RETRACTED ARTICLE: Neferine hinders choriocarcinoma cell proliferation, migration and invasion through repression of long noncoding RNA-CHRF

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\textbf{ABSTRACT}

The comprehensive pathological peculiarities of Neferine (NEF) have been testified in disparate diseases. But, the functions of NEF in choriocarcinoma progression remain unexplored. The research endeavored to uncover the anti-tumour action of NEF in choriocarcinoma cells. NEF at diverse doses was employed to dispose JEG-3 and HTR-8 cells, and cell viability assessment adopted CCK-8 assay. After 60 \mu g/mL NEF management, BrdU-positive cells, apoptosis, migration, invasion and correlative factors were assessed. CHRF expression in choriocarcinoma tumour and choriocarcinoma cell lines was estimated via RT-qPCR. Then, the functions of overexpressed CHRF in NEF-disposed cells were determined. At last, impacts of NEF on PI3K/AKT/mTOR and ERK1/2 pathways were evaluated. Results showed that NEF restrained cell proliferation, triggered apoptosis and repressed migration and invasion in JEG-3 and HTR-8 cells. CHRF was ascended in choriocarcinoma tissues and NEF repressed CHRF expression in choriocarcinoma cell lines. Additionally, overexpressed CHRF abolished the above functions of NEF in choriocarcinoma cells proliferation, apoptosis, migration and invasion. Further, NEF impeded PI3K/AKT/mTOR and ERK1/2 pathways via repressing CHRF. The explorations testified that NEF exhibited the anti-tumour action in JEG-3 and HTR-8 cells via hindering PI3K/AKT/mTOR and ERK1/2 pathways by mediating CHRF.

\section*{Introduction}

Choriocarcinoma is a pernicious trophoblastic cancer, which is usually discovered in the placenta \cite{1}. It pertains to the advanced condition in gestational trophoblastic disease (GTD) and is also classified as a germcell arising in the testis or ovary \cite{2,3}. The clinical symptoms of choriocarcinoma incorporate increased human chorionic gonadotropin (HCG), vaginal bleeding, shortness of breath and the multiple infiltrates of sundy shapes in lungs \cite{4,5}. Placental choriocarcinoma possesses incisively sensitive to chemotherapy; therefore, chemotherapy has become the preferred management for remedying placental choriocarcinoma \cite{6}. Nevertheless, testicular choriocarcinoma is highly resistant to chemotherapy, which has the worst prognosis among all germnomas \cite{7}. Hence, exploration of a neoteric method or valid agent for treating choriocarcinoma is still necessary.

\textit{Nelumbo nucifera}, also known as lotus, is a proverbial traditional medicinal plant encompassing diversified bioactive compounds \cite{8}. The embryo of the mature seed of \textit{Nelumbo nucifera} is a traditional Chinese medicine (TCM), which has been applied for sedative, anti-pyretic and haemostatic purposes \cite{9}. Neferine (NEF) is a dominating bisbenzylisoquinoline alkaloid extracted from the green embryo of the mature seed of \textit{Nelumbo nucifera} \cite{10}. The comprehensive pathological characteristics such as anti-hypertensive, anti-diabetic, anti-arrhythmia and anti-depressant have been disclosed in dozens of diseases \cite{11–13}. In term of cancers, Zhang et al. corroborated that NEF restrained osteosarcoma cells proliferation through activating p38MAPK-regulated p21 stabilization \cite{14}. Moreover, Yoon et al. expounded that NEF potentiated the anti-tumour activity in Hep3B cells via inducing ER stress and apoptosis \cite{15}. Whereas, whether NEF exhibits the anti-tumour action in choriocarcinoma cells is still unexplored.

