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ARTICLE

High expression of nuclear NRF2 combined with *NFE2L2* alterations predicts poor prognosis in esophageal squamous cell carcinoma patients

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Nuclear factor erythroid-2 related factor-2 (*NFE2L2* or *NRF2*) is a frequently mutated gene in esophageal squamous cell carcinoma (ESCC). However, the roles of *NFE2L2* alterations in ESCC remain elusive. In order to elucidate this issue, 130 ESCC patients who underwent esophagectomy were enrolled. The majority of tumor tissues were positive for NRF2, which was significantly enriched in the nucleus of the primary tumor tissues compared with the noncancerous mucosae. Primary ESCC tumors positive for NRF2 tended to be positive for NAD(P)H quinone oxidoreductase 1 (NQO1) as the downstream target of NRF2. There was a positive correlation between NRF2 and NQO1 expression level in primary tumors. NQO1 staining in primary tumors with NRF2 nuclear expression was significantly stronger than that with NRF2 cytoplasmic expression. In addition, high concordance for the status of NRF2 expression between primary tumors and corresponding metastatic lesions was observed. Next, we found high expression of nuclear NRF2 (the proportion of nuclear NRF2 expression >20% or nuclear NRF2 immunohistochemistry score >20) predicted shorter overall survival in patients with dual-positive expression of NRF2 and NQO1. Captured-based targeted sequencing revealed that *NFE2L2* somatic alterations were observed in 52.8% of ESCC patients with dual-positive expression of NRF2 and NQO1. *NFE2L2* amplification and mutations within the DLG/ETGE motifs were seen more frequently in ESCC tumors with nuclear or nucleocytoplasmic expression of NRF2 compared with those with cytoplasmic expression of NRF2. We also found high expression of nuclear NRF2 plus the status of *NFE2L2* alteration exhibited high performance in predicting prognosis of ESCC patients. Our study demonstrated that high nuclear NRF2 expression and *NFE2L2* alterations were associated with poor prognosis of ESCC patients. These findings suggest that NRF2 signaling pathway might play vital roles in ESCC malignancy and the aberrant activation of NRF2 pathway predicts unfavorable prognosis in ESCC.

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INTRODUCTION

Esophageal cancer (EC) is one of the most aggressive gastrointestinal cancers worldwide[1]. Esophageal squamous cell carcinoma (ESCC) is the most common histological subtype of esophageal cancer (EC), and is highly prevalent in East and Southeast Asia[2]. In China, EC is the third most common cancer and the fourth most common cause of cancer death[3]. Despite recent advances in therapy for ESCC, such as chemotherapy, radiotherapy, and immunotherapy, the prognosis of ESCC patients remains poor. Further studies are therefore needed to clarify the molecular pathogenesis of ESCC to explore new therapeutic possibilities.

Nuclear factor erythroid-2 related factor-2 (NFE2L2, also known as NRF2) is a main transcription regulator of stress response. It has been documented to be one of frequently mutated genes in ESCC, mutations in which have been detected in 5.0–11.4% of ESCCs[4–7]. *NFE2L2* mutations also have been detected in about 10.0% of non-small cell lung cancers, 13.0% of laryngeal squamous cell carcinomas, and 6.3% of skin squamous cell carcinomas[4]. Under normal physiological conditions, NRF2 regulates the transcription of antioxidant proteins, including NAD(P)H quinone oxidoreductase 1 (NQO1) and multidrug resistance (MDR) protein family (such as ATP binding cassette subfamily B member 1 [ABCB1], which exerts cytoprotective effects by effluxing toxins and reducing their accumulation in cells) that maintain cellular redox homeostasis and protects against oxidative stress induced by reactive oxygen species (ROS)[8–10]. However, NRF2 may serve as a double-edged sword in cancers. NRF2 is traditionally considered to have tumor-suppressive effects due to its cytoprotective functions[11–13], but accumulating evidence suggest that NRF2 serves as an oncogenic driver in cancers based on that NRF2 hyperactivation may favor the survival of cancer cells by protecting them from excessive oxidative stress, chemotherapeutic agents, or radiotherapy[14–16].

It has been documented that NRF2 accumulation at the protein level induces robust transactivation of cytoprotective genes, such as antioxidative enzyme NQO1[14,17]. Therefore, NQO1 is widely

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used to evaluate NRF2 activity[18,19]. Under normal physiological conditions, NQO1 protects cells against exogenous and endogenous toxins[20]. NQO1 is also implicated in molecular and genetic mechanisms of tumorigenesis, which has been documented to promote cancer progression[21,22]. In addition, ABCB1 is the most extensively studied ABC transporter to confer resistance to cytotoxic and targeted chemotherapy in cancer cells[23,24]. These previous findings suggest that dual-positive expression of NRF2 and NQO1 indicates the activation of NRF2 signaling pathway and is implicated in the malignancy of NRF2-addicted tumors. However, whether *NFE2L2* alterations and the expression level of NRF2/NQO1 are associated with the prognosis of patients remains elusive in ESCC.

In the present study, we investigated the status of NRF2 expression and *NFE2L2* alteration and explored the association between the status of NRF2 expression/*NFE2L2* alteration with overall survival in ESCC patients. This study could pave the way for understanding potential molecular mechanisms and diagnostic/therapeutic perspectives in ESCC.

MATERIALS AND METHODS

Collection of clinical samples

A total of 130 consecutive ESCC patients who underwent esophagectomy without any preoperative treatment at Beijing Chao-Yang Hospital between May 2011 and October 2018 were enrolled in this study. Primary tumors, its adjacent noncancerous mucosae, and matched metastatic lesions were collected from patients. Tumor stage was assessed according to the eighth edition of the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) TNM staging system[25]. One hundred and eighteen patients with stage I-III disease received curative esophagectomy and 12 patients with stage IV disease received palliative esophagectomy. This study was approved by the institutional review boards of Beijing Chao-Yang Hospital, Capital Medical University. Informed consent was waived because of the retrospective and anonymous nature of this study.

