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Research article

Effects of *BRCA1* overexpression via the NRF2 / HO1 / NQO1 pathway on oral cancer cells proliferation, migration, and apoptosis

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A R T I C L E I N F O

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A B S T R A C T

Objective: Herein, we explored the influences of *breast cancer susceptibility gene 1 (BRCA1)* overexpression on oral cancer cells proliferation, migration, and apoptosis via evaluation of its interactions with nuclear factor erythroid 2-like 2 (NRF2).
Design: CAL-27 and DOK cells were transfected with a *BRCA1* overexpressing lentivirus. Next, we utilized Western blot and quantitative real-time polymerase chain reaction (qRT-PCR) analyses to evaluate BRCA1, NRF2, and their target gene expressions. Using cell counting kit-8 (CCK-8) assessment, we assessed cell proliferation and a scratch test detected CAL-27 cell migration. Additionally, flow cytometry was employed used to examine cell apoptosis, while an enzyme-linked immunosorbent assa (ELISA) was employed for evaluation of 8-hydroxy-2′- deoxyguanosine (8-OHdG) expression. An immunohistochemical analysis was employed to determine the NRF2 target genes and Ki-67 expressions.
Results: BRCA1 overexpression increased the NRF2 and its target gene transcript and protein expressions. CCK-8 and scratch test results showed that *BRCA1* overexpression decreased cell proliferation and weakened CAL-27 cell migratory ability. Flow cytometry results showed that *BRCA1* overexpression promoted cell apoptosis in a time-dependent manner, while enzyme-linked immunosorbent assay results showed that *BRCA1* overexpression decreased 8-OHdG expression levels in CAL-27 and DOK cells. Immunohistochemical analysis results showed higher expression of NRF2 target genes and Ki-67 in oral squamous cell carcinoma cells.
Conclusions: Experiments involving oral cancer cells confirmed that *BRCA1* overexpression could up-regulate the NRF2 signalling pathway, reduce oxidative damage, and inhibit cell proliferation and other biological behaviours. The BRCA1 and NRF2 pathways might be associated with oral cancer occurrence and development.

Abbreviations: BRCA1 breast cancer susceptibility gene 1, NRF2 nuclear factor erythroid 2-related factor 2; OLK oral leukoplakia, ROS reactive oxygen species; HO1 heme oxygenase 1, NQO1 NADH quinone oxidoreductase 1; 8-OHdG 8-hydroxy-2′-deoxyguanosine, OSCC oral squamous cell carcinoma.

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1. Introduction

Approximately 90 % of oral cancers are categorized as oral squamous cell carcinomas (OSCCs). OSCCs have the highest incidence rate among oral cancers [1]. Despite significant advances in treatment, the average five-year survival rate following OSCC treatment remains relatively low [2]. Orally potentially malignant disorders (OPMDs), a collective term for diseases affecting the oral mucosa, include conditions like oral lichen planus, leukoplakia (OLK), and submucosal fibrosis, which can induce OSCC progression [3]. *Breast cancer susceptibility gene 1 (BRCA1)*, discovered in 1990 by Hall, is the first tumour suppressor gene identified to be associated with familial breast cancer [4]. *BRCA1* inhibits tumour growth, proliferation, and metastasis through diverse mechanisms [5]. It participates in gene transcriptional activation and inhibition, and has various biological functions [6]. *BRCA1* deletion can promote oxidative damage [7]. Cancer cell proliferation and metastasis often serve as the main contributors to mortality in OSCC patients [8]. *BRCA1* knockout mice can spontaneously develop skin and oral cancer over a lengthy latency period [9]. Exon sequencing studies have shown that *BRCA1* may be involved in the malignant transformation of OLK to OSCC [10].

