

ORIGINAL RESEARCH

DNAJB5 (hsc40) gene as a novel biomarker for cervical cancer

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Abstract

Cervical cancer is among the most common malignant tumor and leading cause of cancer-related mortality in women worldwide. Heat shock proteins (HSPs) are reported increasingly as potential biomarkers and drug targets for cancers. *DNAJB5* has been shown to be involved in HSP binding and response to unfolded proteins, but its role in cervical cancer remains currently unclear. In this study, *DNAJB5* was knocked down in SiHa cells by short hairpin RNA (shRNA) and verified by quantitative real-time polymerase chain reaction (qRT-PCR) as well as Western blotting. Its effects on cell proliferation, apoptosis, viability, and cell cycle were investigated by Celigo assay, Annexin V-Allophycocyanin (Annexin V-APC) assay, tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye (MTT) assay and propidium iodide (PI) staining assay, respectively. shRNA experiments successfully knocked down *DNAJB5* expression, which decreased the proliferation of SiHa cells, reduced the number of viable cells, affected the cell cycle of SiHa cells and increased their apoptosis. The knockdown of *DNAJB5* acted as a tumor suppressor in cervical cancer cells, suggesting *DNAJB5* as a new biomarker candidate and potential target for cervical cancer treatment.

Keywords*DNAJB5*; Cervical cancer; shRNA; HSP

1. Introduction

For female, cervical cancer is still the fourth leading reason of neoplasia worldwide, associated with 311,000 deaths in 2018 [1]. Although its incidence and mortality have decreased due to the human papilloma virus (HPV) vaccine and cytologic screening, they are still much higher in low- and middle-income countries [2]. The treatment of advanced-inoperable cervical cancer is challenging due to increased rates of local recurrence and distant metastasis. Recurrent cervical cancer cases that cannot be radically treated are considered incurable, and those with distant metastatic disease have dismal prognoses. Thus, there is an urgent need to identify new biomarkers to improve the diagnosis and personalized treatment of cervical cancer.

Heat shock proteins (HSPs) are a large family containing several classes of proteins, often classified according to their molecular weight. They act as molecular chaperones to maintain cellular protein homeostasis. The HSP group 40, also known as DNAJ, contains the largest number of members. The *DNAJB5* family has been divided into 3 subclasses that share a highly conserved J structural domain. Many DNAJ members are thought to be associated with tumor development and metastasis in many kinds of organs, such as *DNAJA1* in prostate and glioblastoma cancer [3], *DNAJA3* in melanoma, kidney, osteosarcoma, breast and hematopoietic

cancer [4–8], *DNAJB4* in breast and lung cancer [9–11], *DNAJB6* in breast cancer [12, 13], *DNAJB11* in Kaposi sarcoma-associated herpesvirus-related tumors and ovarian cancer [14, 15], *DNAJC9* in cervical cancer [16], *DNAJC10* in neuroblastoma [17], and *DNAJC15* in ovarian and brain cancer [18–20]. *DNAJB5*, also named hsc40, has been shown to be up- or down-regulated in many abnormal conditions [21–25]. Based on current literature, in cervical cancer setting, there is no research on the effect of *DNAJB5* in oncogenesis, development and treatment.

This study aimed to determine the expression of *DNAJB5* in cervical cancer and its function on the proliferation, viability, apoptosis and cell cycle of cancer cells. We expect the findings to stimulate greater interest in *DNAJB5* as a new biomarker candidate in cervical cancer and the potential development of new therapeutic targets.

2. Materials and Methods

2.1 The procedure of Quantitative real-time (qRT)-PCR

Total RNA was extracted from human cervical cancer SiHa cell lines obtained from Cell bank of Chinese Academy of Science by Trizol Kit (PuFei, Shanghai, China). Then, reverse transcriptions of the isolated RNAs

were performed by the Moloney Murine Leukemia Virus reverse transcriptase (M-MLV) Kit (Promega, Madison, WI, USA). qRT-PCR of *DNAJB5* was operated with SYBR Green Master Mix (TAKARA, Dalian, China, DRR041B) on a Nanodrop spectrophotometer (Thermo 2000/2000C) according to the manufacturer's instructions. Meanwhile, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the internal control. Sequences of the primer were as follows: *GAPDH*, 5'-TGA CTTCAACAGCGACACCCA-3', and 5'-CACCTGTTGCTGTAGCCAAA-3' for forward and reverse respectively. *DNAJB5*, forward, 5'-CCTATGATGTGCTAAGTGACCCC-3', and reverse, 5'-GAAAGGTGTAGTGAAAGGAGCC-3'. There were two steps for qRT-PCR process, which were performed as follows. For the first step, under the temperature 95 °C, and hold for 30 s; for the second step PCR, which had 40 cycles, under the temperature 95 °C for 5 s, 60 °C for 30 s, followed by a dissociation step at the temperature 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. The relative levels of *DNAJB5* transcripts were determined by using the comparative CT method ($2^{-\Delta\Delta CT}$).

