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KEAP1/NRF2 signaling pathway mutations in cervical cancer

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Abstract. – OBJECTIVE: The aim of the present study was to explore the potential involvement of mutations in the KEAP1/NRF2 signaling pathway in Chinese samples with cervical cancer.

PATIENTS AND METHODS: 236 Chinese patients with various types of cervical cancer were recruited, and the coding exons and the corresponding intron-exon boundaries of the KEAP1 and NRF2 genes were analyzed for the potential mutations in the KEAP1/NRF2 signaling pathway.

RESULTS: A novel KEAP1 missense somatic mutation (c.1408C>T, p.R470C) and 5 NRF2 missense somatic mutations (c.72G>C, p.W24C; c.85G>T, p.D29Y; c.101G>A, p.R34Q; c.230A>C, p.D77A and c.242G>A p.G81D) were identified in 187 patients with cervical squamous cell carcinoma, respectively; no mutations were detected in other subtypes. All these mutations were heterozygous and predicted to be pathogenic by PolyPhen-2, MutationTaster programs, and evolutionary conservation analysis. Among these mutations, the KEAP1 (p.R470C) and 3 NRF2 mutations (p.D29Y, p.D77A, and p.G81D) were detected in cervical cancer for the first time. Also, no mutations were identified in our 21 adenosquamous carcinomas or 25 adenocarcinomas.

CONCLUSIONS: We identified 6 potential diseases causing mutations in the KEAP1/NRF2 signaling pathway in 187 (3.2%) Chinese cases with cervical squamous cell carcinoma, implicating KEAP1/NRF2 signaling pathway might play an active role in the pathogenesis of this subtype of cervical cancer. Furthermore, among these detected mutations, the KEAP1 and 3 NRF2 mutations were reported in cervical cancer for the first time.

Key Words:
KEAP1/NRF2 signaling pathway, Mutation, Cervical cancer, Chinese.

Introduction

Cervical cancer is one of the major causes of gynecological cancer-related deaths worldwide[1,2]. In spite of a high incidence, most of the locoregional cervical cancers have good outcomes after the available therapy regimens[3,4]. However, some patients will experience metastasis after the regular therapies and seriously threaten their lives[5,6]. Thus, it is necessary to discern the molecular and biologic mechanisms in the initiation and progression of cervical cancer and, thus, develop novel therapeutic strategies.

Nuclear factor (erythroid-derived 2)-like 2 (NRF2, NFE2L2) is a transcription factor playing crucial roles in cellular defense against electrophilic and oxidative stresses, its primary function involves the regulation of related stress-responsive proteins expression, via binding to the antioxidant response elements (ARE) in the promoters regions of the target genes[7-9]. Under the basal conditions, NRF2 is repressed by a Kelch-like erythroid cell-derived protein with CNC homology [ECH]-associated protein 1 (KEAP1), which could promote the degradation of NRF2[10]; while under oxidative stress, NRF2 could be activated and it regulate the expression of related stress-responsive genes[9,11]. Accumulating evidence has shown that dysregulation of KEAP1/NRF2 signaling pathway, such as gene mutations[12,13], and aberrant expression[14], participates in the initiation and development processes of human cancers.

KEAP1 mutations were identified frequently in multiple human cancers, including lung, renal, and liver cancers[12,15-18]. In addition, ac-

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cumulating evidence has also identified NRF2 mutations with available frequencies in diverse cancers[16,19,20]. A recent large-scale genomic analysis of cervical cancer in Euro-American populations has shown that the frequency of NRF2 somatic mutations was 3.8% (3/79) in cervical squamous cell carcinoma and 50.0% (1/2) in cervical clear cell carcinoma[21]. All the identified NRF2 mutations (p.W24C, p.R34P, p.R34Q and p.E82D) were located in the binding domain of KEAP1, the negative regulator of NRF2[21,22]. The similar findings were also observed in squamous cell carcinoma of lung, esophagus, and larynx[23-25]. These studies suggested that mutations in KEAP1/NRF2 signaling pathway might be a common reason for human cancers.