Immunohistochemistry and evaluation of staining

Formalin-fixed, paraffin-embedded (FFPE) tissue sections were assessed by immunohistochemistry (IHC) staining using an automated tissue immunostainer (Ventana BenchMark ULTRA, Roche Diagnostics, USA) and the following antibodies: NRF2 (dilution 1:100, clone EP1808Y, Abcam, Cambridge, UK), NQO1 (dilution 1:150, clone A-5, Santa Cruz Biotechnology, Santa Cruz, CA), and ATP binding cassette subfamily B member 1 (ABCB1, dilution 1:250, clone D-11, Santa Cruz Biotechnology, Santa Cruz, CA). All histological and immunohistochemical slides were reviewed independently by two pathologists. Staining intensity for NRF2, NQO1 or ABCB1 was rated on a scale of 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). IHC score was calculated by multiplying the staining intensity by the percentage of positive tumor cells, which ranged from 0 to 300. The proportion of NRF2 nuclear expression was defined as the ratio of tumor cells positive for nuclear NRF2 expression (with a IHC staining scale of 1, 2, or 3) to the total number of viable tumor cells. High expression of nuclear NRF2 was defined as the proportion of NRF2 nuclear expression >20% or nuclear NRF2 IHC score >20.

Tissue DNA isolation and capture-based targeted DNA sequencing

Tissue DNA was extracted from FFPE tumor tissues using a QIAamp DNA formalin-fixed paraffin-embedded tissue kit (Qiagen, Hilden, Germany). Next, a minimum of 30 ng was used for library construction. Tissue DNA was sheared to yield 200–400 bp fragments using an M220 ultrafocused sonicator (Covaris, MA, USA), followed by end repair and adaptor ligation for library construction. Next, the DNA library was purified by using an Agencourt AMPure XP Kit (Beckman Coulter, CA, USA), amplified, and selected with magnetic beads. The quality and the size of the fragments were assessed by high sensitivity DNA kit using Bioanalyzer 2100 (Agilent Technologies, CA, USA). Capture-based targeted sequencing was performed using a panel consisting of 168 cancer-related genes, spanning 253 Kb of the human genome (Burning Rock Biotech, Guangzhou, China). Indexed samples were sequenced on MiSeq or NextSeq500 (Illumina, Inc., USA) with paired-end reads and average sequencing depth of 1000×.

Sequence data analysis

Sequence data were mapped to the reference human genome (hg19) using Burrows-Wheeler Aligner (version 0.7.10). Local alignment optimization, duplication marking, and variant calling were performed using Genome Analysis Tool Kit (version 3.2) and VarScan (version 2.4.3). Variants were identified using the VarScan and loci with a depth of less than 100× were filtered out. Base calling required at least eight supporting reads for single nucleotide variations and five supporting reads for insertion-deletion variations. Variants with a population frequency of more than 0.1% in the ExAC, 1000 Genomes, dbSNP or ESP6500SI-V2 databases were defined as single nucleotide polymorphisms and excluded from further analysis. Remaining variants were annotated with ANNOVAR (released on February 1, 2016) and SnpEff version 3.6. Analysis of DNA translocation was performed using Factera version 1.4.3. Copy number variations were analyzed based on the depth of coverage data of capture intervals as previously described[26]. The next-generation sequencing data of this study can be obtained from the corresponding author.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). The continuous variables were described as mean ± standard deviation unless otherwise stated. Differences between two-groups were assessed by *t*-test or Wilcoxon rank sum test for continuous variables and by Fisher's exact test or chi-square test for categorical variables. Kaplan–Meier curves and Cox regression analyses were performed for investigating the association between NRF2 expression/*NFE2L2* alteration and overall survival (OS) outcome. $P < 0.05$ was considered to be statistically significant.

RESULTS

Clinical characteristics

Of the 130 ESCC patients, 116 (89.2%) were males, and 14 (10.8%) were females. The median age was 62.5 years (range, 39-82 years). Eighty-seven patients (66.9%) had a smoking history and 68 patients (52.3%) had a history of heavy alcohol consumption. The majority of patients (74.6%) had stage II/III disease. For histological grade, 9 patients (6.9%) had well differentiated ESCC (Grade 1), 82 (63.1%) had moderately differentiated ESCC (Grade 1), and 43 (30.0%) had poorly differentiated ESCC (Grade 3). The clinical characteristics of patients are presented in Table S1.

The expression of NRF2 in primary tumors and noncancerous mucosae

Total of 130 primary tumors were stained by immunohistochemistry for NRF2. Seventy-seven tumor tissues (59.2%) were positive for NRF2, and the remaining 53 (40.8%) samples were negative for NRF2 (Fig. 1A). Among the 77 NRF2-positive cases, NRF2 staining were localized in cytoplasm in 46 (59.7%) cases, in nucleus in 14 cases (18.2%), and in both cytoplasm and nucleus in 17 cases (22.1%) (Fig. 1A–E). NRF2 IHC scores in both cases with NRF2 nuclear expression and those with nucleocytoplasmic expression were significantly higher than those with NRF2 cytoplasmic expression (both $P < 0.0001$, Fig. 1F).

In order to compare the expression status of NRF2 between tumor tissues and noncancerous mucosae, IHC staining was also performed in 74 patients who had matched noncancerous mucosae. The majority of samples (46/74, 62.2%) were positive for NRF2, the expression of which had strongest staining in the parabasal cell layer and decreased stepwise toward the superficial cell layer (Fig. 2A). Moreover, NRF2 staining predominantly distributed in the cytoplasm (45/46, 97.8%) of the noncancerous mucosae. Compared with the noncancerous mucosae, NRF2 was significantly enriched in the nucleus of the tumor tissues (40.3% vs. 2.2%, $P < 0.0001$, Fig. 2B).

