RESEARCH ARTICLE

LncRNA HEIH regulates cell proliferation and apoptosis through miR-4458/SOCS1 axis in triple-negative breast cancer

Peng Li[1] · Bo Zhou[1] · Yuetao Lv[1] · Qian Qian[1]

Abstract

Hepatocellular carcinoma up-regulated EZH2-associated long non-coding RNA (HEIH) is a newly discovered lncRNA and has been suggested to be dysregulated in human cancers. However, the role of HEIH in triple-negative breast cancer (TNBC) was still unknown. Thus, the aim of our study was to investigate the clinical significance and biological function of HEIH in TNBC. In our study, we found that HEIH was overexpressed in TNBC tissues and cell lines compared with adjacent normal mammary tissues and normal mammary epithelial cell line, respectively. In addition, we conducted bioinformatic analysis, and found that HEIH harbors a potential miR-4458-binding site. Furthermore, we observed that HEIH and miR-4458 had a high correlation score in TNBC tissues, and HEIH directly binds to miR-4458, and negatively regulates miR-4458 expression in TNBC cells. The in vitro cell proliferation and apoptosis assays suggested down-regulation of HEIH inhibited TNBC cell proliferation and promoted apoptosis through regulating miR-4458/SOCS1 axis. Finally, we found that TNBC patients with tumor size ≥ 5 cm or advanced clinical stage had higher levels of HEIH expression than patients with tumor size < 5 cm or early clinical stage. In conclusion, HEIH functions as an oncogenic lncRNA that is overexpressed in TNBC and associated with clinical progression, and regulates TNBC cell proliferation and apoptosis through miR-4458/SOCS1 axis.

Keywords LncRNA · HEIH · MiR-4458 · SOCS1 · Breast cancer · Biomarker

Introduction

Breast cancer is the most common female malignancy and the leading cause of female mortality worldwide, accounting for about 6.6% of global cancer mortality [1]. Breast cancers are highly heterogeneous in nature and can be divided into at least five genetically distinct subtypes: Luminal A, Luminal B, HER2 overexpressing, and triple-negative breast cancers, which generally includes Basal-like and Claudin-low subtypes [2, 3]. Triple-negative breast cancer (TNBC) is characterized by lack of estrogen receptor (ER) and progesterone receptor (PR) expressions and HER2 amplification [4]. TNBC patients had worst clinical outcome among all types of breast cancer due to high metastatic risk and lack of endocrine therapy and targeted therapy [5, 6]. For identifying potential biomarkers and therapeutic targets, it is necessary to sufficiently investigate about TNBC development and progression.

Long non-coding RNAs (lncRNAs) are a set of non-coding transcripts with a length more than 200 nucleotides and no encoding protein ability [7]. Accumulating evidence suggests that lncRNAs have broad functional roles in pathological and physiological processes including tumorigenesis [8–12]. In our previous studies, we found that miR-4458 inhibited TNBC cell proliferation and promoted cell apoptosis through targeting SOCS1 [13, 14]. In lncRNA-miRNA database, we observed potential lncRNA–miRNA interactions, and found that miR-4458 could interact with hepatocellular carcinoma up-regulated EZH2-associated long noncoding RNA (HEIH), which has been showed to function as oncogenic lncRNA in human cancers [15]. We supposed that HEIH enhances TNBC cell proliferation and reduces cell

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apoptosis via counteracting miR-4458-mediated repression of SOCS1. Therefore, the aim of our study was to investigate the clinical significance and biological function of HEIH in TNBC, and elucidate the relationship between HEIH and miR-4458/SOCS1 axis in TNBC cells.

Materials and methods

Clinical tissue specimens

This study cohort comprised 60 fresh TNBC tissue specimens, 30 fresh non-TNBC tissue specimens, and 60 fresh adjacent normal mammary tissue specimens collected from Jining No. 1 People's Hospital [13]. The clinicopathological characteristics of triple-negative breast cancer patients are summarized in Figure S1. The adjacent mammary tissues are at least 5 cm from the tumor edge. The staging was based on the 7th edition of AJCC TNM staging system. For the use of these clinical materials for research purposes, the approval of our study was gained from the Ethics Committees of Jining No. 1 People's Hospital. The informed consent was obtained from each patient.