2.2 Gene knockdown by lentivirus transfecting SiHa cells

Genechem (Shanghai, China) constructed the *DNAJB5* knockdown lentiviral GV115 vector (GV115-*DNAJB5*-shRNA), which was then verified by DNA sequencing. The SiHa cells were cultured in Minimum Essential Medium + 10% fetal bovine serum at 37 °C and 5% carbon dioxide. The cells were laid in humidified atmosphere for 72 h to grow and when they reached 2×10^5 cells, they were transfected with the recombinant plasmid and classified as the experimental group (sh*DNAJB5*), while the empty lentivirus GV115 was used as a control group (shCtrl).

2.3 Western blotting (WB)

The cells were prepared with $2 \times$ Lysis Buffer (100mM 1M Tris-HCL pH 6.8, 2% mercaptoethanol, 20% glycerol and 4% sodium dodecyl sulfate). After centrifugation at 12,000 g at 4 °C for 15 minutes, insoluble material was removed. Then, protein contents were quantified by the Bicinchoninic acid (BCA) protein assay kit (P00105, Beyotime, Shanghai, China) according to the manufacturer's instructions. Then, the proteins were separated in 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride (PVDF) membranes which blocked with Tris-buffered saline (TBS) containing 5% skim milk at the room temperature for 1 h. The membranes were incubated with anti-*DNAJB5* (1:500, ab234992, Abcam, England) as the primary antibody. Incubated the membrane overnight at 4 °C. The membranes were washed with TBS containing 0.05% Tween-20 (TBST) and were incubated with a secondary antibody, named HRP-linked anti-rabbit IgG (1:2000, sc-2004, Santa-Cruz). *GAPDH* was used as a loading control, with anti-*GAPDH* (1:2000, sc-32233, Santa-Cruz, Dallas, TX, United States) as the primary antibody, and anti-Mouse IgG (1:2000, sc-2005, Santa-Cruz) as the secondary antibody. The membranes were then washed with TBST and assayed with the

Pierce™ ECL WB Substrate Kit (Thermo Scientific, Waltham, MA, United States).

2.4 Cell proliferation assay

Cells with *DNAJB5* stable knockdown or non-knockdown were seeded at 600 cells/well in 96-well plates. Cells in each well were counted by Celigo (Nexcelom, Lawrence, MA, United States) starting from the next day for 5 days. The experiments were performed in triplicate for each assay.

2.5 Cell apoptosis assay

The cells were cultured in 6-well plates until they reached a confluency of about 70%. They were collected and then centrifuged at 1300 rpm for 5 minutes. They were washed with binding buffer and centrifuged again. The cells were stained with Annexin V-APC (eBioscience, San Diego, CA, United States, model 88-8007) for 15 minutes at room temperature, and apoptotic cells were detected using fluorescence microscopy. The experiments were performed in triplicate for each assay.

2.6 Cell cycle assay

The cells were cultured in 6cm dishes until they reached a confluency of about 80%. They were then collected and centrifuged at 1300 rpm for 5 minutes. The sediment was collected, washed and centrifuged at 1300 rpm for 5 minutes. The cells were then stained with a staining solution (40 × PI master mix (2mg/mL) (P4170, Sigma, St. Louis, MO, United States): 100 × RNase master mix (10mg/mL) (EN0531, Thermo Fisher Scientific): 1 × DPBS: 25 × Triton X-100 (SLBT4524, Sigma) = 25:10:1000:40). Cells in different cell cycle periods were then examined using fluorescence microscopy. The experiments were conducted in triplicate.

2.7 MTT detection

The cells were grown and collected at log-phase growth. The two groups of cells were placed in five 96-well plates with the density of 2500 cells/well. Then, 20 μL MTT with the concentration of 5 mg/mL (JT343, Genview, United States) was added to each well. After 4 hours, followed by 100 μL of DMSO. The plates were shaken for 2–5 minutes, and the optical density (OD) value of each well was detected at 490 nm on days 1, 2, 3, 4 and 5, using a microplate reader.

