nrf2 would have a significant impact on the prognosis and treatment of cancer in the era of precision medicine. This perspective would provide an insight into the genetic alterations in Nrf2 and suggest the application of small molecules, RNAi, and genome editing technologies, particularly CRISR-Cas9, in therapeutic intervention of cancer in the context of the involvement of Nrf2 mutations.

Key Words Mutation, Gene therapy, Precision medicine

INTRODUCTION

Environmental carcinogens are normally inert chemically and lack ability to modify DNA unless they are converted to reactive species by xenobiotic-metabolizing enzymes [1,2]. Biologically reactive metabolites of carcinogens are electrophilic, in general, and hence can interact covalently with DNA. The resulting DNA adduct formation can cause mutations and consequently carcinogenesis [1,2]. To prevent such deleterious effects of chemical carcinogens, cells mount various defense mechanisms, such as blockade of the formation of reactive intermediates or stimulation of detoxification through a conjugation reaction catalyzed by phase II xenobiotic-metabolizing enzymes [3]. It is important to note that the majority of genes encoding phase II carcinogen-detoxifying enzymes and related cytoprotective proteins contain the consensus sequence 5'-TGCTGAG/CTCAT/C-3' termed the antioxidant responsive element (ARE) or electrophile responsive element in their 5'-flanking regions [4,5], a binding site of a member of Cap' n' Collar (CNC) family transcription factor, nuclear factor-erythroid 2-related factor (Nrf2) [6]. As Nrf2 has an essential role in the expression of antioxidant and carcinogen-detoxifying enzymes, activating this transcription factor has been proposed as a prominent strategy for cancer chemoprevention [7]. Indeed, some compounds that activate Nrf2 have been subjected to clinical trials as well as preclinical studies [8-10].

Paradoxically, cancer cells hijack Nrf2 signaling, and Nrf2 activators turn out to accelerate tumor progression and metastasis in mice [11-13]. In this respect, mounting evidence supports that tumors are addicted to Nrf2 signaling [14,15] and some Nrf2-dependent signaling pathways are linked to clinical aggressiveness of cancer cells [15-18]. Overexpression/hyperactivation of Nrf2 frequently observed in various types of cancer is strongly associated with a poor clinical outcome [19,20]. Nrf2 has an inhibitory partner named Kelch-like erythroid cell-derived protein with CNC homology (ECH) associated protein (Keap1) with which Nrf2 forms an inactive complex in the cytoplasm [21]. Notably, mutations in the region encoding Keap1 binding domain of Nrf2 are commonly observed in cancer, which also contributes to the sustained overactivation of Nrf2 [22-25]. Here in this review, we discuss

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Correspondence to Young-Joon Surh, E-mail: surh@snu.ac.kr, https://orcid.org/0000-0001-8310-1795

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the mechanisms which underlie the overactivation of Nrf2 and its implications in cancer and suggest the desirable therapeutic approaches.

THE Keap1/Nrf2 AXIS

Human Nrf2 protein is composed of 605 amino acids with seven conserved regions named Nrf2-ECH homology (Neh) domain [26] which are distributed along the coding region (Fig. 1A). The Neh1 domain is located in the C-terminal half of Nrf2 and contains the CNC homology region and a basic-leucin zipper domain, which allows Nrf2 to form a heterodimer complex with small Maf and then bind to the ARE region in target genes. The proximal amino terminal Neh2 domain negatively regulates Nrf2 through binding of its DLG and ETGE motifs to Keap1 [27]. The Neh3 domain in the C terminus of Nrf2 allows Nrf2 to interact with a chromo-ATPase/helicase DNA binding protein CHD6, which functions as an Nrf2 transcriptional activator [28]. The Neh4 and Neh5 domains adjacent to Neh2 cooperatively bind to cAMP responsive element binding protein binding protein, which plays an essential role as a coactivator of many transcription factors, conferring the maximal transcriptional activity of Nrf2 [29]. The Neh6 domain contains two phosphorylation-dependent destruction motifs (DSGIS and DSAPGS) recognized by beta-transducin repeats-containing proteins (β-TrCP) of Skp-Cullin1-F-box protein E3 ubiquitin ligase complex. Phosphorylation of a Ser cluster in DSGIS motif by glycogen synthase kinase-3 beta facilitates the recognition of Nrf2 by β-TrCP and promotes its ubiquitination for proteasomal degradation [30-32]. A seventh Neh domain was then identified as a region through which retinoic X receptor alpha binds to and subsequently suppresses the transcriptional activity of Nrf2 [33].