In the present investigation, we analyzed a total of 236 Chinese cases with various types of cervical cancer for the presence of KEAP1/NRF2 signaling pathway mutations. There are two aims of this study: (1) to detect whether the KEAP1 and NRF2 mutations are common in Chinese samples with cervical cancer and (2) to explore whether the KEAP1 and NRF2 mutations are existed in Chinese samples with other types of cervical cancer, besides squamous cell carcinoma and clear cell carcinoma.

Patients and Methods

FFPE Tissue Samples

Only cases with > 40% of cancerous cells and the corresponding adjacent non-cancerous tissues were recruited. In total, the formalin-fixed, paraffin-embedded (FFPE) subjects were recruited from 236 histologically diagnosed cervical cancer cases at Jiangxi Provincial Cancer Hospital and Jiangxi Provincial Maternal and Child Health Hospital, during January 2012 through December 2015 period. All of the samples were reviewed in a blinded manner by two experienced pathologists. The 236 samples included 187 squamous cell carcinomas, 21 adenosquamous carcinomas, 25 adenocarcinomas, and 3 clear cell carcinomas (Table I); the median age was 43 years (range, 22-74 years). The Institutional Ethics Review Boards of Jiangxi Provincial Maternal and Child Health Hospital and Jiangxi Provincial Cancer Hospital approved this study. Each patient signed the informed consent before participating. The research was performed according to the Declaration of Helsinki.

DNA Extraction and Polymerase Chain Reaction (PCR) Amplification

After deparaffinization with xylene, Genomic DNA (gDNA) was extracted from FFPE tissue specimens using Qiagen's QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). The obtained DNA was quantified using a SmartSpec Plus spectrophotometer (Bio-Rad, Hercules, CA, USA). For the PCR amplification, the coding exons and the corresponding intron-exon boundaries of the KEAP1 and NRF2 genes were amplified using a set of primer pairs, respectively (Table II). A total of 200 ng gDNA was used for each of the 19 PCR amplicons in a final volume of 50 µl, containing 2 U of Taq DNA polymerase (TaKaRa Biotechnology Dalian Co. Ltd, Dalian, Liaoning, China), 5 µl of 10 x PCR buffer, 0.2 µM dNTPs (TaKaRa Biotechnology Dalian Co. Ltd, Dalian, Liaoning, China), 0.6 µM of each primer (TaKaRa Biotechnology Dalian Co. Ltd, Dalian, Liaoning, China), and 2.5 mM of $MgCl_2$ (TaKaRa Biotechnology Dalian Co. Ltd, Dalian, Liaoning, China). The PCR amplification was

Table I. The mutation analysis of the *KEAP1* and *NRF2* genes in 236 cases with distinct subtypes of cervical cancer.

Mutation	Squamous cell carcinoma (n = 187)	Adenosquamous carcinoma (n = 21)	Adenocarcinoma (n = 25)	Clear cell carcinoma (n = 3)
KEAP1				
p.R470C (c.1408C > T)	1/187	0/21	0/25	0/3
NRF2				
p.W24C (c.72G > C)	1/185	0/20	0/25	0/3
p.D29Y (c.85G > T)	1/185	0/20	0/25	0/3
p.R34Q (c.101G > A)	1/185	0/20	0/25	0/3
p.D77A (c.230A > C)	1/187	0/21	0/25	0/3
p.G81D (c.242G > A)	1/187	0/21	0/25	0/3

Table II. The PCR primer sequences for KEAP1 and NRF2 mutation analysis.