Long noncoding RNAs (IncRNAs) refer to a sort of RNA that are longer than 200 nucleotides and cannot encode proteins, which participate in adjusting cell cycle, cell apoptosis and cell metastasis in multiple cancers \cite{16,17}. Cardiac hypertrophy related factor (CHRF) is one of the pivotul IncRNAs, has been certified to mediate cardiac hypertrophy via immediately bound to microRNA-489 (miR-489) and mediating MyD88 expression \cite{18}. Nonetheless, whether CHRF is implicated in mediating choriocarcinoma cell growth and metastasis remains unreported. To achieve the purpose of uncovering the anti-tumour action of NEF in choriocarcinoma cells, the cell behaviors of proliferation, apoptosis, migration and invasion were evaluated. The roles of CHRF and PI3K/AKT/mTOR and ERK1/2 pathways in above-mentioned process were further delved to showcase the probable mediatory mechanisms.
Materials and methods

Cell culture and disposition

The choriocarcinoma-derived placental JEG-3 cells (ATCC® HTB-36™) and trophoblast HTR-8 cells (ATCC® CRL-3271™) were both attained from American Type Culture Collection (Rockville, MD). JEG-3 cells were cultivated in ATCC-recommended EMEM (ATCC® 30–2003™) comprising 10% FBS, and HTR-8 cells were cultivated in ATCC-recommended RPMI-1640 medium (ATCC® 30–2001™) with 5% FBS (ATCC® 30–2020™). These cells were cultivated in an incubator at 37 °C constant temperature. For cell administration, NEF bought from Sigma (St. Louis, MO, USA) was dissolved in 0.1% sterile cell culture-grade dimethylsulphoxide (DMSO), and were attenuated to diverse dosages ranging from 5–100 μg/mL. The above cells were then disposed of the above divergent concentrations of NEF for 24 h in the next correlatives experiments.

Clinical specimens

Clinical human choriocarcinoma tissues (n = 22) and the para-carcinoma chorion tissues (n = 20) were procured from the patients with choriocarcinoma aging from 29 to 45 years old (Shandong Provincial ENT Hospital, Shandong Provincial ENT Hospital Affiliated to Shandong University, Jinan, China). The samples were collected from July 2017 to September 2018. All above patients participated in this research unreceived any curative modalities prior to surgery. The informed consents were signed personally. The research was encouraged by the Medical Ethics Committee of the Shandong Provincial Western Hospital.

Cell transfection

The overexpression vector of CHRF (pc-CHRF) and pcDNA3.1 were compounded by GenePharma (Shanghai, China). Above vectors were applied for cell transfection trial through carrying out Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) in line with its specification. The above-transfected cells were gathered after 48 h and were utilized in the next trials.

Cell viability

Cell viability was tested via adopting Cell Counting Kit-8 (CCK-8, Dojindo, Gaithersburg, MD) after transfection with pc-CHRF and management with NEF. These above-managed cells were thereafter co-cultivated with 10 μL CCK-8 solution for additional 2 h at 37 °C. Subsequently, a Microplate Reader (Bio-Rad, Hercules, CA) was executed for analyzing the absorbance at 450 nm.

Bromodeoxyuridine (BrdU) staining assay

Detection of cell proliferation adopted the BrdU staining assay in the actual experiment. After transfection with pc-CHRF and management with NEF, these disposed cells were co-fostered with the BrdU solution (Roche, Alameda, CA) for 40 min. These cells were then immobilized in ethanol for 30 min, meanwhile co-cultivated with anti-BrdU antibody (ab1893, Abcam, Cambridge, UK) for an additional 1 h at 37 °C. After this, above cells were stained with haematoxylin (Sigma, St. Louis, MO) for 20 min, and the BrdU positive cells and total cells were counted via exploiting a microscope (Olympus, Tokyo, Japan).

Detection of cell apoptosis

After transfection with pc-CHRF and management with NEF, these treated cells were laundered with PBS, meanwhile re-suspended in 400 μL 1 × Binding Buffer. Subsequently, 5 μL Annexin V-FITC (Biosea, Beijing, China) was utilized and reacted with cells for 15 min under the absence of the light condition. At the meantime, 5 μL PI (Biosea, Beijing, China) was supplemented and further reacted with above cells for another 15 min. Ultimately, cell apoptosis was appraised via carrying out flow cytometry instrument (FACSArria II, BD, San Diego, CA).