Under a physiological condition, Nrf2 is sequestered in the cytoplasm through its binding to Keap1. Keap1 contains two canonical protein interaction motifs: a Broad-complex C, Tramtrack, and Bric-a-brac (BTB) and a double glycine repeat (DGR) or Kelch repeat, respectively located in the middle and carboxy terminus, typical of *Drosophila* cytoskeleton binding protein Kelch [21]. Two additional domains of Keap1 include intervening region (IVR) and C-terminal region (Fig. 1B) [34,35]. Each Keap1 molecule uses a C-terminal Kelch

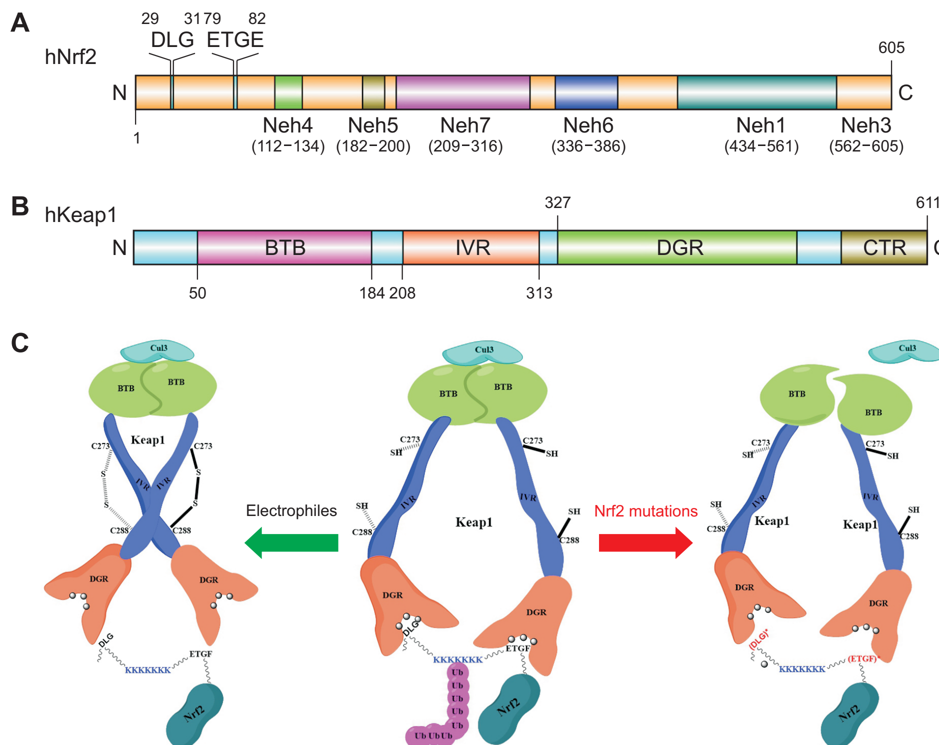

Figure 1. Structures explaining the negative regulation of Nrf2 by Keap1. (A) Structure of Nrf2. The positions of seven Nrf2-ECH homology domains (Neh) are shown. DLG and ETGE motifs by which Nrf2 associates with Keap1 are highlighted. (B) Structure of Keap1. (C) Distortion of Nrf2-Keap1 association. Middle: one Nrf2 molecule uses low-affinity DLG and high-affinity ETGF motifs to associate with two Keap1 molecules through its DGR domain. BTB domain in Keap1 allows two Keap1 molecules to form a homodimer, and through the same BTB domain recruits Cullin (Cul) 3 to ubiquitinate Nrf2 at lysine residues clustered between DLG and ETGE motifs, finally subjecting Nrf2 to 26S proteasomal degradation. Left: Disulfide bonds formed due to the reaction of oxidants with reactive thiol groups to cross link two Keap1 molecules, dislodging the attachment of Nrf2 to Keap1. Right: Any genetically changes in either DLG or ETGE motifs would lead to the dissociation of Keap1-Nrf2 binding. ECH, erythroid cell-derived protein with Cap' n' Collar homology; BTB, broad-complex C, tramtrack, and bric-a-brac; IVR, intervening region; DGR, double glycine rich; CTR, Carboxyl terminal region.

domain (DGR) to interact separately with two Keap1 binding motifs in the Neh2 domain of Nrf2 [27]. The BTB domain allows two Nrf2-bound Keap1 molecules to form a homodimer [36], which serves as an adaptor bridging Nrf2 to Cullin3 (Cul3)-associated E3 ubiquitin ligase complex [37,38]. The binding of Nrf2 to Cul3 anchored by Keap1 promotes ubiquitin conjugation of lysine residues in the Neh2 domain and therefore marking Nrf2 for degradation by 26S proteasomes [37,38].

Due to the constant exposure of mammalian cells to oxidative stresses, cystine residues, particularly C273 and C288, in the IVR domain of Keap1 undergo oxidation to form intermolecular disulfide bonds (most likely one C273 of one monomer and C288 of the other), thus covalently linking two monomers of Keap1. Such intermolecular disulfide linkage subsequently introduces an increase in the distance between the two DGR domains of two Keap1 molecules, which possibly dislodges the weaker binding between DGR of the Kelch domain and the DLG motif in Nrf2. Consequently, the modified Keap1 is no longer able to sequester newly synthesized Nrf2 (Fig. 1C, left), allowing it to translocate into the nucleus and enhance the expression of phase II enzymes for the clearance or inactivation of the reactive oxygen species (ROS) or electrophilic species [27,34,35]. Alterations in DLG and ETGE motifs of Nrf2 would also lessen the interaction between Nrf2 and Keap1. Regulating the Keap1/Nrf2 axis, therefore, would present an important mechanism for the maintenance of intracellular redox homeostasis.