Nuclear factor erythroid 2-like 2 (NRF2) is a transcription factor (TF) that controls antioxidant reactions. It regulates the dynamic redox balance in tumour cells by activating cellular protective antioxidant genes, inhibiting reactive oxygen species (ROS), and repairing oxidative damage via target gene expressions, thus playing a role in cancer inhibition [11]. NRF2 knockdown mice are more susceptible to the toxic or carcinogenic effects of exogenous chemicals than wild-type mice, which results in tumour formation [12,13]. Oxidative stress is brought about by an imbalance in the oxidative and antioxidative reactions within the body, mediated by various components of the oxidive system, resulting in cellular oxidative damage. ROS generated during metabolic processes plays a crucial role in oxidative reactions. Previous studies have confirmed that *BRCA1* can facilitate stable NRF2 activation by binding to the ETGE region of NRF2 in mouse mammary epithelial cells [14]. *BRCA1* deletions or mutations inhibit the NRF2 signalling pathway, leading to an increase in ROS levels, which in turn might be associated with the occurrence of breast cancer [15]. The biomarker 8-OHdG indicates the impact of both endogenous and exogenous factors on oxidative damage to DNA [16]. In breast cancer cell lines, *BRCA1* regulates the DNA region associated with the transcriptional coupling repair of 8-OHdG, and the lack of *BRCA1* activity could increase 8-OHdG content [17]. However, the BRCA1 significance in OSCC is still unclear. In this investigation, we aim to elaborate on the reciprocal regulation of BRCA1 and NRF2 signalling pathways and their effect on oxidative damage and oral cancer cell biology behaviours. Our findings suggest that this interplay may be intricately linked to OSCC occurrence and progression.

2. Materials and methods

2.1. Cells and cell culture

The oral cancer line CAL-27 was obtained from the Beijing Stomatological Hospital Research Institution (Capital Medical University, China). Dysplastic oral keratinocytes (DOK) cells (from the European Collection of Authenticated Cell Cultures data bank) were provided by Dr. Qianming Chen (West China Medical center, China). All cells were grown in augmented-glucose DMEM (BI, New York, NY, USA) medium with 100 U/mL penicillin, 100 U/mL streptomycin, and 10 % fetal bovine serum at 37 °C in a CO_2 (5 %) incubator.

2.2. Lentiviral transfection

BRCA1 overexpressing lentiviral vectors and controls for the lentiviral vectors (reagent solutions without *BRCA1*) were synthesized by Genechem Co., Ltd (Shanghai, China). Cells (CAL-27 at 6×10^4/well and DOK at 2×10^5/well) were seeded in 6-well plates, prior to transfection with 5 μL lentivirus. An appropriate volume of HiTtansG P-enhanced infection solution was introduced to 950 μL medium and pipetted into the wells. After transfection at 37 °C for 12 h, fresh medium was introduced, prior to a 48 h cell incubation. Stable BRCA1 overexpressing cell lines and control cells were obtained by adding 1 μg/mL puromycin (Sigma-Aldrich, MO) for screening upon observing notable fluorescence levels under a microscope, and the expression of green fluorescent protein was about 80%–90 % after the subculture process. Transfection efficiency was verified via experiments involving Western blot and polymerase chain reaction (qRT-PCR) analysis.

Table 1
Primer sequences employed in qRT-PCR assessment.

Gene Name	Primer	Sequence (5'–3')
BRCA1	Forward	CTGAAGACTGCTCAGGGCTATC
	Reverse	AGGGTAGCTGTTAGAAGGCTGG
NRF2	Forward	ATGACAATGAGGTTTCTTCGG
	Reverse	CAATGAAGACTGGGCTCTC
HO1	Forward	AGCGAAACAAGCAGAACCCA
	Reverse	GCCACCAGCAGCTCAGGATG
NQO1	Forward	GCGGTGAGAAGAGCCCTGAT
	Reverse	ATTCGACCACCTCCCATCCT
GAPDH	Forward	GTCGTCCATCTTGCCATTCACTCC
	Reverse	GCTCTCCACCACCTGCTCCTC

2.3. qRT-PCR

Total RNA was extracted employed Trizol (Life Technologies Corporation, Grand Island, NY, USA), with subsequent cDNA generation with a PrimeScript RT reagent kit (Takara, Tokyo, Japan), and subsequent qRT-PCR reaction via a SYBR® Premix Ex Taq™ Kit in a Roche LightCycler 480II system (Switzerland). The following genes were analyzed: *BRCA1*, *NRF2*, *heme oxygenase 1 (HO1)*, *NADH quinone oxidoreductase 1 (NQO1)*, and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*. Primer synthesis was by Sangon Biotech Co. Ltd. (China), with sequences shown in Table 1. The abundance levels were calculated relative to those for *GAPDH*.