Gene/ exon	Forward primer (5′-3′)	Reverse primer (5′-3′)	Annealing (°C)	Amplicon (bp)
KEAP1				
Exon 2-1	GCCAGAGGTGGTGGTGTTG	CTGAGCCGCAGCTCGTTC	50	255
Exon 2-2	ATACCAAGCAGGCCTTTGG	ATGGAGATGGAGGCCGTGT	60	265
Exon 2-3	ATGGAGCGCCTCATTGAA	AGCCCCACTTCCCCGCT	57	263
Exon 3-1	CCGTCCCACTGTCGCCCTC	TGCAGGATCTCGCACTTC	57	260
Exon 3-2	AGCTGCAGAAGTGCGAG	GTTGTTCCTGCCGCCCACG	52	296
Exon 3-3	TGGGCGGGCTGTTGTAC	CCAGGCCCTGCCACTCA	52	235
Exon 4	TCTTACGCCCTTGCAGGT	CTACCGTCCCCACCCAC	52	238
Exon 5	CACCTTCTCTGCATGGTG	ATGGGCTAGTCAGGACTC	55	243
Exon 6	CTCTTGGATGTGGTGTGA	ACTCCCCATTGGACTGTA	52	262
NRF2				
Exon 1	CAGCCGGAACAGGGCCGCC	CTGTCCCTCCCGGGCCGCGG	56	181
Exon 2-1	TCTTAAACATAGGACATG	GCTCCTTTTGGAGTTGTT	60	163
Exon 2-2	CTTGAAAAGGAAAGACA	CAAGAACTGAGTACTCTG	52	174
Exon 3	AATCAATGCCTTATCAA	TTACATTCTATTTTAGTT	50	235
Exon 4-1	TAGTATAAACTTCCTTCT	AGTTCACTGTCAACTGGT	56	239
Exon 4-2	CTCTCCACAGAAGACCC	TGGTTGAAAGCTTTGCAA	58	218
Exon 4-3	TGTTTCTGATCTATCACT	ACAAGGGTTGTACCATATCC	52	269
Exon 4-4	CACCAGTACATTCTTCTGG	GAAGTCAACAACAGGGAG	54	260
Exon 4-5	ATCATTAACCTCCCTGTTG	TTCAGTAGGTGAAGGCT	50	245
Exon 4-6	AAGCCTTCACCTACTGA	TTAGTATAATAGTACAA	52	228

amplified with a denaturation step at 94°C for 60 s, a primer annealing step at 50-62°C for 30 s (Table II), and an elongation step at 72°C for 30 s. The final step at 72°C was extended for 10 min. All PCR reactions were performed in a Thermal Cycler 2720 (Applied Biosystems, Foster City, CA, USA). Agarose gel electrophoresis was performed to confirm the PCR amplification, and the PCR products were sequenced bidirectionally to confirm the presence of the mutations, by using Big Dye terminator chemistry and an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The identified somatic mutations were confirmed by sequencing the paired adjacent non-cancerous tissues.

In Silico Analysis of the KEAP1 and NRF2 Mutations

MutationTaster (http://www.mutationtaster.org) and PolyPhen-2 (http://gen etics.bwh.harvard.edu/pph2) tools were used to predict the disease-causing potentials for the identified NRF2 and KEAP1 mutations. These programs automatically assess each mutation to be either benign or pathogenic, according to the predicted probability score.