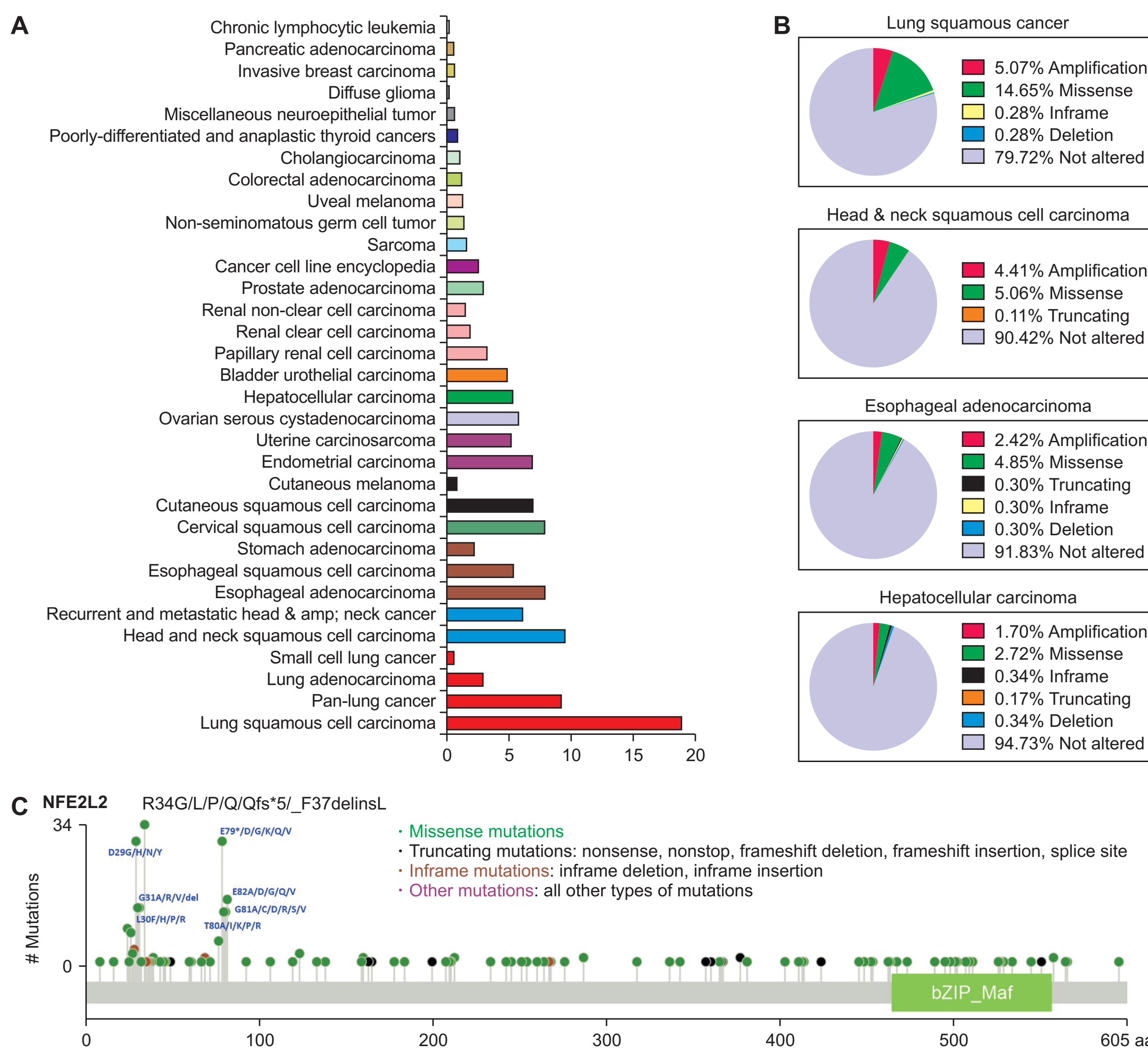

Figure 2. Nrf2 is frequently mutated in various types of cancer. (A) Frequencies of Nrf2 mutations in many types of cancer. (B) Distribution of Nrf2 mutations with amplification and missense mutations occurring at almost the same frequency and accounting for most of Nrf2 genetic alterations. (C) Distribution of Nrf2 substitution mutations along the coding region of Nrf2 with the highest frequency clustered in DLG and ETGE motifs.