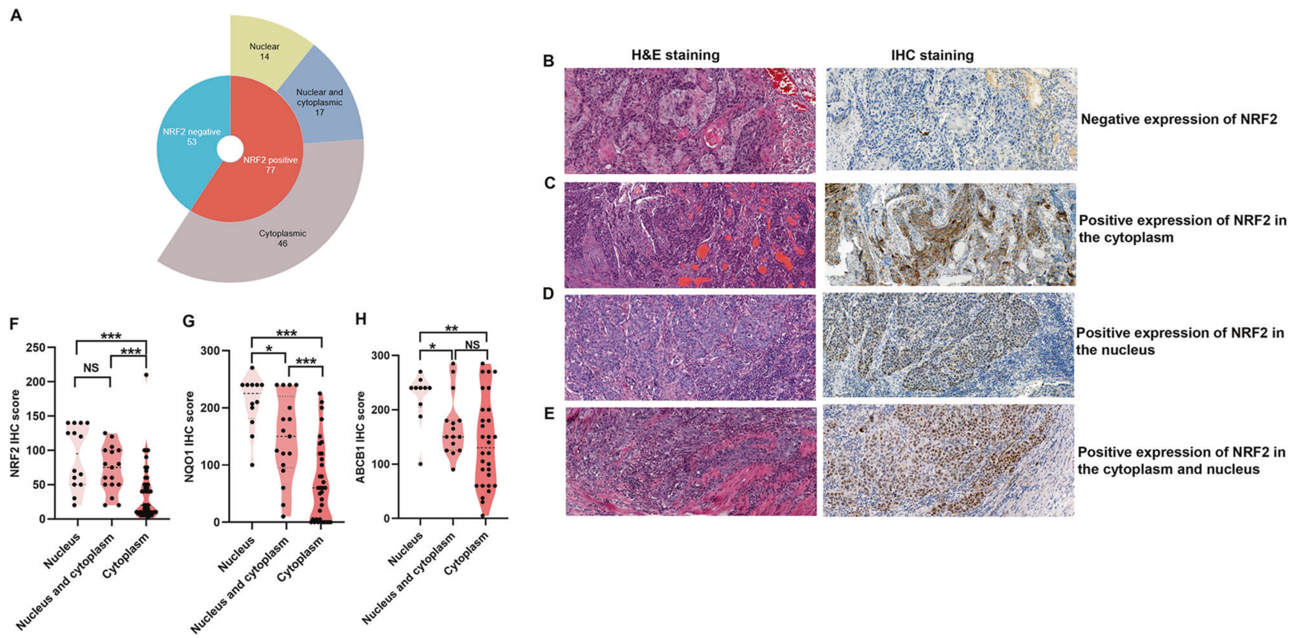

Fig. 1 The expression of NRF2 and its downstream NQO1 and ABCB1 in ESCC tumors. A The distribution of the status of NRF2 expression and NRF2 localization in ESCC patients; (**B**). IHC staining showed in the tumor with negative expression of NRF2; (**C**). IHC staining showed in the tumor with positive expression of NRF2 in the cytoplasm; (**D**), IHC staining showed in the tumor with positive expression of NRF2 in the nucleus; (**E**), IHC staining showed in the tumor with positive expression of NRF2 in the cytoplasm and nucleus; (**F**), The difference of NRF2 IHC score among patients with NRF2 positivity; (**G**), The difference of NQO1 IHC score among patients with NRF2 positivity; (**H**), The difference of ABCB1 IHC score among patients with NRF2 positivity. *, **, and *** indicates $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. NS (not significance) indicates $P > 0.05$. Each dot in Fig. 1F, 1G, and 1H represents a case with NRF2 positivity.

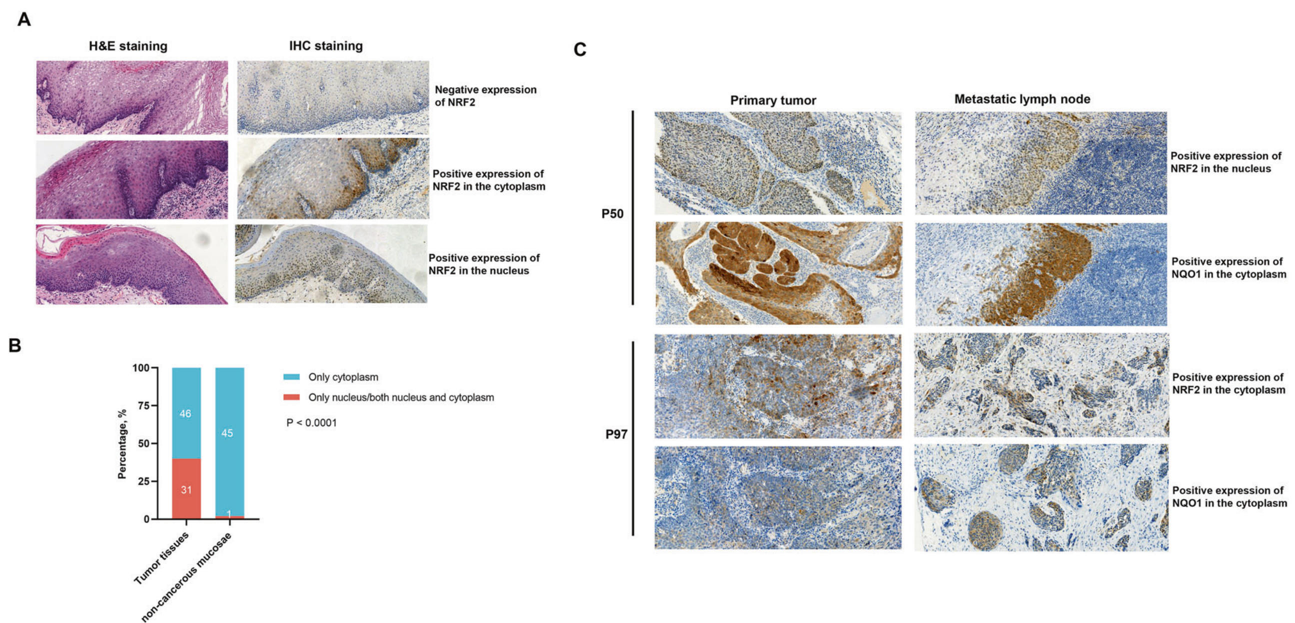

Fig. 2 The expression of NRF2 in the noncancerous mucosae, primary tumors, and metastatic lymph nodes. A IHC staining for NRF2 in the noncancerous mucosae; (**B**) The difference of NRF2 positivity between tumors and noncancerous mucosae; (**C**) Representative IHC staining for NRF2/NQO1 in primary tumors and matched metastatic lymph nodes collected from two patients. P50: patient 50; P97: patient 97.

The expression of NQO1 and ABCB1 in primary tumors

NQO1 and ABCB1 act downstream of NRF2. Next, we also investigated the expression of NQO1 and ABCB1 in patients who had adequate archived tumor tissue. NQO1 immunostaining was performed on 85 cases. NQO1 positivity was observed in the cytoplasm of the tumor cells in 64 of the 85 samples (75.3%). Moreover, 67 cases were subjected to IHC staining for ABCB1, which was stained almost uniformly on the tumor cell membrane and in the cytoplasm (97.0%, 65/67). The NQO1 ($P = 0.000$) or ABCB1 ($P = 0.030$) expression status was associated with NRF2 expression status. In detail, ESCC tumors with NRF2 positivity tended to be positive for NQO1 (56 of 65 NRF2 positive tumors displayed NQO1 positivity)

Table 1. The association between NRF2 localization and the expression status of NQO1/ABCB1.