Quantitative real-time PCR (qRT-PCR)

RNA isolation and qRT-PCR were performed to measure the expression of HEIH and miR-4458 as described previously [13, 14]. Their primers sequences for HEIH are as: 5′-CCT CTTGTGCCCCTTTCT-3′ (forward) and 5′-AGGTCTCAT GGCTTCTCG-3′ (reverse).

Cell lines and cell transfection

The TNBC cell lines (MDA-MB-436 and BT549) and normal mammary epithelial cell line (MCF-10A) were cultured in accordance with the previous description [14]. These small interfering RNAs (siRNAs) for suppressing HEIH expression in vitro were synthesized by GenePharma Co., Ltd (Shanghai, China). Cell transfection performed by Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) in Opti-MEM (Invitrogen, Carlsbad, CA, USA) as described previously [13, 14].

Cell proliferation assays

Cell proliferation was detected by MTT assay and colony formation assay based on the previous description [14].

Apoptosis assay

The Annexin V/Fluorescein Isothiocyanate Apoptosis Detection Kit (Beyotime, Shanghai, China) was used to measure the apoptosis rate at flow cytometry as described previously [14].

Bioinformatics analysis

The Bioinformatics analysis was conducted at starbase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php) for identifying the potential binding sites between miR-4458 and HEIH.

Luciferase reporter assay

The pmirGLO dual-luciferase vectors (Promega, Madison, WI, USA) containing the wild-type or mutant sequences towards the miR-4458 binding of HEIH were constructed, and named as pmirGLO-HEIH-wt and pmirGLO-HEIH-mut. The Dual-Luciferase Reporter System (Promega, Madison, WI, USA) was used to detect the relative luciferase activity in microplate reader as described previously [13].

Western blot

Western blot was carried out to measure the protein expression of SOCS1 according to our previous description [14]. SOCS1 antibody was used at a concentration of 1:2000 (Abcam, Cambridge, MA, USA).

Statistical analysis

SPSS 17.0 software was applied to statistical analysis. Data are presented as the mean ± standard deviation of at least three biological replicates. The difference between two groups was estimated by two-tailed Student t test. The Spearman correlation analysis was utilized to assess the correlation between HEIH and miR-4458 in TNBC tissues. $P < 0.05$ was considered statistically significant.

Results

HEIH is overexpressed in TNBC tissues and cell lines

To investigate the expression status of HEIH in TNBC progression, we detected HEIH expression levels in human TNBC tissues, non-TNBC tissues, adjacent normal mammary tissues, TNBC cell lines (MDA-MB-436 and BT549), and normal mammary epithelial cell line (MCF-10A) through qRT-PCR. We found that the expression level of HEIH was markedly increased in TNBC tissues in comparison with non-TNBC tissues or adjacent normal mammary tissues (Fig. 1a). However, there was no significant difference of HEIH expression between non-TNBC tissues and adjacent normal mammary tissues (Fig. 1a). Meanwhile, HEIH expression levels in TNBC cell lines were also higher
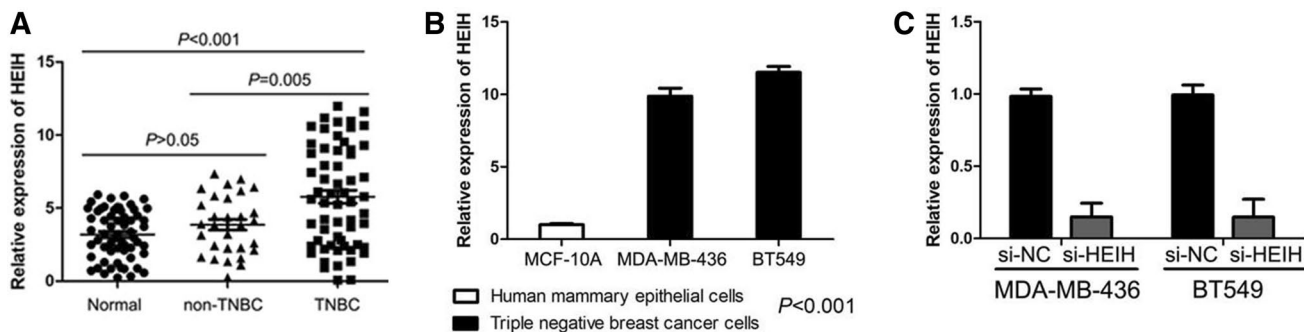
Fig. 1 HEIH is overexpressed in TNBC tissues and cell lines. **a** HEIH expression level was markedly increased in TNBC tissues in comparison with adjacent normal mammary tissues. **b** HEIH expression levels in TNBC cell lines were also higher than in normal mammary epithelial cell line. **c** HEIH expression was obviously reduced after transfection of si-HEIH in MDA-MB-436 and BT549 cells

than in normal mammary epithelial cell line (Fig. 1b). For following loss-of-function study, HEIH expression was obviously reduced after transfection of si-HEIH in MDA-MB-436 and BT549 cells (Fig. 1c).