2.8 Statistical analysis

The data were first tested for normality. Either mean ± standard deviation (SD) or median and interquartile range was used to describe the data. Statistical analysis was performed using the *t*-test. $P < 0.05$ was considered statistically significant. Analysis was performed by the Statistical Product and Service Solutions (SPSS, International Business Machines Corporation, Armonk, NY, United States) 23.0 software.

3. Results

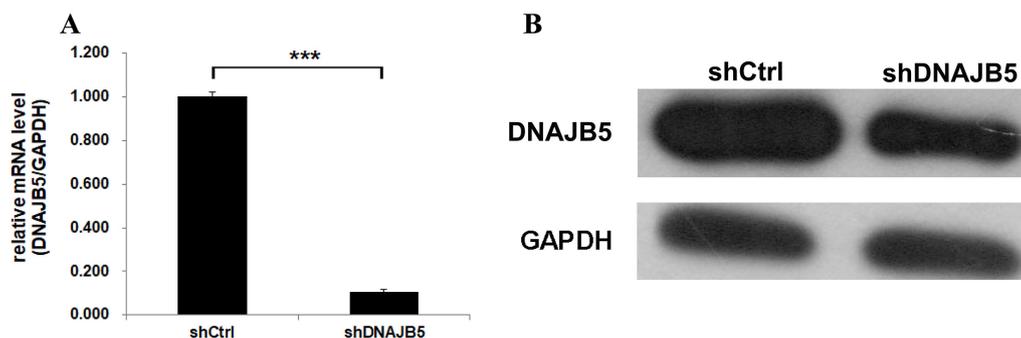


FIGURE 1. Knockdown of *DNAJB5* was verified by qRT-PCR and WB. (A) The relative levels of mRNA (*DNAJB5/GAPDH*) were significantly lower in the shDNAJB5 group than in the shCtrl group ($p < 0.01$). (B) Protein levels were significantly lower in the shDNAJB5 group than in the shCtrl group. *** $p < 0.001$ vs. shCtrl group.

3.1 *DNAJB5* gene was successfully knocked down

qRT-PCR was used to detect the mRNA level of SiHa cells after 3 days of lentivirus transfection. As shown in Fig. 1A, the relative mRNA level in the shDNAJB5 group was lower than in the shCtrl group significantly (0.11 ± 0.01 vs. 1.00 ± 0.02 , $p < 0.001$), demonstrating a knockdown efficiency of 89.4%. The protein levels were then detected by WB, which showed significantly lesser protein levels in the shDNAJB5 group than in the shCtrl group (Fig. 1B). The above results indicated that the endogenous expression of target genes was successfully reduced.

3.2 *DNAJB5* knockdown inhibited proliferation and promoted apoptosis of SiHa cells

As shown in Fig. 2A–B, both the cells observed under fluorescent microscopy and the absolute value of the cells in the shDNAJB5 group were significantly less than in the shCtrl group. With time, the difference became more and more obvious (2597 ± 118 vs. 808 ± 50 ($p < 0.001$) on the 4th day and 3475 ± 177 vs. 860 ± 120 ($p < 0.001$) on the 5th day). On the 4th and 5th days after planking, the number of cells in the shCtrl group increased by 5 times compared with the initial state, while that in the shDNAJB5 group increased by less than 1 time (fold: 5.01 ± 0.19 vs. 1.52 ± 0.03 , $p < 0.01$ on the 4th day; 6.70 ± 0.29 vs. 1.61 ± 0.13 , $p < 0.001$ on the 5th day) (Fig. 2C), suggesting that the proliferation of SiHa cells in the shDNAJB5 group was significantly inhibited.

The results of apoptosis showed that the shDNAJB5 group had a significantly higher apoptosis rate than in shCtrl group 5 days after shRNA lentivirus transfection ($6.07\% \pm 0.29\%$ vs. $0.43\% \pm 0.06\%$, $p < 0.01$) (Fig. 2D–E).