2.4. Western blot assay

Lentiviral-incorporated cells (with and without *BRCA1*) underwent lysis in radioimmunoprecipitation assay (RIPA) buffer, prior to a 15 min centrifugation at 12000 rpm at 4 °C. Protein quantification employed the BCA process. Then, the protein standard solution was diluted in a gradient as directed in the kit, and the protein concentration standard curve was drawn to calculate protein concentrations. For Western blotting, 25 μg/well proteins obtained from the supernatant were separated via SDS-PAGE, before transfer to PVDF membranes via the Bio-Rad system (Bio-Rad Laboratories, Munich, Germany). Membranes then underwent a 2 h blocking in 5 % BSA for 2 h and overnight (ON) incubation with primary antibodies against BRCA1 (rabbit polyclonal, 1:1000, ab131360), NRF2 (rabbit monoclonal, 1:1000, ab62352), HO1 (rabbit monoclonal, 1:1000, ab68477), NQO1 (rabbit monoclonal, 1:1000, ab80588), and GAPDH (rabbit monoclonal, 1:1000, ab181602) at 4 °C, and a subsequent 1 h exposure to a secondary antibody (goat anti-rabbit, 1:1000, ab6721) at 37 °C. All antibodies were from Abcam (UK). Protein bands were detected via a chemiluminescence kit (Millipore, Billerica, MA, USA). Protein quantification utilized Image J, and was normalized to GAPDH levels.

2.5. CCK-8 evaluation

BRCA1-overexpressing and control cells were plated onto 96-well plates (0.25×10^5 cells/well) for 24, 48, and 72 h, and 5 accessory wells were set in each group. Subsequently, cells were 2 h treated with a serum-free medium with 10 % CCK-8 solution at 37 °C. Absorbance was recorded at 450 nm via a microplate reader (Tecan Austria GmbH 5082, Austria). Cellular viability was quantified as a percentage of values for controls.

2.6. Scratch test

CAL-27 cells from individual groups were plated in 6-well plates. When 80 % confluency was achieved, three straight scratches were generated with a 200 μL pipette tip. The tip was inserted vertically into all the wells, to ensure that the width of each scratch was almost identical. Scratches were photographed using a microscope at 0, 24, 48, 72, and 96 h.

2.7. Annexin V/7-AAD staining

Cells were detached with a trypsin solution that did not contain ethylenediaminetetraacetic acid. After replenishing the solution twice with PBS, 5 μL PE Annexin V and 5 μL 7-AAD (BD Biosciences, Bedford, MA, USA) were introduced to the cell suspension prior to a 30 min incubation with the dye in the dark. Finally, cells were analyzed via flow cytometry.

2.8. ELISA

Logarithmically growing were inoculated in 6-well plates in serum-free media. Following a 24-h incubation, supernatant was generated by centrifuging for 5 min at 3500 rpm. The 8-OHdG content was determined via a specific ELISA kit (ab201734, Abcam). Absorbances at 450 nm were read in a microplate reader (Tecan, Austria).

2.9. Immunohistochemistry

Samples were obtained from 50 patients with OLK ($n = 25$) and OSCC ($n = 25$) from The Affiliated Hospital of Qingdao University (reference number: QYFY WZLL 26924). Following sectioning, immediate fixation was performed in 4 % neutral formalin, followed by embedding in traditional paraffin. Immunohistochemical analysis was performed by treating sections (4 μm) with mouse anti-HO1 (1:100, ab189491, Abcam), mouse anti-NQO1 (1:100, ab28947, Abcam), and rabbit anti-Ki-67 (1:150, 05278384001, Roche) antibodies. Image capture was done under a light microscope (Leica, Germany) at 200× magnification. Three fields were randomly selected from each partition. The area and gray value was calculated to assessed the expression of the HO1 and NQO1, and Ki-67 positive cell rate was examined to evaluated the proliferation. Immunohistochemical images calculations were performed using Image J software.