The relationship between HEIH and miR-4458 in TNBC

As a molecular mechanism of lncRNAs, lncRNAs could combine with specific miRNAs to interfere with their functions [16, 17]. The bioinformatic analysis revealed that HEIH harbors a potential miR-4458-binding site (Fig. 2a). The Spearman correlation analysis suggested that HEIH and miR-4458 had a high correlation score

in TNBC tissues (Fig. 2b). The luciferase reporter assay was utilized to further confirm the directly regulatory relationship between HEIH and miR-4458, and showed that co-transfection of miR-4458-mimics and pmirGLO-HEIH-wt reduced the luciferase activity but not that of miR-4458-mimics and pmirGLO-HEIH-mut, and co-transfection of miR-4458-inhibitor and pmirGLO-HEIH-wt increased the luciferase activity but not that of miR-4458-inhibitor and pmirGLO-HEIH-mut (Fig. 2c). Moreover, we also found that down-regulation of HEIH increased miR-4458 expression in TNBC cells (Fig. 2d). The above results showed that HEIH directly combines with miR-4458, and negatively regulates miR-4458 expression in TNBC.

Fig. 2 The relationship between HEIH and miR-4458 in TNBC. **a** The bioinformatic analysis revealed that HEIH harbors a potential miR-4458-binding site. **b** HEIH and miR-4458 had a high correlation score in TNBC tissues. **c** Luciferase reporter assay was utilized to verify the prediction binding sites between HEIH and miR-4458. **D** Down-regulation of HEIH increased miR-4458 expression in TNBC cells (+ present; − absent)
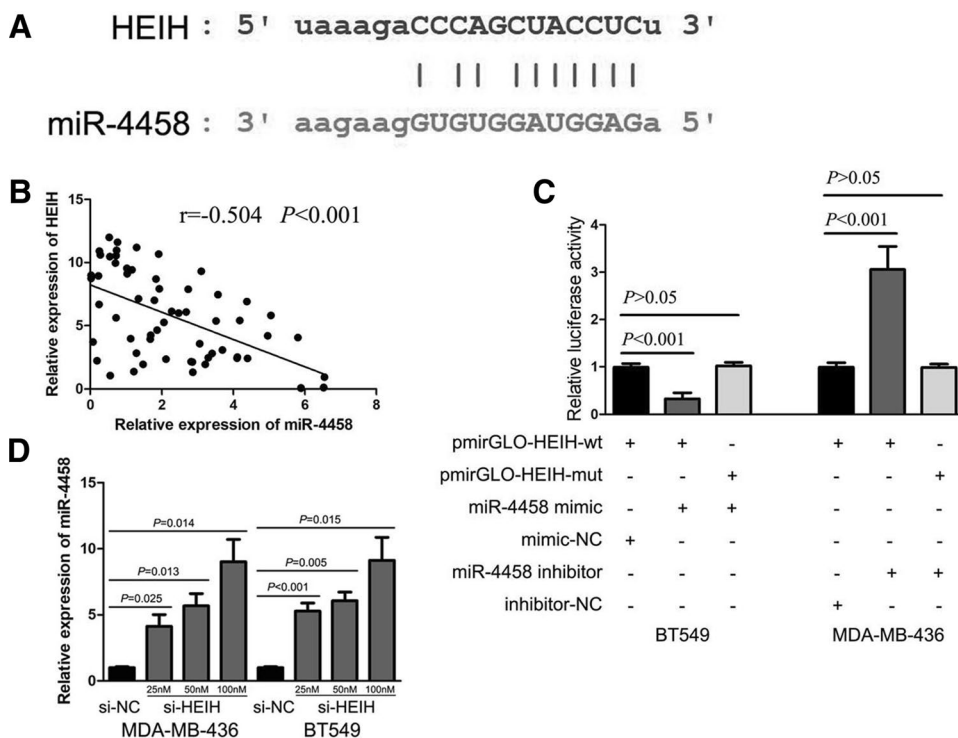
HEIH promotes TNBC cell proliferation and inhibits apoptosis through down-regulating miR-4458