Nrf2 MUTATIONS IN CANCER

The first study demonstrating the hyperactivation of Nrf2 in cancer was reported in 2004 by Ikeda et al. [39]. In the study, mRNA levels of Nrf2 and placental glutathione *S*-transferase (GST-P), an Nrf2-regulated carcinogen detoxifying enzyme that is expressed in cancerous but not normal tissue, were highly elevated in hyperplastic rat liver tissue and during the development of hepatocellular carcinoma. The Nrf2-dependent defense response was also found to be activated in various types of malignancy including head and neck squamous cell carcinoma [40] and lung cancer [41]. Subsequently, it came to be recognized that the Keap1-Nrf2 signaling pathway is largely dysregulated, which would account for the overactivation of Nrf2 [22,24,25,42]. Keap1 somatic mutations accompanied by loss of heterozygousity at the gene locus are common in non-small-cell lung carcinoma (NSCLC), leading to inactivation and consequently the loss of Nrf2 repression by Keap1 [42,43]. The reduction of inhibitory effects of Keap1 on Nrf2 activation in cancer could also be ascribed to hypermethylation of CpG island in the Keap1 gene promoter in lung cancer [22,44]. Moreover, gain-of-function of Nrf2 in cancer commonly arises from genetic alterations in Nrf2. The very first line of evidence by Shibata et al. [22] uncovered frequent mutations in Nrf2 in lung cancer cell lines as well as in primary cancer tissue of lung or head and neck cancer. Nrf2 mutations which are mostly of somatic origin were subsequently identified in other types of cancer including oesophageal and skin cancers [23].

Data provided by The Cancer Genome Atlas database (http://cancergenome.nih.gov/) and publicly available at Bioportal [45,46] have unfolded that Nrf2 mutations are common in primary tumors arising from approximately 20 types of organs (Fig. 2A), which have been found to be correlated with its enhanced transcriptional activity [22,23]. Among them, lung squamous cell carcinoma carries the highest frequency of Nrf2 mutations (~19%), followed by head and neck squamous cell carcinoma (~10%), and esophageal adenocarcinoma (~8.5%). Mutations of Nrf2 are rare in breast, thyroid, and brain cancers (Fig. 2A). Of note, genetic alterations of Nrf2 are sorted into DNA amplification and mis-sense mutations with almost the same percentage (Fig. 2B). Substitution mutations account for most of missense mutations, and are distributed all along the coding region of Nrf2. It is noticeable that mutations occurring with the highest frequency are clustered in either DLG (D29, L30, G31, and R34) or ETGE (E79, T80, G81, and E82) motifs of the Neh domain (Fig. 2C and Table 1). Genetic alterations in the ETGE motif were experimentally shown to result in the failure in the recognition of Nrf2 by Keap1 regardless of the absence of an altered DLG motif, whereas changes in the DLG motif without alterations in the ETGE motif only affected the binding of the DLG motif with the Kelch domain. However, mutations in the DLG motif confer Nrf2 the resistance to ubiquitination, resulting in the impairment of Keap1-directed proteasomal degradation of Nrf2 [22,47].

By contrast, substitutions are rarely found in the DNA binding domain of Nrf2 (known as bZIP-Maf or Neh1, Fig. 2C), which allows ETGE- or DLG-mutant Nrf2 to maintain its transcriptional activity.

Consistently, Nrf2 with aforementioned mutations was preferentially localized in the nucleus and dramatically more active than wild-type Nrf2, which represents constitutive activation of Nrf2 in an irreversible fashion [22,23]. Noticeably, the frequency of substitutions in the Neh6 domain, which in addition to the Neh2 domain negatively regulates Nrf2 stability [48], is not distinctive (Fig. 1C). Hence it is likely that cancer cells evolve to have substitutions exclusively clustered in hotspot regions encoding the DLG and ETGE motifs of Nrf2, which allows Nrf2 to bypass the suppression by Keap1, resulting in the overactivation of this transcription factor.

THE ONCOGENIC PROPERTIES OF CONSTITUTIVELY ACTIVATED Nrf2

The fact that Nrf2 activates the expression of phase II and antioxidant enzymes to eliminate excessive electrophiles and ROS and favor the survival of normal cells would be logically extended to cancer cells [49]. Mild oxidative stress plays a role as an important intracellular signaling molecule to promote cell proliferation and is involved in the maintenance of the oncogenic phenotypes [50], whereas ROS accumulated in excess would be a strategy to target cancer cells [51]. Hence evolving to have Nrf2 activated should be an elegant tactic of cancer cells to survive imbalanced redox homeostasis, which arises from an aberrantly high metabolic rate [50]. It has been reported that endogenous expression of oncogenic alleles of *Kras* (K-Ras^{G12D}), *Braf* (B-Raf^{V619E}), and *Myc* (Myc^{ERT2}) transcriptionally upregulates the expression of Nrf2, which then results in a favorable intracellular environment for tumor cell survival with a lower level of ROS [52]. As a consequence, genetic deletion of the Nrf2 gene greatly impaired K-Ras^{G12D}-induced murine lung and pancreatic cancers [52].

Chio et al. [15] showed that Nrf2 was also utilized by pancreatic cancer cells to protect the cysteine residues in protein components of the mRNA translation machinery from oxidative stress, thereby securing cancer cell proliferation and maintenance. It was reported that Nrf2 could be hijacked by cancer cells to redistribute glucose and glutamine towards the anabolic pathway to meet energy demand for rapid growth of cancer cells [53].