Evolutionary Conservation Analysis

To evaluate the evolutionary conservation of the mutated amino acids in KEAP1 and NRF2, the protein sequences of KEAP1 and NRF2 were obtained from 17 different species from GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). The 17 protein sequences of KEAP1 include Homo sapiens (NP_036421), Pan troglodytes (NP_001266890), *Rattus norvegicus* (NP_476493), *Mus musculus* (NP_001103775), Bison bison bison (XP_010826665), *Bos taurus* (NP_001094612), *Canis lupus familiaris* (XP_005632954), *Sus scrofa* (NP_001108143), *Castor canadensis* (XP_020029887), *Cricetulus griseus* (XP_01682981), *Ictalurus punctatus* (XP_0173377), *Neomonachus schauinslandi* (XP_021560619), *Phascolarctos cinereus* (XP_020850693), *Pteropus vampyrus* (XP_011378579), *Gallus gallus* (XP_015129501), *Xenopus tropicalis* (NP_00100802), and Drosophila melanogaster (NP_788685). The NRF2 protein sequences of the 17 species include Homo sapiens (NP_006155), Pan troglodytes (XP_001145876), *Rattus norvegicus* (NP_113977), *Mus musculus* (NP_035032), *Oryctolagus cuniculus* (XP_002712351), *Equus caballus* (XP_001497042), *Bos taurus* (NP_001011678), *Canis lupus familiaris* (XP_005640409), *Sus scrofa* (XP_013839757), *Castor canadensis* (XP_02001632), *Gavialis gangeticus* (XP_019378544), *Felis catus* (XP_003990942), *Gallus gallus* (NP_990448), *Rhinolophus sinicus* (XP_019594849), *Chrysemys picta bellii* (XP_005300513), *Xenopus tropicalis*

(NP_001007490), and *Danio rerio* (NP_878309). Multiple sequence alignment was performed with the "ClustalW" tool of the alignment function in the Molecular Evolutionary Genetics Analysis (MEGA) software.

Statistical Analysis

Two-tailed Fisher's exact test was used to evaluate the difference of NRF2 mutation frequency in the present study and the prior observation[21]. A p-value < 0.05 was considered as statistically significant.

Results

KEAP1 Mutations

A KEAP1 heterozygous missense mutation, p.R470C (c.1408C>T), was identified in 1 out of 187 (0.5%) cervical squamous cell carcinomas (Figure 1). The mutated sample was a 48-year-old woman and displayed no other gynecological condition. No mutations were detected in the 21 adenosquamous carcinomas, 25 adenocarcinomas or the 3 clear cell carcinomas.

NRF2 Mutations

A total of 187 cervical squamous cell carcinomas, 21 adenosquamous carcinomas, 25 adenocarcinomas and 3 clear cell carcinomas were analyzed for the presence of NRF2 mutations. Herein, 5 somatic missense mutations in NRF2 were identified in 5 out of 187 (2.7%) patients with cervical squamous cell carcinoma: p.W24C (c.72G>C), p.D29Y (c.85G>T), p.R34Q (c.101G>A), p.D77A (c.230A>C) and p.G81D (c.242G>A). All of these mutations were heterozygous and restricted to the binding domain of KEAP1 (Figure 1). Among