2.10. Statistical analyses

Data are shown as mean ± SD values of three individual experiments. Normal distribution was assessed via the Levene test. Subsequently, inter-group assessments utilized the Student's t-test and multi-group assessments used one-way ANOVA and appropriate

adjustments. $P < 0.05$ indicated the significance cut-off. All data analyses were conducted on GraphPad Prism 8.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. BRCA1 overexpression up-regulated the NRF2 signalling pathway

Using Western blot and qRT-PCR assays, we determined the BRCA1 overexpressing efficiency. Our analysis revealed that, relative to controls (the lentiviral vectors without *BRCA1*), the mRNA expression levels of BRCA1 in CAL-27 ($P < 0.0001$, $t = 14.87$) and DOK ($P = 0.0016$, $t = 7.557$) cells were significantly increased after lentivirus transfection, the protein expression quantity increased, suggesting that CAL-27 and DOK cell lines with BRCA1 overexpression were successfully constructed (Fig. 1A–C). When the BRCA1 overexpression, the NRF2, HO1, and NQO1 transcript and protein expressions were considerably enhanced relative to transfected cells (without *BRCA1*) (Fig. 1D–F). This suggests that *BRCA1* overexpression can activate the NRF2/HO1/NQO1 pathway in *BRCA1* overexpression CAL-27 and DOK cells.

3.2. BRCA1 overexpression inhibited cell proliferation and migration, induced cell apoptosis, and reduced oxidative damage

Using CCK-8 assay, we assessed the CAL-27 and DOK cells proliferation. We revealed that relative to transfected cells (without *BRCA1*), the CAL-27 and DOK cells proliferation was significantly inhibited after stable transfection of *BRCA1* overexpression lentivirus, and the alteration became significance with increasing duration ($P < 0.01$, Fig. 2 A). The CAL-27 cells migration ability was evaluated via the Scratch test. We demonstrated that *BRCA1* overexpression could slow down the migration distance of CAL-27 cells to the other side, and the migration ability of CAL-27 cells was weakened in a time-reliant fashion ($P < 0.01$, Fig. 2 B). PE Annexin V/7-AAD staining was employed for CAL-27 and DOK cells apoptosis analysis. Our findings confirmed that *BRCA1* overexpression could significantly promote CAL-27 and DOK cells apoptosis. ($P < 0.01$, Fig. 2 C). Moreover, 8-OHdG expression in *BRCA1* overexpression cells were reduced (Fig. 2 D). Therefore, *BRCA1* overexpression can inhibit the oral cancer and dysplasia cellular malignant biological behavior and diminish oxidative damage.