Detection of cell migration and invasion

Transwell assay was implemented to test cell migration and invasion. In cell invasion trial, Matrigel (BD Biosciences, San Diego, CA, USA) was employed to cover the upper chamber of Transwell. After transfection with pc-CHRF and management with NEF, these administrated cells were re-suspended in 200 μL serum-free medium and replenished to the upper chamber of Transwell. At the same time, the below chamber was supplemented by 600 μL complete medium. After cultivation for 24 h in a CO₂ incubator at 37 °C, the cells on the top surface of the Transwell filter were cleared away via utilizing a moist cotton swab. The methyl alcohol was exploited for the fixation of migrated or invaded cells, meanwhile, these cells were then dyed with 0.5% crystal violet (Sigma, St. Louis, MO, USA) for 20 min. After this, the traversed cells were counted under inverted microscopy (Olympus, Tokyo, Japan).

Real-time quantitative PCR (RT-qPCR)

TRIzol reagent (Invitrogen, Carlsbad, CA) was adopted to elicit total RNA from cells transfected with pc-CHRF/pDNA3.1 and stimulated with NEF. For compounding the cDNA, the PrimeScript 1st Strand cDNA Synthesis kit (TaKaRa, Siga, Japan) was conducted in accordance with its specification. For PCR amplification, the Applied Biosystems™ TaqMan (ABI, Foster City, CA,USA) was carried out. β-actin served as a housekeeping gene for detecting CHRF expression. The associative data were computed through utilizing 2−DDCt method [19].

Western blot assay

After transfection with pc-CHRF and pDNA3.1, choriocarcinoma cells received NEF management. The RIPA lysis buffer (Beyotime, Shanghai, China) comprising protease inhibitor (Roche, San Francisco, CA) were utilized for the preparation
of the protein samples. Assessment of the total protein concentration adopted BCA™ Protein Assay Kit (Beyotime, Shanghai, China). These above-involved protein samples were resolved by SDS-PAGE, meanwhile were shifted to nitrocellulose membranes (Millipore, Billerica, MA, USA). Afterward, above nitrocellulose membranes were sealed with 5% BSA, and co-cultivated with the correlative primary antibodies of p53 (ab32389), CyclinD1 (ab16663), Cleaved-Caspase-3 (ab49822), Cleaved-Caspase-9 (ab2324), MMP-2 (ab92536), MMP-9 (ab76003), Vimentin (ab16700), t-AKT (ab18785), p-AKT (ab38449), t-mTOR (ab32028), p-mTOR (ab137133), t-ERK1/2 (ab17942), p-ERK1/2 (ab214362), β-actin (ab227387, Abcam, Cambridge, UK) and p-PI3K (#13857, CST, Beverly, MA) for all night at 37 °C. Additionally, the membranes were strained with the interrelated second antibody (ab205718, Abcam) for extra 1 h at surrounding temperature. For visualizing the bands, the ECL reagent (GE Healthcare, Braunschweig, Germany) was carried out. The signals were analyzed via adopting ImageJ software (Bio-Rad, Hercules, CA, USA).

Statistical analysis
The outcomes from this study were depicted as the mean ± SD. Adoption of SPSS 19.0 statistical software (IBM, Armonk, NY, USA) to compute statistical results was conducted. The data in diverse groups were computed via Student t-test and ANOVA following with Tukey post-hoc test. The significant difference consequence was ascertained when the p values were less than .05.

Results
NEF hindered cell proliferation and triggered apoptosis in choriocarcinoma cells
The disparate dosages of NEF (5, 10, 20, 40, 60, 80 and 100 μg/mL) were utilized for disposition of choriocarcinoma cells (JEG-3 and HTR-8), and cell viability was subsequently appraised. The reduced viabilities of JEG-3 and HTR-8 cells were observed after stimulation with NEF at the concentrations of 20, 40 (p < .05), 60, 80 (p < .01) and 100 μg/mL (p < .001, Figure 1(A,B)). Next, 60 μg/mL NEF was elected to dispose JEG-3 and HTR-8 cells in the follow-up experiments due to the inhibitory impacts of NEF on cell viability was the closest to 50% at this concentration. We observed that the percentage of BrdU positive cells was evidently declined by NEF administration (p < .001, Figure 1(C)). The p53 and CyclinD1 protein levels were respectively elevated (p < .001) and restrained (p < .01) by NEF disposition (Figure 1(D–F)). For cell apoptosis assessment, we discovered that NEF apparently evoked cell apoptosis (p < .01 or p < .001), concurrently elevated Cleaved-Caspase-3 and Cleaved-Caspase-9 expression (p < .001, Figure 1(G–J)). We summarized the above