Alterations in Nrf2 including amplification and substitutions clustered in a gene region coding for amino acids in DLG and ETGE motifs are closely related to a poor clinical outcome. Squamous cell lung carcinoma patients with these activating genetic alterations in Nrf2 showed a shorter disease-free survival compared to their cohort with wild-type Nrf2 [18,22]. The similar segregation was observed in esophageal squamous

Table 1. A list of commonly found substitutions in Nrf2

Mutations in aa 29-31			Mutations in aa 79-82			Other mutations		
Mutations	Cancer type	N	Mutations	Cancer type	N	Mutations	Cancer type	N
D29H	Lung squ	4	E79Q	Lung squ	8	W24R	NSCLC	1
	NSCLC	6		NSCLC	7		Head & neck	1
	Head & neck	6		Head & neck	8		Esophagus	1
	Esophagus	1		Esophagus	2		Bladder	2
	Cervical	2		Bladder	2		nccRCC	1
	Uterine	2		ESCC	1	W24C	Lung squ	2
	ccRCC	2	E79K	NSCLC	1		NSCLC	2
D29Y	Lung squ	2		Head & neck	5		Head & neck	2
	NSCLC	2		Esophagus	1	W24_D29delinsY	Liver	1
	Head & neck	1		Bladder	4	Q26P	Lung squ	1
	Cervical	1		Cervical	1		NSCLC	1
	nccRCC	1	E79V	Lung adeno	2		Liver	1
D29N	Lung squ	2		NSCLC	1	Q26K	ESCC	1
	NSCLC	5		Liver	1		Uterine	2
	Head & neck	1	E79G	NSCLC	1	Q26L	Lung squ	3
	ESCC	1		Esophagus	1		NSCLC	1
D29G	Lung squ	2	E79D	Cervical	1	Q26R	NSCLC	1
	NSCLC	3	E79*	Liver	1		Liver	1
L30F	Lung squ	4	T80K	Lung adeno	1	Q26H	Liver	1
	NSCLC	4		Lung squ	2	R34G	Lung squ	2
	Head & neck	3		NSCLC	3		NSCLC	7
	Esophagus	1		ccRCC	1		Head & neck	3
	ESCC	1		pRCC	1		Esophagus	1
L30H	Head & neck	2	T80A	NSCLC	1		Bladder	5
	ccRCC	2		Head & neck	1		Uterine	4
L30P	Head & neck	1		Liver	1		ESCC	3
	Liver	1	T80R	Esophagus Squ	1		ccRCC	2
L30R	Bladder	1		Uterine	2	R34Q	Lung squ	6
	pRCC	1	T80I	Liver	1		NSCLC	5
G31A	Lung squ	4	T80P	Uterine	4		Cervical	1
	NSCLC	6	G81S	Lung squ	4		Head & neck	3
	Esophagus	2		NSCLC	2	R34P	Lung squ	2
	Bladder	3		Esophagus	1		NSCLC	5
G31R	NSCLC	1		Liver	1		Head & neck	2
	Bladder	2	G81V	Lung squ	2	R34L	NSCLC	1
G31V	NSCLC	1		NSCLC	1	R34_F37delinsL	NSCLC	1
	Esophagus	1		Uterine	2	R34Qfs*5	Esophagus	1
G31del	NSCLC	1		pRCC	1	D77G	Lung squ	2
			G81C	Head & neck	2		NSCLC	1
				Liver	1		ESCC	1
			G81D	Breast	2		Head & neck	1
				ccRCC	2	D77E	ESCC	1
			G81A	Liver	1	D77H	Cervical	1
			G81R	Liver	1	D77Y	NSCLC	1
			E82D	NSCLC	1	A124G	Breast	2
				Bladder	3	A124V	Colorectal	3
				Uterine	4			
				Cervical	1			
				ccRCC	2			
				pRCC	1			
				Liver	1			
				Cholangiocarcinoma	1			
			E82G	Esophagus	1			
				pRCC	1			
				Liver	1			
			E82A	Esophagus	1			
				Liver	1			
			E82Q	NSCLC	1			
			E82V	Head & neck	1			

Note that these somatic alterations cluster in gene coding regions coding for two hot-spot motifs of Nrf2, through which Nrf2 associate with its most prominent endogenous inhibitor Keap1 to negatively control its stability. The frequencies of presented substitutions in total 275 samples analyzed are shown. Data are publically available on http://www.cbioportal.org/ [45,46]. A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine; NSCLC, non-small cell lung cancer; lung squ, lung squamous cell carcinoma; lung adeno, lung adenocarcinoma; ESCC, esophageal squamous cell carcinoma; ccRCC, clear cell renal cell carcinoma; nccRCC, non-clear cell renal cell carcinoma; pRCC, papillary renal cell carcinoma.

cancer [54]. This suggests the oncogenic potential of activated Nrf2 and puts chronically sustained activation of Nrf2 on high alert. This urgently calls for an approachable intervention in cases of cancer with overactivated Nrf2.