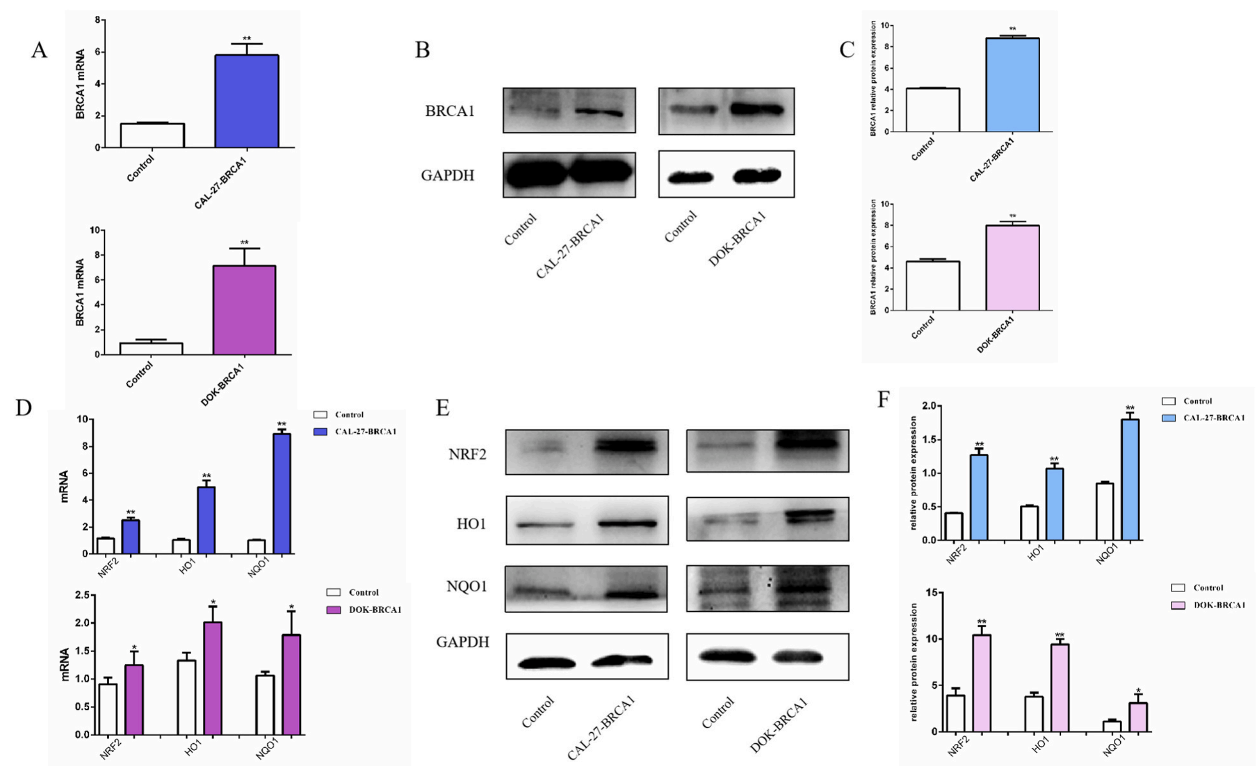

Fig. 1. BRCA1 overexpression up-regulates the NRF2/HO1/NQO1 axis in CAL-27 and DOK cells. (A–C) BRCA1 mRNA (A) and protein (B–C) in cells. **(D–F)** NRF2, HO1, and NQO1 mRNA (D) and protein (E–F) in *BRCA1* overexpression cells (Student's t-test, $*P < 0.05$; $**P < 0.01$). All experiments were conducted three separate times.