3.3 *DNAJB5* knockdown influenced cell cycle in SiHa cells

Five days after shRNA lentivirus transfection, the shDNAJB5 group showed a decrease in the G1 phase of the cells (77.13%

$\pm 0.15\%$ vs. $75.30\% \pm 0.26\%$, $p < 0.05$) and an increase in the S phase ($14.16\% \pm 0.20\%$ vs. $14.73\% \pm 0.06\%$, $p < 0.05$) and G2 phase ($8.53\% \pm 0.12\%$ vs. $9.63\% \pm 0.12\%$, $p < 0.05$) (Fig. 3), indicating that *DNAJB5* was associated with the cell cycle distribution of SiHa cells.

3.4 *DNAJB5* knockdown reduced cell viability

After 3 days of shRNA lentivirus transfection, the cells were spread on 96-well plates. After 5 days of continuous detection, we found the OD490 absolute value of the shDNAJB5 group was significantly lower than in the shCtrl group (Fig. 4), indicating the proliferation activity of the shDNAJB5 group was significantly lower than the shCtrl group.

4. Discussion

In this study, we successfully constructed knocked down *DNAJB5*, which was confirmed by qRT-PCR and WB. Our results showed that the proliferation of SiHa cells in the shDNAJB5 group decreased, and the apoptosis increased significantly compared with the shCtrl group.

Compared with the results detected by Celigo, the proliferation of the shDNAJB5 group detected by the microplate reader at 490 nm wavelength seemed to be higher. Due to the lentivirus genome containing the fluorescent gene, the Celigo method only detected the proliferation of cells those were successfully transfected, while the microplate reader detected the proliferation of all cells (whether transfected or not). Therefore, the results detected by the two methods were slightly different but demonstrated a similar trend. These results indirectly demonstrated that the knockdown of *DNAJB5* could inhibit the proliferation of SiHa cells.

Members of the DNAJ/HSP40 proteins are highly conserved and expressed in several tissues. The members of the DNAJ family interact with HSP70 and act as co-chaperones for the ATPase activity of HSP70. DNAJ proteins have been shown to play a crucial role in identifying specific targets, recruiting them to HSP70, and translocating folded proteins to the nu-