	NRF2 expression				*P* value
	Negative positivity (G1)	Cytoplasmic positivity (G2)	Nucleocytoplasmic positivity (G3)	Nuclear positivity (G4)	
NQO1 expression status					$P1 = 0.0001$
Positive	8	27	17	12	$P2 < 0.001$
Negative	12	9	0	0	$P3 < 0.010$
ABCB1 expression status					$P4 = 0.030$
Positive	10	30	15	10	$P5 = 0.525$
Negative	2	0	0	0	$P6 = 1.000$

P1, P2, P3 indicated the difference of the expression status of NQO1 between G1 and G2/G3/G4, between G1/G2 and G3/G4 group, and between G2 and G3/G4 group, respectively; P4, P5, P6 indicated the difference of the expression status of ABCB1 between G1 and G2/G3/G4, between G1/G2 and G3/G4 group, and between G2 and G3/G4 group, respectively.

NRF2 Nuclear factor erythroid-2 related factor-2, *NQO1* NAD(P)H quinone dehydrogenase 1, *ABCB1* ATP binding cassette subfamily B member 1.

and ABCB1 (all 55 NRF2 positive tumors displayed ABCB1 positivity) (Table S2, Figure S1). Next, the association between NRF2 localization and NQO1/ABCB1 expression status was investigated. ESCC tumors with nuclear and nucleocytoplasmic NRF2 expression displayed a significantly higher rate of NOQ1 positivity (100% vs. 75%, $P < 0.010$) and a comparable rate of ABCB1 positivity (100% vs. 100%, $P = 1.000$) compared with tumors with cytoplasmic NRF2 expression (Table 1). Furthermore, there was a positive correlation between NRF2 IHC score and NQO1 (Spearman $r = 0.670$, $P < 0.0001$) or ABCB1 IHC score (Spearman $r = 0.493$, $P < 0.0001$). Our data revealed that the expression status of NQO1 was significantly associated with NRF2 localization. Both NQO1 (Fig. 1G, $P < 0.001$) and ABCB1 IHC score (Fig. 1H, $P < 0.010$) were significantly higher in tumors with NRF2 nuclear expression than that with NRF2 cytoplasmic expression.

The associations between NRF2 expression and clinical characteristics in primary tumors and noncancerous mucosae

Next, statistical analyses were performed to assess the associations between NRF2 expression and clinicopathological characteristics in patients with ESCC. For noncancerous mucosae, NRF2 expression was not associated with clinical characteristics except for smoking history (Table S3). Our analyses showed that smokers showed a trend of having NRF2 overexpression in noncancerous mucosae ($P = 0.054$). For primary tumors, we failed to find associations between NRF2 expression (Table S4) or localization (Table S5) and clinicopathological characteristics.

NRF2/NQO1 expression in matched metastatic lymph node lesions

In order to investigate the concordance for the status of NRF2 expression between primary and metastatic tumors, surgical specimens from 19 cases with primary tumors and matched metastatic lymph node lesions were obtained and performed for IHC staining (Fig. 2C). NRF2 positivity was observed in both primary and metastatic lesions (12/19 *vs.* 10/19). The concordance for the status of NRF2 expression between primary tumors and corresponding metastatic lymph node lesions achieved 89.5% (17/19) with a kappa value of 0.787 (Table S6). In two patients, the primary tumors were positive for NRF2 staining in cytoplasm, while the paired metastatic lesions were negative. In ten NRF2-positive metastatic lymph node lesions, the subcellular localization of NRF2 was consistent with that in paired primary tumors, five were cytoplasmic staining, three were nuclear and cytoplasmic staining, and 2 were nuclear staining. For NQO1, its expression status in both primary and metastatic lesions was also investigated (Fig. 2C). We found that the concordance for the status of NQO1 expression between primary tumors and corresponding metastatic lymph node lesions achieved 88.9% (8/9) with a kappa value of 0.800 (Table S7). Collectively, metastatic lymph node lesions might be the alternative to primary tumors in detecting the expression status of NRF2 and NQO1 in ESCCs.

The associations between NRF2 expression/localization and overall survival

Next, we analyzed the associations between NRF2 expression/localization in primary tumors and OS of ESCC patients. Our analysis revealed that neither the status of NRF2 (*P2* = 0.303, Figure S2A) nor NQO1 expression (*P2* = 0.877, Figure S2B) was associated with OS, after adjusting for gender, age, tumor stage, smoking, and drinking history. Subsequently, we analyzed the associations between the status of NRF2 subcellular localization and OS. Of the 64 patients with NRF2 positivity, patients with cytoplasmic ($N = 37$, OS = 39.0 months), nucleocytoplasmic ($N = 15$, OS = 40.0 months) or nuclear ($N = 12$, OS = 24.0 months) NRF2 expression had comparable median OS (P2 = 0.105, Figure S2C), after adjusting for gender, age, TNM stage, smoking, and heavy alcohol consumption history.

Intriguingly, our analysis revealed that the proportion of NRF2 expression in the nucleus more than 20% predicted statistically significantly shorter median OS in patients with dual-positive expression of NRF2 and NQO1 without (25.0 months *vs.* not reached [NR]), *P1* = 0.021) or with (25.0 months vs. NR, *P2* = 0.042) adjusting for age, gender, tumor stage, smoking, and drinking history (Fig. 3A). We also found that NRF2 IHC score in the nucleus more than 20 predicted a trend of shorter median OS in patients with dual-positive expression (Fig. 3B). Collectively, our analyses demonstrated that the expression level of NRF2 in the nucleus of ESCC tumors had an impact on OS.