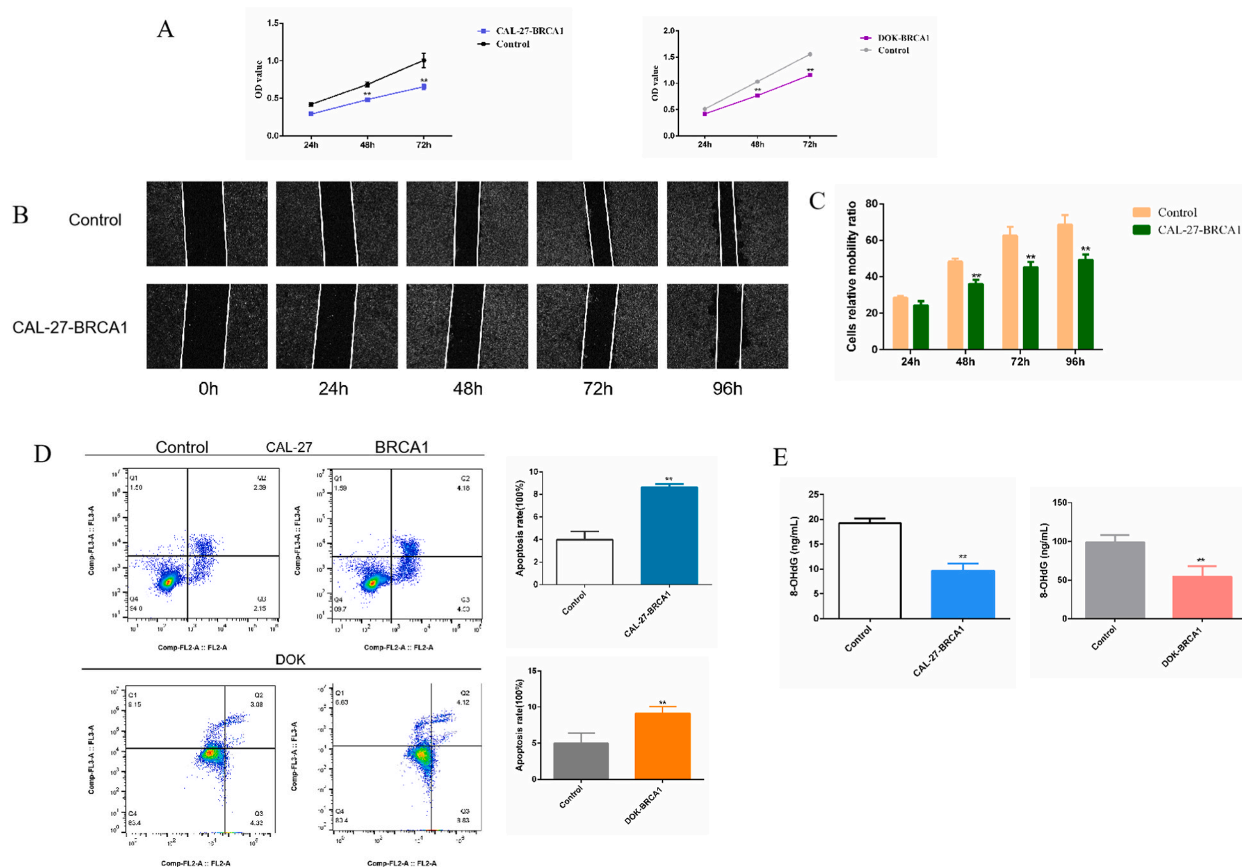

Fig. 2. Influences of BRCA1 overexpression on cellular biological behavior and oxidative damage. (A) *BRCA1* overexpression cells proliferation rates over various time points. **(B–C)** CAL-27 cells migration rates over various time points. **(D)** *BRCA1* overexpression and control cells (the lentiviral vectors without *BRCA1*) apoptosis rates (summation of early and late apoptotic rate). **(E)** 8-OHdG expression following *BRCA1* overexpression (Student's t-test and One-way ANOVA, $*P < 0.05$; $**P < 0.01$). All experiments were carried out three separate times.

3.3. Detecting the expression of HO1, NQO1, and Ki-67 contents in oral leucoplakia and oral squamous cell carcinoma cells

Using immunohistochemistry, we detected the HO1, NQO1, and cell proliferation index Ki-67 contents in tissues obtained from patients with OLK and OSCC, the cells were stained brown in the nucleus or cytoplasm were defined as positive cells. HO1 and NQO1 were expressed in the cytoplasm of epithelial cells, and Ki-67 was expressed in the nucleus. The area and gray value was calculated to assessed the expression of the HO1 and NQO1. We evaluated the Ki-67 positive cell rate to examine the proliferation. The HO1, NQO1, and Ki-67 contents were elevated in OSCC (Fig. 3A–D).

4. Discussion

OSCC is characterized by squamous differentiation, nuclear polymorphism, and aggressive growth [18]. A comprehensive understanding of OSCC pathogenesis and identification of tumour suppressor genes are crucial for enhancing and supplementing clinically significant treatment strategies. Certain tumour suppressor genes, such as *BRCA1*, may be associated with the occurrence of OSCC, but the mechanism of action is still unclear. The tumour suppressor gene *BRCA1* aids in DNA repair, cell cycle modulation, and genome stability. It expresses a protein known as the BRCA1-associated genome surveillance complex (BASC), which forms a complex of proteins in combination with other tumour suppressors. This complex regulates recombination, transcription, and DNA repair [19]. BRCA1 resides on human chromosome 17 q21, and the entire *BRCA1* gene length is about 100 kb. The gene encodes a multifunctional nucleoprotein encompassing 1863 amino acids, containing 24 exons and 22 introns, of which 22 are encoded as transcripts [20]. Cytological studies have shown that after DNA damage was induced, BRCA1 and other proteins involved in repair are present at damage-induced foci. This might be one of the earliest indications that *BRCA1* could be associated with DNA repair machinery [21]. It is thought that BRCA1 has E3 ubiquitin ligase activity, and might promote ubiquitination at the sites of DNA injury, thus aiding in DNA damage repair [22]. As a crucial tumour suppressor gene, *BRCA1* is closely linked to the occurrence of upper gastrointestinal and head and neck tumours, including oral and oesophageal cancers [23]. *BRCA1* knockdown can development of head and neck tumours in mice [24]. Studies report that higher BRCA1 expression contributes to worsened disease-specific survival, independent of other