For estimating the impact of HEIH on TNBC cell proliferation, MTT assay and colony formation assay were performed. The MTT assay indicated that down-regulation of HEIH significantly depressed the viability of TNBC cells after 48, 72, and 96 h transfection (Fig. 3a). Meanwhile, colony formation assay showed down-regulation of HEIH markedly reduced the number of colonies of TNBC cells (Fig. 3b). Moreover, we estimated the influence of HEIH on cell apoptosis at flow cytometry, and observed down-regulation of HEIH elevated the percentage of apoptotic TNBC cells (Fig. 3c).

Further determining whether HEIH promotes TNBC cell proliferation and inhibits cell apoptosis by regulating miR-4458, si-HEIH, and miR-4458-mimic/miR-4458-inhibitor were cotransfected into TNBC cells. We found that miR-4458-inhibitor could rescue the tumor-suppressive effect of si-HEIH on cell proliferation and apoptosis in TNBC cells, and miR-4458-mimic did not further enhance the tumor-suppressive effect of si-HEIH on cell proliferation and apoptosis in TNBC cells (Fig. 3a–c). Therefore, we thought that HEIH promotes TNBC cell proliferation and inhibits apoptosis through down-regulating miR-4458.

HEIH positively regulates SOCS1 expression through modulating miR-4458 in TNBC cells

The previous study showed that miR-4458 inhibited TNBC cell proliferation and promotes cell apoptosis through targeting SOCS1. Thus, we conducted western blot to measure the impact of HEIH on SOCS1 expression. We observed down-regulation of HEIH decreased SOCS1 expression in TNBC cells. Furthermore, we found that miR-4458-inhibitor could rescue the inhibitory effect of si-HEIH on SOCS1 expression

Fig. 3 HEIH promotes TNBC cell proliferation and inhibits apoptosis through down-regulating miR-4458. The influence of HEIH and miR-4458 on TNBC cell proliferation and apoptosis was assessed by MTT assay (**a**), colony formation assay (**b**), and apoptosis assay (**c**)
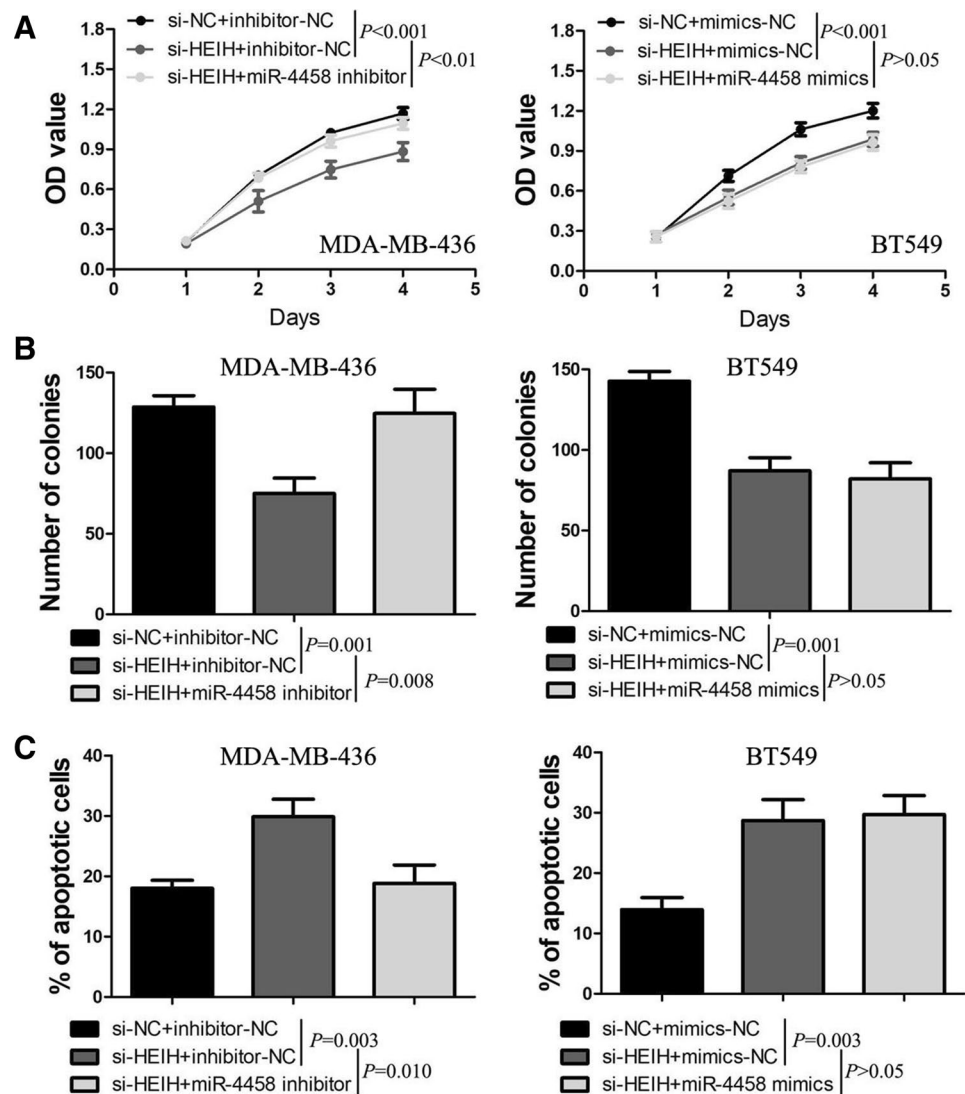
in TNBC cells, and miR-4458-mimic did not further promote the inhibitory effect of si-HEIH on SOCS1 expression in TNBC cells (Fig. 4).