Figure 1. Functions of NEF in choriocarcinoma cell proliferation and apoptosis. (A and B) After management with NEF (5, 10, 20, 40, 60, 80 and 100 μg/mL), assessment of cell viability via executing CCK-8 assay. After disposition with 60 μg/mL NEF, evaluations of (C) the percentage of BrdU positive cells and (D–F) p53 and CyclinD1 via implementing BrdU and western blot assay. Estimations of (G) cell apoptosis and (H–J) Cleaved-Caspase-3/-9 expression via exploiting flow cytometry and western blot assays. *p < .05, **p < .01, ***p < .001.
elementary findings that NEF exerted the repressive ability in cell growth of choriocarcinoma cells.

**NEF restrained choriocarcinoma cells migration and invasion**

The influences of NEF in JEG-3 and HTR-8 cells migration and invasion were estimated via executing Transwell and western blot assays. The repression of cell migration and invasion evoked by NEF administration was obviously displayed in JEG-3 and HTR-8 cells (p < .001, Figure 2(A,B)). In the meantime, the protein levels of MMP-2, MMP-9, and Vimentin were all impeded by NEF in JEG-3 and HTR-8 cells (p < .001, Figure 2(C–E)). All these above-involved data implied that NEF exerted the prohibitive impacts on choriocarcinoma cell migration and invasion.

**Repression of CHRF was triggered by NEF administration**

The twenty-two clinical human choriocarcinoma tissues and twenty para-carcinoma chorion tissues were gathered for the determination of the expression level of CHRF in these tissues. In Figure 3(A), we discovered that CHRF expression was dramatically ascended in choriocarcinoma tissues as contrasted to that in para-carcinoma chorion tissues (p < .001). After management of NEF, we observed that the relative CHRF expression was evidently impeded in both JEG-3 and HTR-8 cells (p < .001, Figure 3(B)). These observations uncovered that CHRF expression was elevated in choriocarcinoma tissues and restrained by NEF disposition in choriocarcinoma cells. Therefore, we speculated that CHRF might be a vital controller joined in mediation of the functions of NEF in choriocarcinoma cells growth, migration and invasion.

**NEF prohibited choriocarcinoma cell growth, migration and invasion via repression of CHRF**

To certify the above-mentioned speculation, pc-CHRF and pcDNA3.1 expression vectors were transfected into JEG-3 and HTR-8 cells to further probe the latent functions of CHRF. The elevation of CHRF was observed in pc-CHRF-transfected cells as relative to that in pcDNA3.1-transfected cells (p < .001, Figure 4(A)). The repression of the percentage of BrDU positive cells triggered by NEF management was obviously overturned by overexpressed CHRF (p < .01 or p < .001, Figure 4(B)). Moreover, induction of cell apoptosis triggered by NEF administration was also reversed by overexpressed CHRF (p < .001, Figure 4(C)). Otherwise, the functions of NEF in the protein levels of p53, CyclinD1, Cleaved-Caspase-3 and Cleaved-Caspase-9 were all abrogated by overexpressed CHRF (p < .001, Figure 4(D–I)). Beyond that, we also discovered that overexpressed CHRF augmented cell migration and invasion (p < .05 or p < .001) in NEF-disposed cells (Figure 5(A,B)). At the same time, MMP-2, MMP-9 and Vimentin expression levels were all ascended by overexpressed CHRF (p < .001) in NEF-disposed cells (Figure 5(C–F)). These explorations confirmed aforementioned conjecture and implied that the suppressive impacts of NEF on choriocarcinoma cell growth, migration and invasion might be adjusted by CHRF.