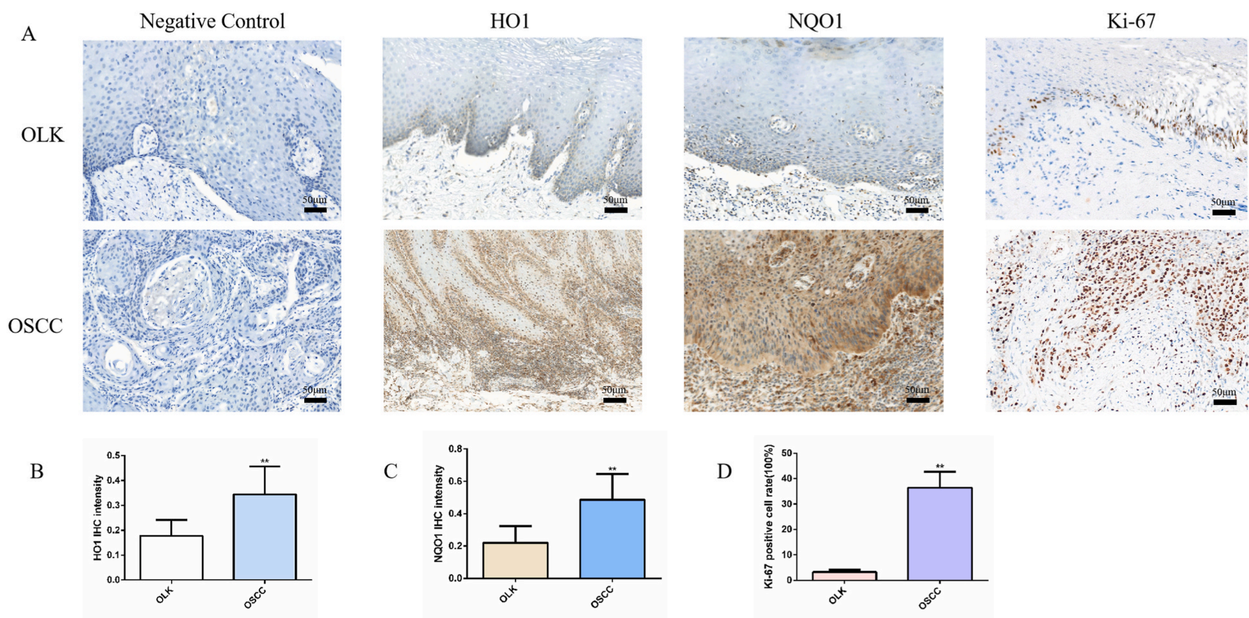

Fig. 3. HO1, NQO1 and Ki-67 contents were augmented in OSCC versus OLK. (A) Immunohistochemistry staining of HO1, NQO1 and the proliferation marker Ki-67 in OLK and OSCC (200× magnification). **(B–D)** Immunohistochemical analysis of HO1, NQO1, and Ki-67 (Student's t-test, $*P < 0.05$; $**P < 0.01$) ($n = 25$).

variables [25]. Beyond *BRCA1*, NRF2 is a major transcription factor regulating cellular responses to oxidative stress. It can modulate the expression of antioxidant genes and enhance cellular defence against inflammatory stimulants and chemical carcinogens. Several signalling pathways have been identified to protect healthy tissues from oxidative damage, with NRF2 emerging as a key regulator that triggers the activation of related genes such as *HO1* and *NQO1* [26]. The reduction of quinones and other organic molecules is catalyzed by NQO1 [27]. HO1 is essential to the anti-inflammatory and antioxidant systems in the body. The breakdown of heme molecules, which results in the production of free iron ($Fe2+$), biliverdin (BV), and carbon monoxide (CO), is mediated by HO1 [28]. In liver cancer cells, promoting the repositioning of BRCA1 to chromatin facilitates the activation of NRF2, and its downstream signalling targets, such as NQO1 and HO1. This activation of BRCA1-NRF2 enhances the oxidative response and reduces ROS, thereby protecting hepatocarcinoma cells from oxidative damage [29]. In our cytological study, we found that CAL-27 cell lines with *BRCA1* overexpressing lentiviral vectors have a higher expression of BCRA1 mRNA and protein levels. Similar findings were observed with DOK cells. Additionally, NRF2, HO1, and NQO1 contents were also augmented in *BRCA1* overexpression CAL-27 and DOK cells. This suggests that *BRCA1* overexpression up-regulates the NRF2/HO1/NQO1 signalling pathway.