The comprehensive genomic profiling of primary tumors with dual-positive expression of NRF2 and NQO1

In order to investigate the underlying genetic mechanism of NRF2 overexpression in ESCC, capture-based targeted sequencing using a panel consisting of 168 cancer-related genes was performed on 36 primary ESCC tumors with dual-positive expression of NRF2 and NQO1, including 12 with nuclear NRF2-positivity, 16 with nuclear and cytoplasmic NRF2-positivity and 8 with cytoplasmic NRF2-positivity. All these tumors were positive for NQO1. We achieved a mean coverage depth of 1,567× across all targeted regions on all tissue samples. For all samples, the mapped reads percentage was over 99.0%. Collectively, we identified 211 mutations spanning 53 genes, including 100 single nucleotide variants (SNVs), 17 insertions or deletions (Indels), 89 copy-number amplifications (CNAs), four copy number deletions, and 1 translocation. Of the 36 samples, 35 (97.2%) had mutations

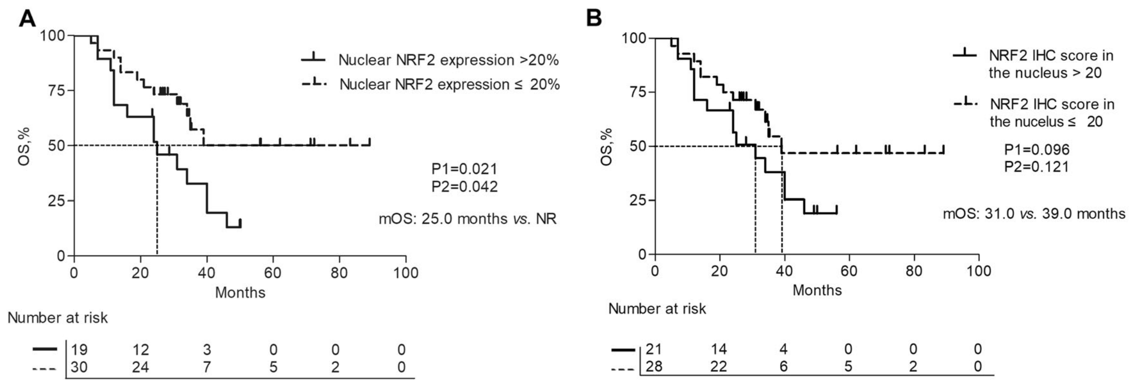

Fig. 3 Overall survival by expression level of NRF2 in ESCC patients with dual-positive expression of NRF2 and NQO1. **A** Comparison of overall survival between patients with the proportion of NRF2 expression in the nucleus more than 20% and those with less than 20%; **B** Comparison of overall survival between patients with NRF2 IHC score in the nucleus more than 20 and those patients with less than 20. P1 and P2 indicated *P*-value respectively calculated without and with adjusting for age, gender, tumor stage, smoking, and heavy alcohol consumption.

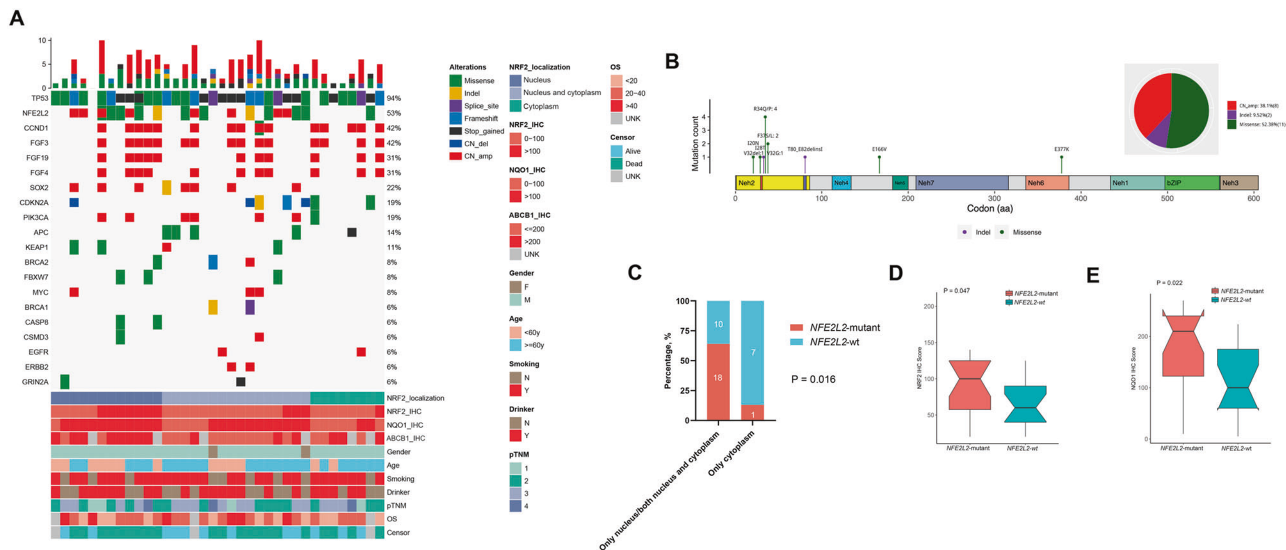

Fig. 4 The association between *NFE2L2* somatic alteration status and NRF2 expression in ESCC patients. **A** Genomic profiling of patients with dual positive expression of NRF2 and NQO1; (**B**) *NFE2L2* alterations identified in the present work; (**C**) The difference of NRF2 localization between patients with and without *NFE2L2* alteration; (**D**) The difference of NRF2 IHC score between patients with and without *NFE2L2* alteration; (**E**) The difference of NQO1 IHC score between patients with and without *NFE2L2* alteration.

detected from this panel (Fig. 4A). The most frequently mutated gene was *TP53*, occurring in 94.4% (34/36) patients; followed by *NFE2L2*, occurring in 52.8% (19/36) of patients. The most commonly seen mutation in *NFE2L2* was p.R34Q/P, which was in an agreement with previous studies[5]. Of the 19 patients harboring *NFE2L2* alterations, 10 had SNVs, 2 had Indels, and eight had CNAs (one patient had concurrent SNV and CNA) (Fig. 4B). Focal amplification of chromosome region 11q13 (*CCND1*, *FGF3*, *FGF4*, and *FGF19* amplification) was observed in 44.4% (16/36) of patients.

NRF2 activity is regulated by the oxidative-stress sensor molecule Kelch-like ECH-associated protein 1 (KEAP1) under unstressed conditions. *KEAP1* alterations p.R272C, p.R470H, p. D294Y, and amplification were also identified in tumor samples. Of four patients with *KEAP1* alterations identified, 1 patient harbored *KEAP1* p.R272C and *NFE2L2* amplification, 1 harbored *KEAP1* p. R470H plus *NFE2L2* p.I20N and *NFE2L2* amplification, 1 harbored *KEAP1* p.D294Y and *NFE2L2* amplification, and the remaining 1 patient harbored *KEAP1* amplification.