HEIH expression is associated with tumor size and clinical stage in TNBC patients

To investigate the clinical value of HEIH expression in TNBC patients, all cases were classified into two groups based on the clinicopathological characteristics. As shown in Fig. 4a, b, TNBC patients with tumor size ≥ 5 cm or advanced clinical stage had higher levels of HEIH expression than patients with tumor size < 5 cm or early clinical stage.

Discussion

HEIH, a newly discovered lncRNA, has been suggested to be up-regulated or down-regulated in human cancers depending on tumor type. Initially, Yang et al. identified a specific differentially high expressed lncRNA in hepatitis B virus-related hepatocellular carcinoma through microarray and qRT-PCR, and named HEIH [18]. Subsequently, Zhang et al. found that HEIH expression was increased in serum and exosomes of hepatitis C virus-related hepatocellular carcinoma [19]. Moreover, Zhang et al. reported that hepatocellular carcinoma cell lines had higher HEIH expression than human normal hepatocyte line [20]. Besides, high HEIH expression levels were also observed in lung cancer [21], colorectal cancer [22], and melanoma [23]. On the contrary, Haque et al. found that HEIH expression was decreased in

head-and-neck squamous cell carcinoma tissues compared normal tissues through RNA-sequencing data and qRT-PCR [24]. In addition, Bawa et al. also observed an isoform of HEIH which is reduced expression in prostate cancer tissues compared with normal prostate tissues [25]. The expression status of HEIH in TNBC was still unknown. In our study, we found that HEIH was overexpressed in TNBC tissues and cell lines compared with adjacent normal mammary tissues and normal mammary epithelial cell line, respectively. Furthermore, we investigated the clinical value of HEIH expression in TNBC patients through analyzing the relationship between HEIH expression and clinicopathological characteristics, and found that TNBC patients with large tumor size or advanced clinical stage had higher levels of HEIH expression than patients with small tumor size or early clinical stage. Similarly, Cui Chunhui et al. showed that HEIH overexpression was associated with large tumor size and high T classification in colorectal cancer patients [22]. In addition, Zhao Haiying et al. also found that high HEIH expression was correlated with advanced clinical stage in melanoma patients [23]. In hepatitis B virus-related hepatocellular carcinoma patients, Yang et al. suggested that patients with liver cirrhosis had higher HEIH expression than patients with no liver cirrhosis [18]. However, there was no correlation between HEIH expression and clinicopathological characteristics of patients with neck squamous cell carcinoma [24] (Fig. 5).