It has been shown that the NRF2 content in OSCC tissues and cell lines was substantially elevated relative to non-cancerous tissues and healthy oral keratinocytes. Patients with higher NRF2 content experienced reduced survival rate than relative to those with diminished NRF2 content, indicating that elevated NRF2 expression regulates OSCC occurrence and development and might serve as a robust prognostic indicator [30]. Similar research showed that as compared to healthy tissues, the expression levels of MCU, NRF2, and MICU1 contents were upregulated in OSCC tissues [31]. Our prior investigation showed that the expression of BRCA1 and NRF2 contents in OSCC was elevated compared to that in OLK [32]. This study shows that the expression levels of HO1 and NQO1 contents in OSCC are elevated relative to those in OLK during histopathological examination. This suggests that the increased expression of NRF2 and its key genes in the pathway may be a predictor of the transformation of OPMDs into OSCC. These findings suggest that the NRF2/HO1/NQO1 axis may regulate OSCC occurrence and development, and interactions with *BRCA1*.

Numerous studies have shown that cell proliferation is one of the most important biological mechanisms associated with tumorigenesis that has important prognostic significance in various tumours [33–36]. Immunohistochemical testing has been used to assess factors associated with oral cancer, such as Ki-67, a cell proliferation marker whose levels increase with disease progression [37]. We also observed a significantly higher positive expression rate of Ki-67 in OSCC than in OLK. To maintain the balance between cell oxidation/antioxidation, Ki-67 plays a protective role in cells. The eighth carbon atom of guanine in DNA molecules is particularly susceptible to attack by ROS, resulting in the formation of the oxidative adduct 8-OHdG [38]. Therefore, 8-OHdG is an essential biomarker of DNA oxidative damage that is widely used in experiments and clinical practice [39]. The level of 8-OHdG in cells can generally reflect the degree of oxidative damage to nDNA and mtDNA [40]. In our study, *BRCA1* overexpression abrogated cell proliferation, migration ability, and 8-OHdG expression, and promoted cell apoptosis. This seems to indicate that while there is a lower level of oxidative stress, there is a higher apoptosis rate in cancer cells. Although cancer cells are undergoing apoptosis, they are simultaneously upregulating antioxidant mechanisms. It is suggested that BRCA1 is closely related to OSCC development.

In conclusion, BRCA1 overexpression up-regulated the NRF2 pathway, thereby inhibiting malignant biological behaviour and reducing oxidative damage in oral cancer cells. This suggests that cell proliferation and oxidative damage can be regulated through the

BRCA1 and NRF2/HO1/NQO1 signalling pathways, impacting oral cancer occurrence and development. Additional investigations are warranted to test and confirm these findings. Ongoing research shall elucidate the mechanism of BRCA1 more clearly, providing new insights and a theoretical foundation for the preventing and treating of oral cancer. This study only conducted a preliminary exploration of some clinical samples from a cytological perspective; thus further investigation into the interplay between BRCA1 and the NRF2 pathway and the influence in OSCC will continue to be a focus of our research.

CRediT authorship contribution statement

Xiaofei Yu: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Jing Deng:** Writing – review & editing, Supervision, Conceptualization. **Hui Zhang:** Writing – original draft, Methodology, Investigation, Formal analysis. **Junjie Tong:** Writing – original draft, Software, Methodology, Investigation. **Chunyan Wan:** Writing – review & editing, Methodology, Investigation, Data curation. **Yao Liu:** Methodology, Investigation, Data curation. **Zheng Sun:** Writing – review & editing, Resources, Conceptualization. **Zhengyi Shan:** Methodology, Investigation, Data curation. **Pei Sun:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

Data availability statement

Data will be made available on request.

Ethics approval and consent to participate

This work was consented by The Affiliated Hospital of Qingdao University ethics committee with the ethical approval number QYFY WZLL 26924.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38977.

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