We then examined whether the status of *NFE2L2* alteration was associated with NRF2 expression/localization. Of 19 patients with dual-positive expression of NRF2 and NQO1 who harbored *NFE2L2* alteration, 15 patients had the proportion of NRF2 nuclear expression >20% and/or NRF2 IHC score in the nucleus >20 (Table 2). Mutations within the DLG and ETGE motifs and *NFE2L2* amplification were observed in 64.3% (18/28) of ESCCs with nuclear or nucleocytoplasmic expression of NRF2, whereas mutations not in DLG and ETGE motifs were only found in 1 of 8 patients (12.5%, 1/8) who had cytoplasmic localization of NRF2 ($P = 0.016$) (Fig. 4C). The median NRF2 (100 vs. 60, $P = 0.047$, Fig. 4D) and NQO1 IHC score (210 vs. 100, $P = 0.022$, Fig. 4E) in *NFE2L2*-mutant cases were significantly higher than those in *NFE2L2*-wide type (wt) cases. There was no significant difference for *NFE2L2* alteration status in terms of tobacco and heavy alcohol consumption, tumor stage, or histological grade (Table S8).

Next, we examined whether the status of *NFE2L2* alteration was associated with OS in patients with dual-positive expression of NRF2 and NQO1. The OS of patients with and without *NFE2L2*

alterations was comparable (Fig. 5A). Remarkably, when combining the status of *NFE2L2* alterations and the result of IHC stanning for NRF2, we found that patients with a proportion of NRF2 expression in the nucleus more than 20% and *NFE2L2* alterations (Fig. 5B) or those with a NRF2 IHC score in the nucleus more than 20 and *NFE2L2* alterations (Fig. 5C) exhibited unfavorable OS, with or without adjusting for gender, age, tumor stage, smoking, and drinking history.

DISCUSSION

Despite the advancement of knowledge in epidemiology, etiology, and pathogenesis of ESCC, the incidence and mortality rates of ESCC remain high over the past few decades. Therefore, a deeper understanding of ESCC pathogenesis is an unmet need to promote the development of feasible therapeutic strategies. In the present study, we explored the associations between the status of NRF2 expression and/or *NFE2L2* alteration and prognosis in ESCC patients. We found that *NFE2L2* alterations were significantly enriched in patients with NRF2 nuclear expression, which suggest IHC might be a feasible tool for identifying patients who harbored *NFE2L2* alterations based on nuclear NRF2 positivity in tumor cells. This study also demonstrated that high nuclear NRF2 expression combined with *NFE2L2* alterations showed high performance in predicting the clinical outcomes of ESCC patients with dual-positive expression of NRF2 and NQO1. These findings suggest that *NFE2L2* mutations within DLG and ETGE motifs or *NFE2L2* amplification could result in high nuclear expression of NRF2 and might play vital roles in promoting ESCC tumorigenesis.

Consistent with previous studies, our study revealed that NRF2 was expressed both in primary tumors and matched noncancerous mucosae[27]. Our analysis showed that smokers showed a trend of significantly higher NRF2 overexpression level in noncancerous mucosae. It has been documented that NRF2 signaling is substantially upregulated in response to cigarette smoke exposure in the airway epithelium of a *Drosophila* model[28]. Compared with noncancerous mucosae, NRF2 was significantly enriched in the nucleus in primary tumors (40.3% vs. 2.2%). Although the difference of NRF2 expression level between primary tumors and matched noncancerous mucosae was not investigated in this study, it has been described in several published papers with conflicting results[5,27]. In addition, obvious difference of NRF2 localization between primary tumors and noncancerous mucosae was observed in the present study, which suggested that NRF2 localization might play vital roles in ESCC pathogenesis. We also found that the concordance for the status of NRF2 expression between primary tumors and matched metastatic lymph node lesions was high. These findings suggest that metastatic lymph node lesions might be a surrogate for primary tumors in detecting NRF2 expression status to guide treatment option for patients with ESCC in the future.

In the present work, the expression status of NQO1/ABCB1 were highly consistent with that of NRF2, which indicated that NRF2 might play vital roles in ESCC via activating downstream target genes. ABCB1 has been reported to be related to multidrug resistance[29,30]. These findings suggested that NRF2 might be associated with drug resistance in cancers. Our study also revealed that NRF2 expression level or status had no association with clinicopathological characteristics, including age, gender, smoking, heavy alcohol consumption, clinical stage, and histological grade among ESCC patients. Similar results were observed in a recent study indicating that there was no significant relationship between the expression level of nuclear NRF2 and clinical characteristics,

Table 2. *NFE2L2* mutations and/or amplification occurring in patients with the proportion of nuclear NRF2 expression >20% and/or nuclear NRF2 IHC score >20.

Patient no.	Alteration(s)
P20	*NFE2L2* amp (CN = 4.4)
P21	*NFE2L2* p.R34Q
P50	*NFE2L2* p.V32G
P61	*NFE2L2* p.I20N
	NFE2L2 amp (CN = 3.0)
	KEAP1 p.R470H
P67	*NFE2L2* p.R34Q
P68	*NFE2L2* p.T80_E82delinsI
P80	*NFE2L2* amp (CN = 3.0)
	KEAP1 p.R272C
P87	*NFE2L2* amp (CN = 4.8)
P88	*NFE2L2* p.R34P
P98	*NFE2L2* p.V32del
P102	*NFE2L2* p.I28T
P114	*NFE2L2* p.R34Q
	NFE2L2 p.E377K
P116	*NFE2L2* amp (CN = 3.5)
	KEAP1 p.D294Y
P126	*NFE2L2* p.F37L
P128	*NFE2L2* p.F37S

NRF2 (NFE2L2) Nuclear factor erythroid-2 related factor-2, *IHC* Immunohistochemistry, *amp* Amplification, *del* Deletion, *delins* Deletion and insertion, *CN* Copy number, *KEAP1* Kelch-like ECH-associated protein 1.