Nrf2 AS A THERAPEUTIC TARGET FOR PRECISION CANCER MEDICINE

Tumors are highly heterogeneous with cancer cells within the same tumor being not all the same (intratumor heterogeneity). Tumors of a given cancer type could be also drastically different from patient to patient (intertumor heterogeneity) in terms of genetic alterations [55-57]. Intratumor heterogeneity might arise from clonal evolution, a process through which selection upon exposure to given therapies gives rise to a pool of mutations [57,58]. The phenotype hence could explain the development of polyclonal resistance in the course of treatment in patients who initially have a good response to a given cancer drug, but subsequently develop the relapse of cancer [58-60]. Whilst, the variations among individuals with the same type of cancer determine which targeted therapies a subpopulation would be sensitive to [57,61]. Current advances in genomics, proteomics, and metabolomics and the availability of large-scale clinical genomic databases have enabled characterizing patients' tumors and the identification of variations specific to each patient thereby allowing the determination of a more precise treatment with maximal efficacy and minimal side effects [62-64], which is the basis of precision cancer medicine.

The regularity of genetic alterations in Nrf2 in cancer as well as their association with a poor clinical outcome suggests this transcription factor as a potential biomarker in precision cancer medicine [17,65,66]. In this context, profiling the gene encoding Nrf2 would be recommended in cancer management, encompassing initial diagnosis, risk stratification, disease prognosis, predicting responses to treatment, and finally deciding more precise medications for corresponding individuals (Fig. 3).

A drop in the cost and advances in high-throughput next-generation sequencing technology have made characterization of genetic aberrations in the Nrf2 gene easier than ever [67,68]. Nevertheless, the pitfall remains to be addressed here is that the Nrf2 gene profiling has been so far conducted on a fraction of cells collected through tumor removal [22,69], which might not represent the whole tumor genomic landscape due to the intratumor heterogeneity [57]. As a result, there are chances that the changes in Nrf2 might be overlooked although they could be possibly an important feature of the tumor. Conversely, a wrong therapy could be indicated if the Nrf2 mutations, regardless of being detected, are not common in the tumor. Single-cell DNA and RNA sequencing (SCS) methods [70-72] would be applied to narrow our understanding of alterations in Nrf2 in every tumor cell rather than in the entire tumor mass [70,71]. SCS methods for characterizing the Nrf2 geen could be also extended to circulating tumor cells (CTCs), which are sloughed from primary tumors and carry even additional acquired mutations as a result of exposure to therapeutic treatment in a metastatic context [73,74]. In addition to CTC, tumor-derived circulating-tumor DNA (ctDNA) present in blood or other body fluids [75], which has been noticed to well correlate with changes in tumor load [76-78], should be encouraged to be subjected to the Nrf2 gene profiling. The use of CTC and ctDNA would be attractive noninvasive alternatives to tumor biopsies and may reflect real-time tumor burden during the course of cancer treatment [79-81].

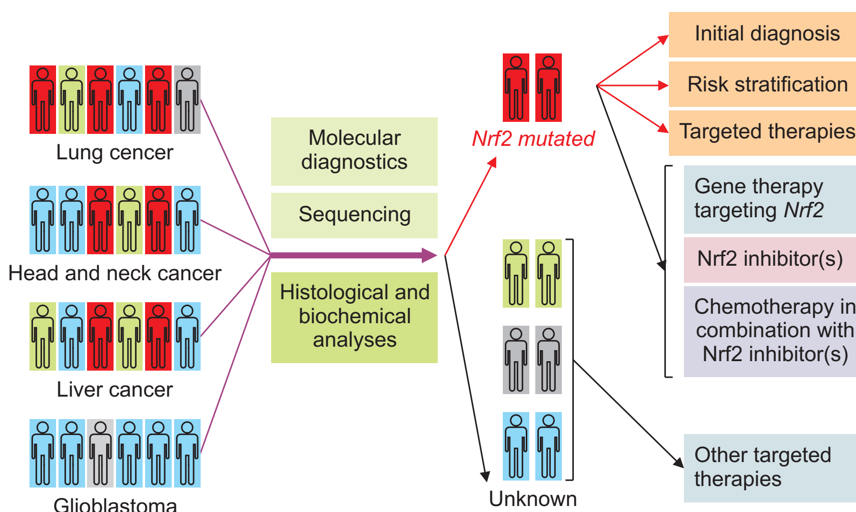

Figure 3. The introduction of Nrf2 as a target for precision cancer therapy. Tumor tissue from various types of cancer can be subjected to a series of molecular diagnostics including histological and biological analyses to evaluate Nrf2 levels as well as the magnitude/frequency of genetic alterations. Individuals with genetically altered Nrf2 then would be distinguished and grouped to guide the selection of more appropriate drugs.