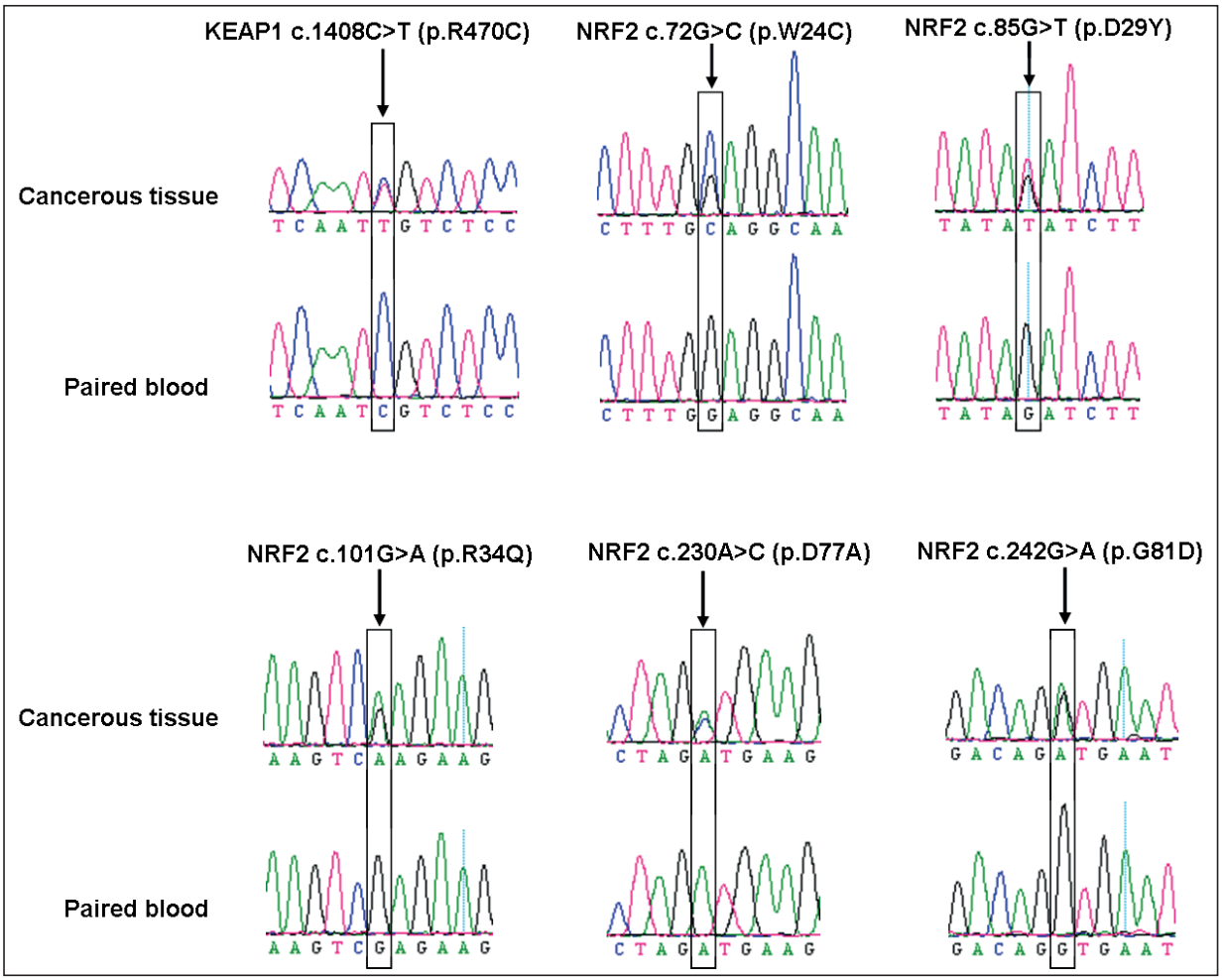

Figure 1. The sequencing electropherograms of KEAP1 and NRF2 mutations, the arrow refers to locations of the mutation.

these cases with NRF2 mutations, 2 individuals were also diagnosed with uterine leiomyoma, 1 case was diagnosed with endometriosis, while the remaining 2 cases had no other evident gynecological conditions.

The Potential Pathogenic Roles of the KEAP1 and NRF2 Mutations

For the KEAP1 p.R470C mutation, PolyPhen-2 predicted it to be possibly damaging with a damaging score of 0.882 (sensitivity: 0.82; specificity: 0.94). MutationTaster software gave a probability of "disease causing" over 0.9999 and a pathogenic score of 180, and showed that this mutation had not been reported in 1000G (https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/) or in ExAC (http://exac.broadinstitute.org/) databases. For all of the 5 NRF2 mutations, namely, p.W24C (c.72G>C), p.D29Y (c.85G>T), p.R34Q (c.101G>A), p.D77A (c.230A>C), and p.G81D (c.242G>A), PolyPhen-2 predicted that these mutations are "probably damaging" with a score of 1.000 (sensitivity: 0.00; specificity: 1.00); while MutationTaster gives a probability of "disease causing" over 0.9999 and a pathogenic score of 180, and shows that all of these mutations were neither found in 1000G nor in ExAC databases. In addition, the evolutionary conservation analysis results suggested that all of the mutated amino acid residues in KEAP1 and NRF2 were highly conserved among the 17 species ranging from *Homo sapiens* to *Drosophila melanogaster* or *Danio rerio* (Figure 2A-C).