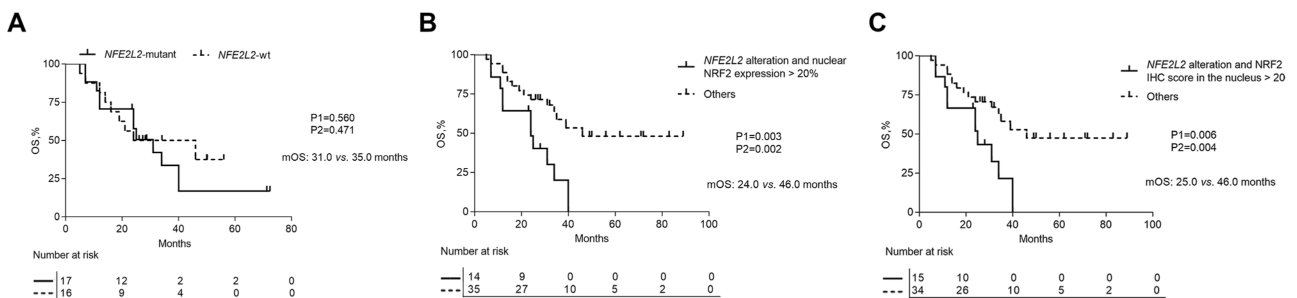

Fig. 5 Overall survival by expression level of NRF2 and *NFE2L2* somatic alteration status in ESCC patients with dual-positive expression of NRF2 and NQO1. A The difference of overall survival between patients with and without *NFE2L2* alteration; (**B**) Comparison of overall survival between patients with the proportion of NRF2 expression in the nucleus more than 20% plus *NFE2L2* alteration and those with less than 20% regardless of *NFE2L2* alteration status; (**C**) Comparison of overall survival between patients with NRF2 IHC score in the nucleus more than 20 plus *NFE2L2* alteration and those patients with less than 20 regardless of *NFE2L2* alteration status. P1 and P2 indicated *P*-value respectively calculated without and with adjusting for age, gender, tumor stage, smoking, and heavy alcohol consumption.

including age, sex, smoking history, location of primary tumor, and clinical stage in patients with inoperable locally advanced ESCC[31].

NFE2L2 somatic alterations occurred in 52.8% (19/36) of patients with dual-positive expression of NRF2 and NQO1. The frequency of *NFE2L2* alterations identified in the present study was significantly higher than that in unselected ESCC patients as previously reported[4–7], which might be attributed to several factors. First, the selected ESCC patients were included in this study that only those cases displaying dual-positive expression of NRF2 and NQO1 were sequenced. Second, copy number variations were not detected in the previous studies.

NFE2L2 hotspot mutation p.R34Q/p.R34P within the DLG motif (4/21) was observed in this study, which was consistent with previous studies[5]. Both DLG and ETGE motifs are within Neh2 domain of NRF2 and bind to Kelch-like ECH-associated protein 1 (KEAP1)[32]. Previous studies have demonstrated that KEAP1 could negatively regulates NRF2 activity by targeting it to cullin-3 (CUL3)-mediated ubiquitination and proteasomal degradation and constitutively suppresses NRF2 activity in the absence of stress[32]. Alterations occurring in *KEAP1* or *CUL3* could lead to oncogenesis in different types of cancers[33]. However, the status of *CUL3* alteration remains elusive in this study due to the fact that CUL3 was not included in the targeted sequencing panel. It has been reported that *NFE2L2* alterations are mutually exclusive with alterations in *CUL3* and *KEAP1*[34]. In this work, one patient harboring *KEAP1* amplification and three patients harboring *KEAP1* point mutations were observed. These four patients had the aberrant expression of NRF2 and three of four patients harbored concurrent *NFE2L2* alterations. These findings suggest that *NFE2L2* alterations can co-occur with *KEAP1* alterations.

We also found that patients with *NFE2L2* alterations had a significantly higher expression level of nuclear NRF2. It is well documented that both NQO1 and ABCB1 act downstream of NRF2 and the expression of NQO1 and ABCB1 is regulated by NRF2 transcription factor[35]. Consistent with these studies, the expression level of NQO1 or ABCB1 had a positive correlation with that of NRF2 in this study. These findings demonstrated that *NFE2L2* somatic mutations were implicated in the activation of NRF2 signaling pathway.

NRF2 associated with tumorigenesis has been documented in several previous studies[36,37]. In normal esophagus, several driver genes of ESCC are under positive selection, such as *NOTCH1/2*, *TP53*, and *NFE2L2*, conferring a competitive advantage on mutant cells over wild-type cells[38]. Furthermore, previous studies have demonstrated that NRF2 involved in tumorigenesis is related to mutant *KRAS* or *PTEN*[36,37]. In this work, NRF2 nuclear expression was also observed in those tumors with dual-positive expression of NRF2 and NQO1 and negative *NFE2L2* alterations, which might be resulted from the presence of alterations in other oncogenes, such as *CUL3, KRAS*, and *PTEN*. In details, two tumor samples with dual-positive expression of NRF2 and NQO1 carried *KRAS* amplification and two samples carried *PTEN* alterations. The alteration status of *CUL3* in ESCC samples negative for *NFE2L2/KEAP1* alterations should be further investigated.

NRF2 acting as an oncogenic driver or a tumor suppressor in EC remains debatable. Kitano et al. have demonstrated that NRF2 promotes EC cell proliferation through metabolic reprogramming and detoxification of ROS in vitro and leads to a poor clinical outcome in patients with EC[27]. Xia and colleagues have revealed that NRF2 promotes the radiation resistance of ESCC and is closely related to a poor survival of ESCC patients[15]. Unlike mutations in tumor-suppressor genes, including *TP53*, *KMT2D*, and *ZNF750*, oncogenic *NFE2L2* mutations were late events during tumor evolution in ESCC[39]. However, a recent in vivo study has revealed that *NFE2L2* may act as a tumor suppressor in ESCC based on the data that NRF2-loss promotes ESCC cell proliferation[5]. In the present study, we found that high nuclear expression of NRF2 combined with the status of *NFE2L2* alteration exhibited high performance in predicting prognosis of ESCC patients with dual-positive expression of NRF2 and NQO1, which indicated that NRF2 might act as an oncogenic driver in ESCC. Our analyses suggest that the expression level of NRF2 combined with the status of