**NEF impeded PI3K/AKT/mTOR and ERK1/2 pathways via restraining CHRF expression**

The influences of NEF in PI3K/AKT/mTOR and ERK1/2 pathways were eventually probed to disclose the regulatory mechanisms. We observed that NEF management hindered p-PI3K, p-AKT and p-mTOR protein levels in JEG-3 and HTR-8 cells as contrasted to control group (p < .001, Figure 6(A–D)). After transfection with pc-CHRF, the repressive impact of NEF on PI3K/AKT/mTOR was evidently eliminated (p < .001,
Figure 3. Functions of NEF in CHRF expression. (A) Twenty two clinical human choriocarcinoma tissues and twenty para-carcinoma chorion tissues were gathered, determination of the expression level of CHRF in these tissues via adopting RT-qPCR assay. (B) After administration with 60 μg/mL NEF, detection of the expression of CHRF in both JEC-3 and HTR-8 cells via employing RT-qPCR assay. ***p < .001.

Figure 4. Functions of CHRF overexpression in choriocarcinoma cell proliferation and apoptosis. (A) After transfection with pcDNA3.1 and pc-CHRF, determination of CHRF expression in these transfected cells via utilizing RT-qPCR assay. After stimulation with 60 μg/mL NEF and transfection with pc-CHRF, assessments of (B) the percentage of BrdU positive cells and (C) cell apoptosis via adopting BrdU and flow cytometry assays. Evaluations of (D–F) p53/CyclinD1 and (G–I) cleaved-caspase-3/-9 in JEC-3 and HTR-8 cells via exploiting western blot assay. *p < .05, **p < .01, ***p < .001.
Figure 6(A–D)). Analogously, p-ERK1/2 was restrained by NEF disposition ($p < .05$) in JEG-3 and HTR-8 cells (Figure 6(E–H)). But, the suppressive impact of NEF on ERK1/2 pathway was also abrogated by overexpressed CHRF ($p < .05$ or $p < .001$, Figure 6(E–H)). There was no appreciable impact of NEF on t-PI3K, t-AKT, t-mTOR and t-ERK1/2 in both JEG-3 and HTR-8 cells (Figure 6(A–H)). These observations illuminated that NEF impeded PI3K/AKT/mTOR and ERK1/2 pathways through repression of CHRF in choriocarcinoma cells.

Discussion

Herein, the research delineated the anti-tumour impacts of NEF on choriocarcinoma cells. The particular results testified that NEF evidently repressed JEG-3 and HTR-8 cells proliferation, migration and invasion, meanwhile triggered apoptosis. Up-regulated CHRF was observed in choriocarcinoma tissues and down-regulated CHRF evoked by NEF was discovered in choriocarcinoma cells, which implied that CHRF was likely to
be a pivotal regulator in the anti-tumour action of NEF in choriocarcinoma cells. Further experiments revealed that overexpressed CHRF overtly overturned the functions of NEF in choriocarcinoma cell proliferation, apoptosis, migration and invasion. Beyond that, we discovered that NEF hindered PI3K/AKT/mTOR and ERK1/2 pathways through repression of CHRF in choriocarcinoma cells.

Chemotherapy is recognized as a valid method for remediating choriocarcinoma, but the drugs used can trigger great impair to the normal tissues of the human body [20]. Additionally, the large dosage of chemotherapeutics may produce more serious toxic and side effects. Recently, a substantial amount of researches attested that TCM therapy can not only prevent and alleviate the toxic and side effects of chemotherapy but also ameliorate the disease resistance and conduct to kill cancer cells in the patients with diverse cancers [21,22]. Evidence from Huang et al. disclosed that shikonin could repress choriocarcinoma JEG-3 cells proliferation and evoke apoptosis via mediating ERK and JNK pathways [23]. NEF as a momentous TCM has been extensive utilized for cure of diverse cancers. Research from Poormima et al. testified that NEF could repress lung cancer cell growth via activation of MAPK pathway and cell cycle arrest [24]. Otherwise, the anti-tumour actions of NEF in hepatocellular carcinoma cell invasion and oxaliplatin sensitivity were testified in the research from Deng et al. [25]. But, whether NEF exerts the anti-tumour activity in choriocarcinoma cells is still vague. The study disclosed that NEF prohibited JEG-3 and HTR-8 cells proliferation, migration, invasion and triggered apoptosis, apparent hinting the anti-tumour impact of NEF on choriocarcinoma cells.