The prognostic significance of HEIH expression was investigated in several types of cancer such hepatocellular carcinoma [18], colorectal cancer [22] and melanoma [23], and neck squamous cell carcinoma [24]. Yang et al. reported that high HEIH expression was correlated with

Fig. 4 HEIH positively regulates SOCS1 expression through modulating miR-4458 in TNBC cells. The influence of HEIH and miR-4458 on SOCS1 expression was assessed by western blot
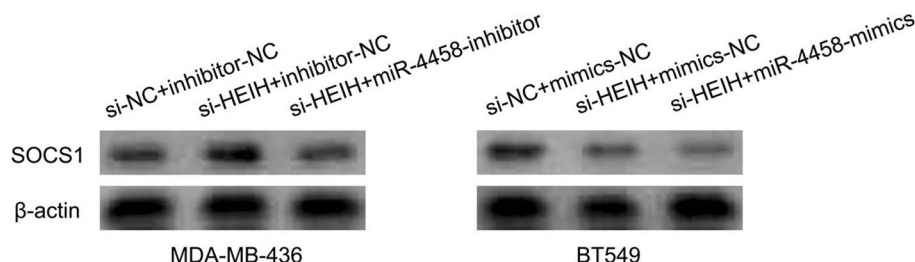
Fig. 5 HEIH expression is associated with tumor size and clinical stage in TNBC patients. **a** TNBC patients with tumor size ≥ 5 cm had higher levels of HEIH expression than patients with tumor size < 5 cm. **b** TNBC patients with advanced clinical stage had higher levels of HEIH expression than patients with early clinical stage
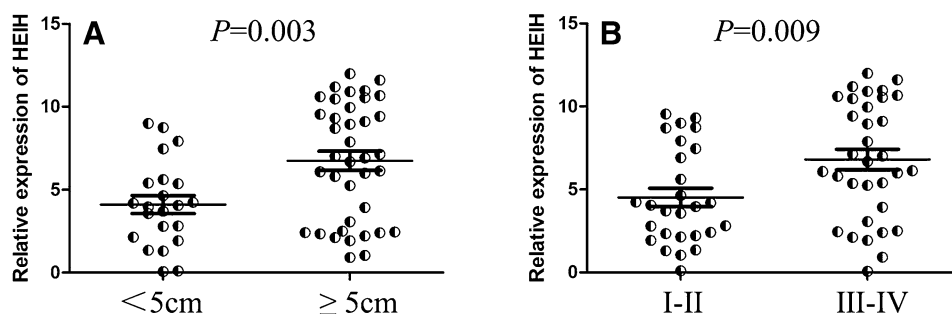
tumor recurrence, and acted as an independent prognostic factor for recurrence-free survival [18]. Moreover, Cui et al. indicated that colorectal cancer patients with high HEIH expression had short overall survival compared with patients with low HEIH expression [22]. Besides, Zhao et al. showed that HEIH overexpression predicted an unfavorable prognosis in melanoma patients [23]. In patients with neck squamous cell carcinoma, there was no significant association between HEIH expression and clinical outcomes including overall survival and relapse-free survival [24]. In breast cancer patients, we tried to analyze The Cancer Genome Atlas, and found that HEIH expression had no correlation with overall survival and disease free survival.

HEIH has been suggested to be oncogenic lncRNA to regulate tumor cell proliferation, apoptosis, cell cycle, migration, and invasion. In colorectal cancer, Chunhui et al. found that HEIH promoted cell proliferation and inhibited apoptosis through countering miR-939-mediated transcriptional repression of Bcl-xL [22]. In addition, Yang et al. showed that HEIH promoted tumor growth in vitro and in vivo, and plays a role in G0/G1 arrest through regulating p16, p21, and p27 [18]. Moreover, Ma et al. found that silence of HEIH inhibited liver cancer cell growth and metastasis via down-regulating CyclinD1, Bcl-2, MMP-2, MMP-8, Vimentin expression, and up-regulating p53, Bax, and cleaved caspase-3 [26]. In our previous study, showed miR-4458 inhibited TNBC cell proliferation and promoted cell apoptosis through targeting SOCS1 [13]. In addition, the bioinformatic analysis revealed that HEIH harbors a potential miR-4458-binding site. Thus, we explored the relationship between HEIH and miR-4458/SOCS1 axis in TNBC cells, and found that down-regulation of HEIH inhibited TNBC cell proliferation and promoted apoptosis through regulating miR-4458/SOCS1 axis. Taken together, it appears that just a small part of the biological function of HEIH was explored in our study. More studies are necessary to investigate the possible biological function and molecular mechanism of HEIH in TNBC.

In conclusion, we preliminarily revealed that HEIH expression is increased in TNBC tissues and cells, and down-regulation of HEIH inhibits TNBC cell proliferation and promotes apoptosis through regulating miR-4458/SOCS1 axis.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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