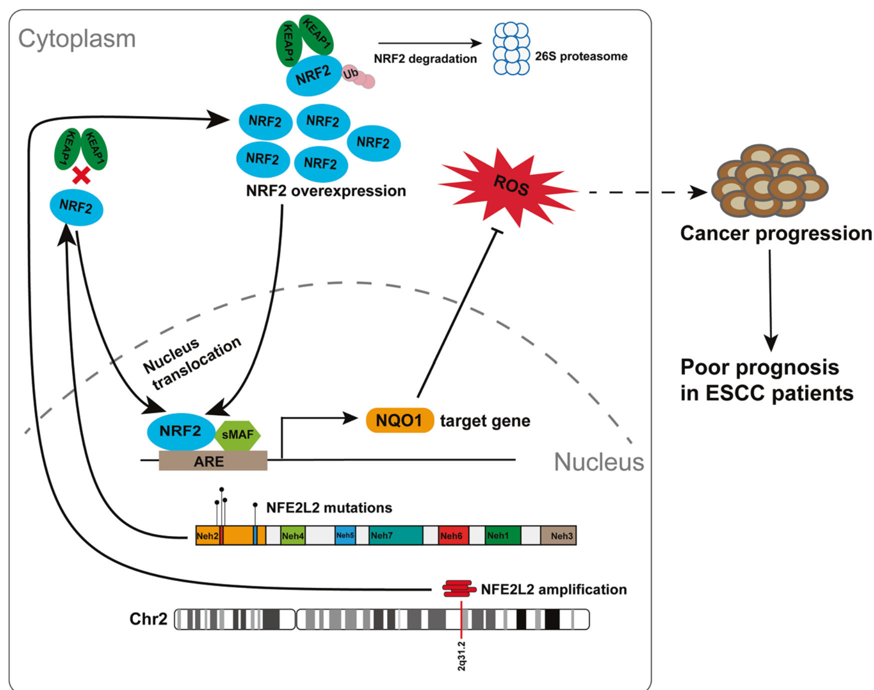

Fig. 6 Schemic diagram of *NFE2L2* somatic alterations on the prognosis of ESCC patients. NFE2L2 somatic mutations within DLG and ETGE motifs impair KEAP1-NRF2 interaction and inhibit KEAP1-mediated degradation of NRF2, and NFE2L2 amplification results in overexpression of NRF2 and may overwhelm KEAP1 roles, allowing NRF2 to be translocated into the nucleus and activating the NQO1, then resulting in ROS detoxification and protecting ESCC cells against ROS following cancer progression and a poor prognosis in ESCC patients.

NFE2L2 alterations might be a robust predictor of survival outcomes in ESCC and *NFE2L2* mutations should be taken into account when devising appropriate therapeutic strategies.

A recent study has demonstrated that *NFE2L2* wild type overexpression suppresses tumor growth, while *NFE2L2* mutants (p.R34Q, p.E79K) within DLG/ETGE motifs significantly promote tumor growth in xenograft mouse model[5]. Another study has revealed that both *NFE2L2* mutation (including G31R, G31V, G31A, N160S, T80K) and *NFE2L2* wild type overexpression are tumorigenic and could induce tumor formation in mice models[40]. In this study, we found that both *NFE2L2* mutation (majority of mutations were with DLG/ETGE motifs) and *NFE2L2* amplification could result in NRF2 nuclear/nucleoplasmic expression. Based on these findings, we speculated that both *NFE2L2* mutations within DLG/ETGE motifs and *NFE2L2* amplification could promote ESCC tumorigenesis. We also proposed a model to speculate the roles of NRF2 in promoting ESCC tumorigenesis in our study (Fig. 6). In detail, *NFE2L2* somatic mutations occurring in DLG and ETGE motifs impair KEAP1-NRF2 interaction and inhibit KEAP1-mediated degradation of NRF2, and *NFE2L2* amplification results in overexpression of NRF2 and may overwhelm KEAP1 roles, allowing NRF2 to be translocated into the nucleus and activating the transcription of target antioxidant and redox genes (such as *NQO1*), then resulting in ROS detoxification and protecting ESCC cells against exogenous and endogenous toxins (such as ROS). Our findings suggest that *NFE2L2* alterations and nuclear expression of NRF2 might play vital roles in promoting ESCC tumorigenesis.

With the advancement and improvement of sequencing technology, next-generation sequencing (NGS) has been applied increasingly in cancer genomics research fields. However, high cost and the relatively long turnaround time might hinder the utilizing of NGS to detect the status of *NFE2L2* alteration for predicting the prognosis of ESCC patients in routine clinical practice. This study suggests that IHC staining for NRF2 and its downstream target genes might be a feasible, convenient, fast, and economical tool to identify ESCC patients who have a poor prognosis, with additional advantages of IHC staining directly reflecting the expression status/localization of NRF2 (status of NRF2 activation) and its downstream target genes.

There were some limitations in the study. First, the associations between *NFE2L2* mutations occurring in DLG and ETGE motifs and the nuclear expression of NRF2 in ESCCs should be confirmed in in vitro and in vivo studies. Second, the sample size of this study was relatively small which might weaken the statistical significance of our conclusions. A large, multi-center cohort of ESCC patients is needed to further verify the associations between *NFE2L2* alterations and survival outcomes. Third, NRF2 negative expression cases were not performed for the next-generation sequencing. The prognostic significance of *NFE2L2* mutations in NRF2-negative cases should be further determined.

Taken together, this study demonstrated that increased NRF2 nuclear expression (>20% or IHC score >20) and the presence of *NFE2L2* somatic alterations were associated with shorter median overall survival in ESCC patients. The present study suggests that activation of NRF2 signaling pathway contributes to the malignancy of ESCC. Furthermore, IHC for NRF2 and NQO1 may be useful as surrogate markers for predicting aberrant activation of NRF2 signaling pathway in ESCC.

DATA AVAILABILITY

Data used to support the results of this study can be obtained from the corresponding author.

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AUTHOR CONTRIBUTIONS

X.J., M.J. and W.J. performed study concept and design; X.J., M.J. and W.J. performed development of methodology and writing, review, and revision of the paper; X.J., X.Z., X.Y., X.C., X.H., J.L., and Y.Y. provided acquisition, analysis and interpretation of data, and statistical analysis; H.Z., Q.C. and Y.G. provided technical and material support. All authors read and approved the final paper.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the institutional review boards of Beijing Chao-Yang Hospital, Capital Medical University. Informed consent was waived because of the retrospective and anonymous nature of this study.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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