Discussion

Increasing evidence has identified KEAP1 mutations in multiple cancer types[12,17,26,27]; meanwhile, NRF2 mutations were also found with available frequency in diverse cancer types[16,19,20], including cervical cancer[21]. These studies raised the possibility that the KEAP1/NRF2 signaling pathway mutations might be a common reason for human cancers. Herein we analyzed the mutation frequencies of KEAP1/NRF2 signaling pathway in 236 Chinese samples with distinct subtypes of cervical cancer.

Although a prior high-throughput genomic analysis of 115 cervical cancers with distinct subtypes (79 squamous cell carcinomas, 24 adenocarcinomas, 7 adenosquamous carcinoma, 2 neuroendocrine carcinoma, 2 clear cell carcinoma, and 1 serous carcinoma of cervix) did not detect any KEAP1 mutations[21], the present study identified a heterozygous somatic KEAP1 mutation (c.1408C>T, p.R470C) in 1/187 (0.5%) samples with cervical squamous cell carcinoma. This mutation was predicted to be pathogenic by both PolyPhen-2 and MutationTaster online programs. To the best of our knowledge, this is the first report showing KEAP1 somatic mutation was existed in cervical cancer, albeit with a low frequency.

The frequency of NRF2 mutations in cervical squamous cell carcinoma in our sample cohort was 2.7% (5/187), similar to a prior observation where the NRF2 mutation frequency in cervical squamous cell carcinoma was 3.8% (3/79) (p=0.69)[21]. Among these mutations, p.W24C (c.72G>C) and p.R34Q (c.101G>A) mutations were reported previously in cervical cancer[21]; while the remaining 3 mutations were identified in cervical cancer for the first time, albeit they were reported in other cancer types: p.D29Y (c.85G>T) in kidney[28] and lung cancer[29], p.D77A (c.230A>C) in lung caner[25], while p.G81D (c.242G>A) in lung[30] and esophageal cancers[20]. The mutated amino acid residues in the present work located in the binding domain of KEAP1 protein[22], consistent with the observation in the prior investigation[21]; furthermore, in silico prediction and evolutionary conservation analysis results suggested these mutations were damaging. These results implicated that these NRF2 mutations might possess functional roles in the development of cervical cancer. In contrast to previously identified high frequency of NRF2 mutation (50.0%, 1/2) in clear cell carcinoma[21], we failed to detect any NRF2 mutations in the 3 patients with clear cell carcinoma. We speculated that the limited sample size in both studies might be the main reason for this difference in mutation frequency.

Accumulating evidence has shown that mutations in the KEAP1/NRF2 signaling pathway were frequently observed in human cancers, mainly in squamous cell carcinomas from different tissue types[21,23-25]. In the current study, we failed to identify any KEAP1 or NRF2 mutations in either 21 adenosquamous carcinomas or 25 adenocarcinomas; similarly, a recent report[21] found that KEAP1 and NRF2 mutations were absent in 24 adenocarcinomas, 7 adenosquamous carcinoma, 2 neuroendocrine carcinoma, and 1 serous carcinoma of cervix.