Three clinical trials, KEAPSAKE, CX-839-014, and BeGIN study investigated mutations in the Nrf2/Keap1 pathway to tailor cancer treatment [82-84]. The CX-839-014 trial was aimed to identify patients with stage IV nonsquamous NSCLC that carry mutations in Keap1 and/or Nrf2 genes. CtDNA present in blood samples from patients was analyzed by next generation sequencing (NGS) for the presence of genetic alterations in Keap1 and/or Nrf2 [84]. The eligible patients who are positive for Keap1 and/or Nrf2 gene mutational status will be were then subjected to KEAPSAKE and BeGIN study [82,83]. KEAPSAKE is a phase 2 randomized, multicenter, double-blinded trial designed to test the hypothesis that telaglenastat hydrochloride (CB-839 HCl), an inhibitor of glutaminase catalyzing amino acid glutamine to glutaminase, would work better in treating patients with Keap1/Nrf2 gene mutated, stage IV, NSCLC [83]. The efficacy of this drug in patients bearing Keap1/Nrf2 gene mutation have also been studied in other types of solid tumors or malignant metastatic peripheral nerve sheath tumors in BeGIN study [82].

POTENTIAL STRATEGIES FOR TARGETING Nrf2 IN CANCER

Small molecules to modulate mutagenic activation of Nrf2 in cancer cells

A major hurdle toward precision cancer medicine is the development of therapeutic agents that are paired with validated biomarkers [63,85]. The characterization of Nrf2 gene mutations as a new biomarker in a plethora of cancer types might pave the way for further classification of cancer into subtypes negative or positive for Nrf2 aberrations, which are distinctive in clinical outcomes as well as response to treatment. In such cases of subclasses addicted to Nrf2, small molecules targeting Nrf2 would be considered of particular interest. Some Nrf2 inhibitors have been tested for their antitumor effects in cancers that heavily rely on the Nrf2/Keap1 pathway. Brusatol, a quassinoid from the seeds of *Brucea sumatrana*, was shown to provoke rapid and transient depletion of Nrf2 in a Keap1-independent fashion, thereby sensitizing cancer cells to chemical stress [86,87]. In search of new Nrf2 inhibitors.

Tsuchida and colleagues [88] performed high throughput screening with inhibition of Nrf2 transcriptional activity being a readout on A549 lung adenocarcinoma cells which constitutively express Nrf2 as a result of Keap1 loss-off function mutations. The assay identified febrifugine, a quinazolinone alkaloid from *Dichroa febrifuga*, and its less toxic derivative halofuginone as hit compounds. Halofuginone was found to decrease Nrf2 accumulation in an amino acid starvation response mechanism, which resulted in decreases in resistance of Nrf2-overexpressing cancer cells to anticancer drugs [88]. However, compared with numerous verified Nrf2 activators, which have been long tested in an attempt to activate Nrf2 in normal cells for a purpose of preventing cancer [7], only a small number of Nrf2 inhibitors have been identified so far [89,90], of which none of them is available for clinical use currently. A lack in fully resolved crystal structure of Nrf2 might be the reason why developing Nrf2 inhibitors is still in its infancy. As the cytoprotective effects of Nrf2 are applied to both normal and cancer cells, undesired side effects that might be caused by delivering Nrf2 inhibitors systemically should also be considered when developing drugs targeting Nrf2 [91].

GENE-BASED THERAPIES TO TARGET MUTANT Nrf2 IN CANCER CELLS

Strategies to attenuate Nrf2 overactivation

It is noteworthy that sustained activation of Nrf2 lies in its genetic alterations, which encompass DNA amplification and somatic point mutations, suggesting the potential application of gene therapy targeting the gene for Nrf2. Gene-based therapies are basically the introduction of exogenous nucleic acids into cancer cells in order to restore the functions of genes that are missing or to halt the expression of tumor-promoting genes [92]. RNA interference (RNAi) with use of either small interfering RNAs (siRNAs) or microRNAs, which selectively inhibits translation of mRNA transcript of a target gene into a protein, has been being broadly harnessed for clinical applications and particularly in cases considered 'undruggable' [93,94]. RNAi hence could hold promises for cancer with Nrf2 amplified. It has been demonstrated that Nrf2-addicted cancer cells become more sensitive to chemotherapeutic agents when Nrf2 gene was knockdown by siRNA [41,95,96]. Pharmaceutical companies have used lipid nanoparticles and conjugates to overcome siRNA drug delivery challenges, resulting in five siRNA therapies approved by the US FDA for metabolic diseases and many in clinical trials [97]. The success of siRNA drugs in other applications has opened new avenues for innovative treatments for cancer with the overactivation of Nrf2.

Correction of Nrf2 mutations by programmable nuclease-based genome editing technologies

As a fascinating addition to the RNAi therapy, the introduction of programmable nuclease-based genome editing technologies has paved the way for easier and more efficient deletion or correction of genetic aberrations. The principle is to introduce DNA-double stranded breaks (DSBs) by nucleases at a genomic locus of interest. Thereafter, either nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) in the presence of a single-stranded oligonucleotide (ssODN) homologous to the targeted region is activated to seal the breaks. NHEJ results in random insertion/deletion mutations [98,99], which disrupts the transcription of the target genes. DSBs followed by NHEJ, in consequence, could be introduced to eradicate amplified Nrf2 in cancer. In contrast, HDR allows the exchange of nucleotide sequences between targeted DNA strands and ssODN donor templates, leading to

the introduction of point mutations desired or insertion of sequences of choice [98,100]. DSBs with ssODNs, on the one hand, would allow the insertion of additional mutations to the DNA-binding domain-coding region bZIP-Maf in Nrf2, which thereby attenuates its aberrantly elevated transcriptional activity in cancer. This also would facilitate the correction of activating substitutions in DLG and ETGE motifs of Nrf2, turning Keap1-resistant activated Nrf2 to the wild-type that becomes tightly regulated by Keap1.