Current researches concentrated on lncRNAs almost cover all physiological and pathological processes, incorporating the occurrence and development of cancers [26,27]. Emerging evidence uncovered that lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) could accelerate choriocarcinoma JEG-3 and JAR cell proliferation and trigger cell cycle arrest [28]. As a neoteric lncRNA, CHRF has been testified to participate in aggrandizing colorectal cancer cell metastasis through regulating Twist and epithelial–mesenchymal transition (EMT) pathways [29].

Further, several attractive researches disclosed that the active ingredients extracted from traditional Chinese medicine exert the anti-tumour functions via adjusting lncRNA expression. For example, Ma et al. corroborated that Dioscin restrained gastric tumour growth through the mediation of lncRNA HOTAIR expression [30]. Moreover, Zhu et al. depicted that the extraction from the root of Dandelion root could dampen gastric cancer cells proliferation and migration via modulating lncRNA-CCAT1 [31]. These researches can generate the neoteric guess that whether NEF presented the anti-tumour activity in choriocarcinoma cells via mediating CHRF. In our study, we discovered that CHRF expression was elevated in choriocarcinoma tissues. Moreover, NEF management obviously declined CHRF expression in both JEG-3 and HTR-8 cells. More interestingly, overexpressed CHRF augmented cell proliferation, migration, invasion and restrained apoptosis in NEF-disposed cells. These data hinted that CHRF eliminated the anti-tumour action of NEF in choriocarcinoma cells.

PI3K/AKT/mTOR is a representative survival pathway, which is constitutively activated in sundry types of cancers [32,33]. Crucial research disclosed that NEF could strengthen cisplatin-triggered autophagic cancer cell death via dampening PI3K/AKT/mTOR pathway in lung adenocarcinoma A549 cells [34]. ERK1/2 pathway is a member of MAPK family, which is intimately linked to cell proliferation, apoptosis and metastasis in diverse cancers [35,36]. In context of choriocarcinoma, Lim et al. discovered that chrysophanol could trigger choriocarcinoma cell apoptosis via mediation of AKT and ERK1/2 pathways [37]. Likewise, Lim et al. also verified that apigenin could abate the progression and metastasis of choriocarcinoma cells via adjusting ERK1/2 pathway [38]. In this study, we observed that NEF evidently hindered PI3K/AKT/mTOR and ERK1/2 pathways in JEG-3 and HTR-8. This phenomenon was predominantly overturned by overexpressed CHRF. The explorations insinuated that PI3K/AKT/mTOR and ERK1/2 pathways might join in mediating the function mechanism of NEF-affected choriocarcinoma progression.

The innovativeness of this research has certified the involvement of lncRNA-CHRF in the anti-tumour functions of NEF in choriocarcinoma cells. More interestingly, PI3K/AKT/mTOR and ERK1/2 pathways were blocked by NEF via adjusting CHRF expression. The actual research was also first to corroborate the functional importance of CHRF in choriocarcinoma, which implied that CHRF functions as a positive adjustor to expedite the progression of choriocarcinoma. Nevertheless, more efforts are still necessary to develop CHRF as a neoteric therapy to remedy choriocarcinoma. These new explorations might offer a new idea for the remedy of choriocarcinoma in clinical.

Taken together, the study certified the anti-tumour activity of NEF on choriocarcinoma cells via impeding PI3K/AKT/mTOR and ERK1/2 pathways through repression of CHRF. These discoveries hinted that NEF might be a protective agent for remediying choriocarcinoma. The important relevance among NEF, CHRF and PI3K/AKT/mTOR and ERK1/2 pathways in choriocarcinoma cells were emphasized for the first time. Nevertheless, further researches are still necessary for probing the mechanisms of choriocarcinoma to develop neoteric treatments.

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Disclosure statement

Authors declare that there are no conflicts of interests.

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