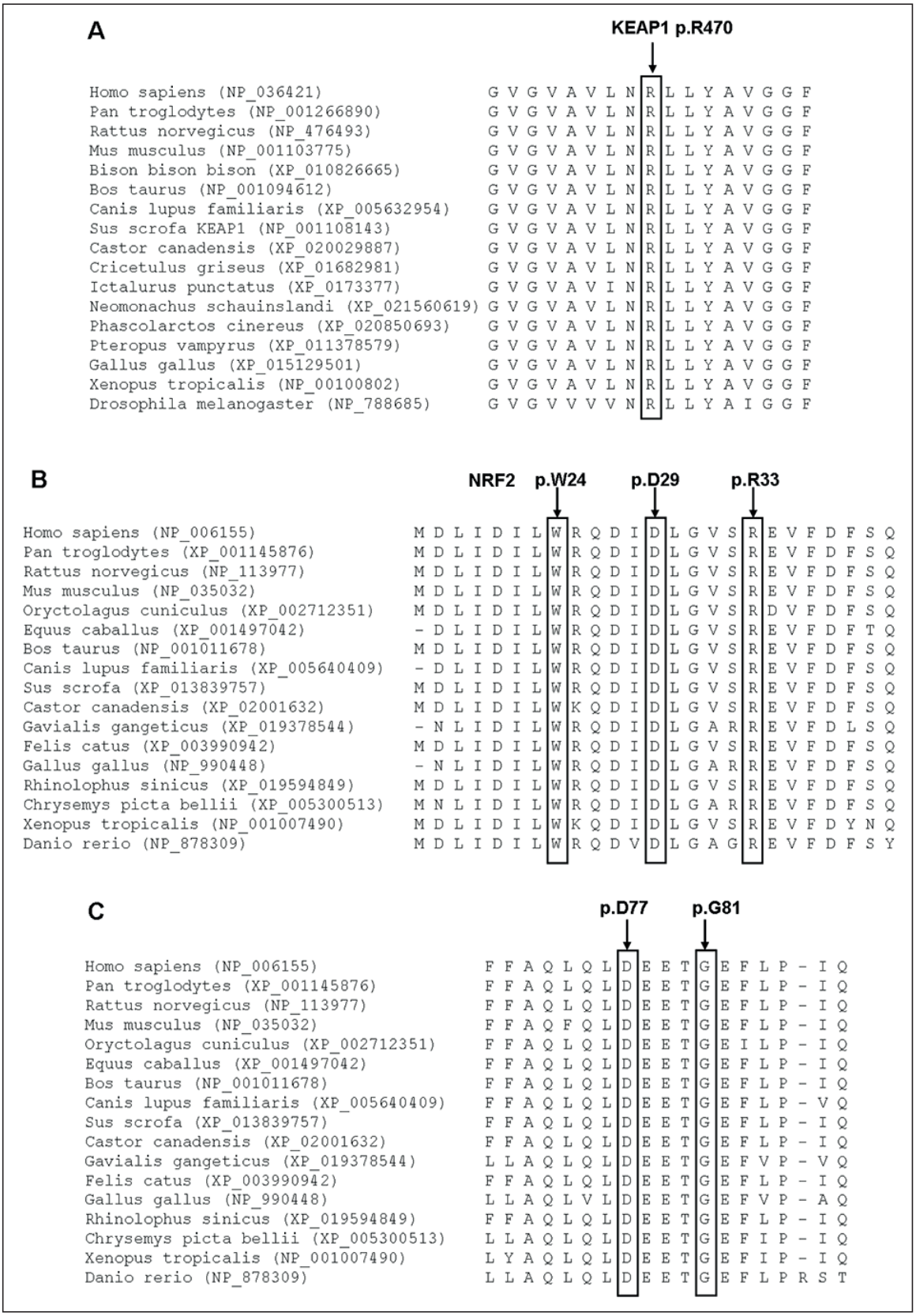

Figure 2. The evolutionary conservation analyses of the mutated amino acids in the KEAP1 (2A) and NRF2 (2B and 2C) genes in the present study, the mutated amino acids were indicated.

Conclusions

We detected 6 potential disease causing mutations in the KEAP1/NRF2 signaling pathway in 187 (3.2%) cases with cervical squamous cell carcinoma, implicating KEAP1/NRF2 signaling pathway might play an active role in the pathogenesis of cervical squamous cell carcinoma. Furthermore, among these identified mutations, a novel KEAP1 and 3 novel NRF2 mutations were detected in cervical cancer for the first time.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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