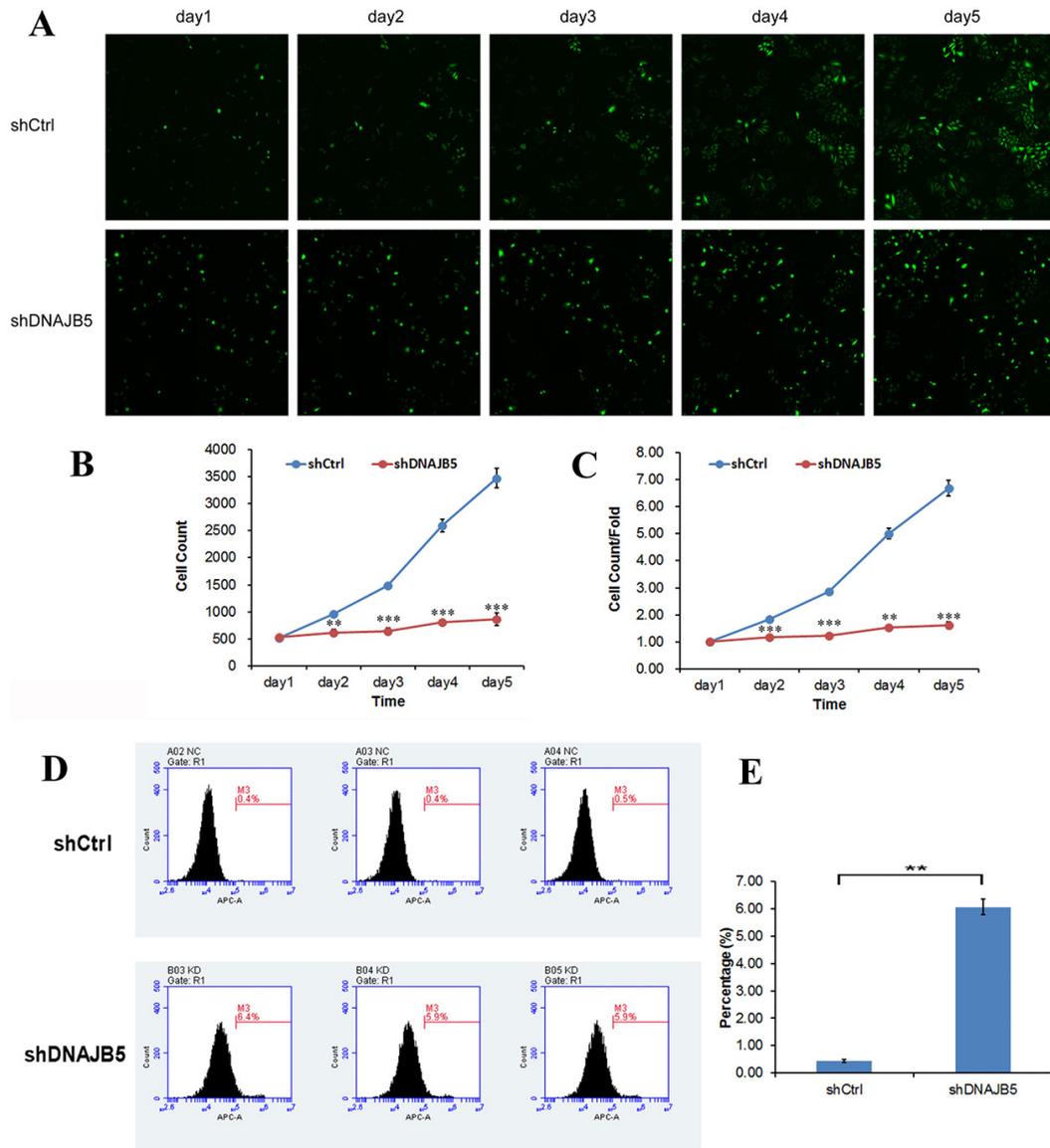


FIGURE 2. Knockdown of *DNAB5* inhibited the proliferation of SiHa cells and promoted apoptosis. (A) Cells in the shDNAB5 group are more diluted than in the shCtrl group. The number of cells in the shDNAB5 group (B) and the rate of increases (C) were significantly lower than in the shCtrl group. (D, E) 5 days after shRNA lentivirus transfection, the number of apoptotic SiHa cells in the shDNAB5 group increased significantly compared with those in the shCtrl group. $**p < 0.01$, $***p < 0.001$ vs. shCtrl group.

cleus and mitochondria [21].

Many DNAJ family members have been extensively studied in several tumors. Several homologs in HSP were reported to be related to cervical cancer, such as HSP70, whose expression was associated with a worse prognosis and correlated with the malignancy marker: c-myc [22, 23]. HSP10, which was found to be overexpressed during carcinogenesis [24], and HSP60, which was detected at higher levels during carcinogenesis [25]. However, few studies have investigated the significance of *DNAB5* in oncogenesis and treatment. Several studies have also been performed on *DNAB5* in non-neoplastic setting. Ago *et al.* [26] suggested that *DNAB5* was upregulated by Thioredoxin1 (Trx1) and served to connect Trx1 and class II histone deacetylases, which were important negative regulators of cardiac hypertrophy. Phenethyl isothiocyanate (PEITC), a

potential cancer suppressor, was shown to significantly upregulate *DNAB5* [27]. Some studies revealed that *DNAB5* was involved in neurological diseases such as Alzheimer associated with or not mitogen-activated protein kinase signaling [28, 29]. Schulze *et al.* [30] found that *DNAB5* was down-regulated in intestinal biopsy samples of ulcerative colitis compared with normal controls by microarray analysis and real-time PCR.

In this study, we investigated the role of *DNAB5* in cervical cancer cell lines. The results showed that *DNAB5* demonstrated oncogene effects in cervical cancer by promoting the proliferation of SiHa cells and reducing their apoptosis. However, the underlying mechanism of how *DNAB5* knockdown affected the cell function has not yet been explained and should be further researched and discussed.