Currently, four classes of nucleases are available. The first three classes are protein-based systems. These include meganucleases, zinc finger nucleases and transcription activator-like effector nucleases that recognize the specific genome sites based on protein-DNA interactions [101,102]. Therefore, different specific nucleases need to be complicatedly engineered for different target sequences [102]. The forth class of nucleases, clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (Cas) 9 system, alternatively works on the basis of the interaction between an engineered short RNA guide and the target DNA site, which is much more flexible and ready-to-use [98,100]. Hence the RNA-based CRISPR-Cas9 system would be strongly urged to be considered as a powerful tool to achieve the desired changes in the Nrf2 gene including elimination and correction of point mutations. In fact, CRISPR-Cas9 technology has been extensively utilized by researchers to manipulate oncogenes [103-105] the Nrf2 gene [106] in various types of cancer and showed very promising therapeutic potentials. In a proof of concept study, Bialk et al. [106] presented that disabling the Nrf2 gene in A549 lung carcinoma cells by CRISPR-directed gene editing slowed down cell proliferation and increased sensitivity of cancer cells to chemotherapy, which brings the technology a step closer to clinical application. US FDA has recently approved the first CRISPR therapy for sickle cell disease [107], which offers hope for developing novel therapies based on the technology for cancer with activating mutations of Nrf2.

A need for drug combinations and drug evaluation to precisely target Nrf2 for cancer treatment

A tumor bulk is heterogeneous with a myriad of mutations that arise in the course of tumor evolution [55,108]. Concordantly, in various types of cancer, genetic alterations in Nrf2 are temporally accompanied by changes of other genes such as *PIK3CA*, *CMYC*, and *KRAS*, which might occur at different stages of tumor development [26,109,110]. It might be plausible that behaviors of mutated Nrf2 in cancer would be dictated by a genetic landscape as well as co-coexisting mutations, which is termed epistatic interactions [108]. Understanding genetic background would hence provide an elegant platform for optimizing the combination of 'drugs' targeting activated Nrf2 and other concomitant aberrations to achieve the desired therapeutic effects [56].

Although Nrf2 could serve as an attractive therapeutic target for cancer treatment, the Nrf2-targeting therapies would not be given precisely to the right patients unless the vulnerabilities of live patient cancer cells to potential drugs are verified [111]. To date, there have been many methods developed to obtain a sufficient number of patient-derived live cancer cells for 'drug' testing. These include conditional reprogramming, which involves the use of an enriched environment to expand the human tissues, cultivating patient cancer cells in a semi-solid extracellular matrix three-dimensional condition to allow formation of organoids [112,113], and enrichment of CTCs on an advanced microfluidic chip [114]. Various functional analyses have also been invented for measuring drug effects on patients' live cancer cells such as utilizing patient-derived xenograft mouse models [111]. This functional testing should be included to facilitate the development of therapies targeting Nrf2 and the guidance on precisely matched therapies.

CONCLUSION

Despite the controversy about Nrf2 being a tumor suppressor or an oncogene has been an unsolved conundrum, it is becoming apparent that Nrf2 actively secures maintenance of fully malignant cells and therefore exacerbating cancer progression [15,49]. Constitutive overactivation of Nrf2 is speculated to regulate metabolic reprogramming to meet the anabolic needs for rapid proliferation of cancer cells [14,53], and to create a favorable microenvironment with less oxidative stress for cancer cell growth [52]. These tumorigenic potentials of Nrf2 lie in its sustained activation in cancer, which arises from two 'hot-spots' gain-of-function mutations located in the DLG and ETGE motifs in the Neh2 domain [115]. As these aberrations of the Nrf2 gene are so ubiquitous and involves in behaviors and vulnerability to chemotherapies in a medley of cancer types, they could be supposed to be an attractive target for precision cancer therapy. However, it still remains questionable that to what extent these genetic changes and 'hot-spot' mutations of Nrf2 contributes to cancer development and progression. For efficiently targeting Nrf2, it is critical to profile the genotype of this transcription factor in every single cell from either tumor bulks or CTCs, and not to mention, to consider not only Nrf2 but the entire genetic background as they might define overall behaviors of Nrf2. Targeting Nrf2 by use of small molecules or gene-based therapies, particularly CRISPR-Cas9, would be an important strategy of cancer intervention in the era of precision medicine.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

ORCID

Hoang Kieu Chi Ngo, https://orcid.org/0000-0003-2151-5401
Hoang Le, https://orcid.org/0000-0001-8427-3230
Young-Joon Surh, https://orcid.org/0000-0001-8310-1795

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