HPV infection is one of the most prominent causal factors

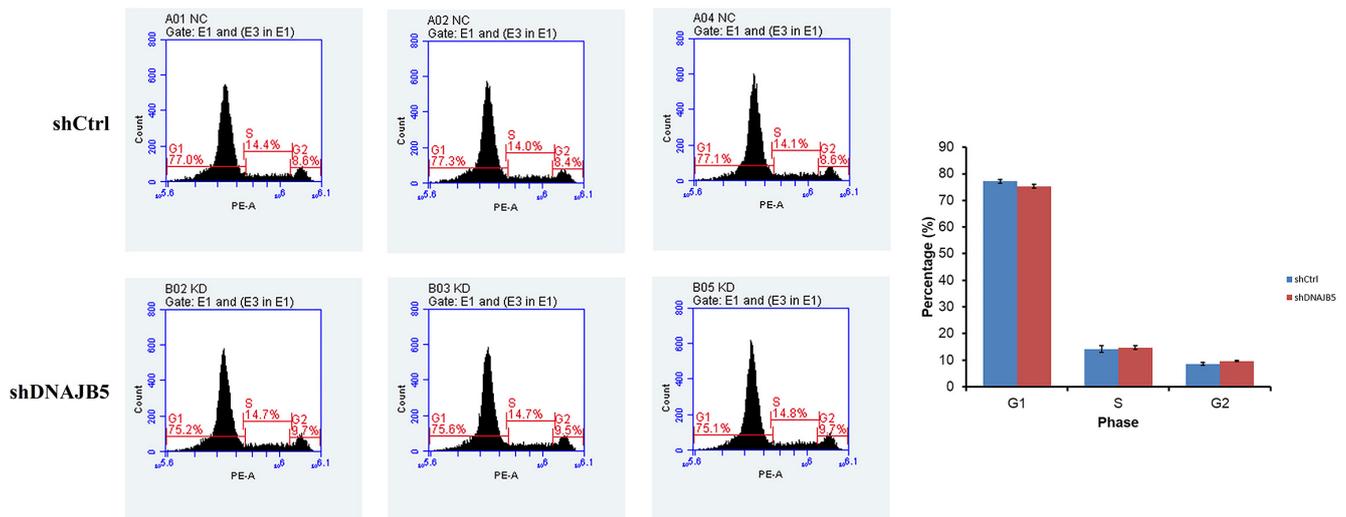


FIGURE 3. Five days after shRNA lentivirus transfection, the percentage of cells in the G1 phase decreased, while the percentage of cells in the S and G2 phases increased. $*p < 0.05$ vs. shCtrl group.

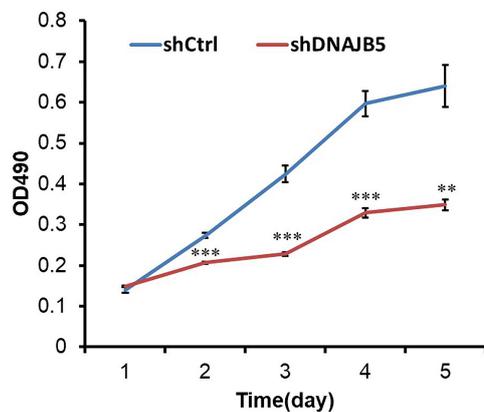


FIGURE 4. The cell proliferation activity of the shDNAJB5 group was significantly lower than the shCtrl group. The OD490 value in the shDNAJB5 group was significantly lower than the shCtrl group when tested for 5 consecutive days. $**p < 0.01$, $***p < 0.001$ vs. shCtrl group.

of cervical cancer. HSP40 has been confirmed to be associated with HPV infection. *DNAJB5* contains an N-terminal J-domain, a glycine/phenylalanine (G/F) rich region and a C-terminus [31]. The J-domain is essential for stimulating the adenosine triphosphate (ATP) hydrolysis of HSP70. J-domain containing host factors was used by HPV to facilitate its replication and cellular transformation. HSP40 acts directly as a chaperone to help virus to bind E1 to the replication origin [32]. We speculated that the carcinogenic effect of *DNAJB5* could be related to HPV infection. Lampis *et al.* [33] reported that miRNA21 could mediate the resistance of cholangiocarcinoma cells to HSP90 inhibitors by reducing the levels of *DNAJB5*, which might be potential mechanism also.

Based on the promising effects of *DNAJB5* in diseases, especially in cervical cancer where it remains underinvestigated, we hope that these new findings could stimulate more focus on

the potential of *DNAJB5* as a novel biomarker and treatment target. In this regard, we aim to conduct more validation studies to confirm our study results and further investigate its underlying mechanisms in cervical cancer. In the meantime, future functional, epigenomic and clinical studies could also be performed to further determine the role of *DNAJB5* in the pathogenesis of cervical cancer and, importantly, to assess its potential as a diagnostic marker and target for personalized cancer treatment.

5. Conclusions

The role of *DNAJB5* in the proliferation and apoptosis of cervical cancer cells was evaluated. Our findings showed that *DNAJB5* has the potential to be a new biomarker and potential target for the treatment of cervical cancer.

AUTHOR CONTRIBUTIONS

HY and KW designed the research study. HY performed the research. YC, GZ and HW analyzed the data. HY and GZ wrote the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

ACKNOWLEDGMENT

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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