

CHI3L1 Inhibits Expression of NIS by Activating MEK/ERK1/2 Signal Pathway in Thyroid Cancer

Yunjie Li

The First Affiliated Hospital of Chongqing Medical University

Fengqiong Hu

The First Affiliated Hospital of Chongqing Medical University

Yifan Wu

The First Affiliated Hospital of Chongqing Medical University

Jie Deng

The First Affiliated Hospital of Chongqing Medical University

Xin Huang

The First Affiliated Hospital of Chongqing Medical University

Chunyan Zhou

The First Affiliated Hospital of Chongqing Medical University

Mengxue Wu

the first hospital affiliated to AMU(Southwest Hospital, Army Medical University)

dong Duan (✉ duandong26@163.com)

The First Affiliated Hospital of Chongqing Medical University

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Abstract

Purpose To study the differentially expressed protein between thyroid cancer and radioactive iodine refractory differentiated thyroid cancer (RR-DTC), which presents highly aggressive and unfavorable prognosis. Meanwhile, to search for a potential radiotherapeutic target for patients suffer from RR-DTC.

Methods Totally 6 metastatic lymph nodes of RR-DTC and DTC were collected during lymph node dissection for proteomics studies. Immunohistochemical staining was performed to verify the expression of Chitinase-3-like 1 (CHI3L1) in RR-DTC and DTC tissues. Western blotting was used to detect the expression of sodium-iodine symporter (NIS), MEK, and ERK1/2 in CHI3L1 over-expression stable transfectants, control stable transfectants, and PTC-K1 cells.

Results CHI3L1 was demonstrated to be significantly up-regulated in RR-DTC by immunohistochemical staining. Besides, CHI3L1 over expression would inhibit expression of sodium-iodine symporter, a key protein for iodine accumulation, by activating MEK/ERK1/2 signal pathway.

Conclusion CHI3L1 might be a potential molecular target for RR-DTC due to its over expression in RR-DTC and membrane location.

Introduction

Tumor cell dedifferentiation, reversing cells from well differentiated states to less differentiated states, is considered to be a specific characteristic of highly aggressive. Generally, poor differentiation tumor cells present faster proliferation and more invasive[1]. Previous studies demonstrated that lots of proteins (such as COL2A1, CD44, and ALDH) and intricate signal pathways (including p38 MAPK, ERK1/2, PI3K signal pathways) are involved in dedifferentiation process[2-6]. In this case, dedifferentiated tumor cells would develop therapy escape and become resistant to traditional therapies, including radiotherapy and chemotherapy[7]. Studies on breast cancer have shown that dedifferentiated breast cancer cells, which is also known as breast cancer stem cells (BCSCs), were more invasive, less sensitive to chemotherapy, and more likely to recur comparing to well differentiated breast cancer cells[8]. Another case of tumor cell dedifferentiation is radioactive 131-iodine refractory differentiated thyroid cancer (RR-DTC), which has become a great challenge for clinical treatment since such cells have lost ability of iodine accumulation.

The mobility of thyroid cancer is increasing at the rate of 3.6% per year in recent decades[9]. Thyroid cancer includes four pathological types: papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), medullary thyroid cancer (MTC), and anaplastic thyroid cancer (ATC). PTC and FTC are also called differentiated thyroid cancer (DTC), which has the ability of radioactive 131-iodine uptake. Most patients with DTC enjoy favorable prognosis after standard treatment, consisting of totally thyroidectomy to remove primary focus, radioactive 131-iodine treatment to clear residue and oral thyroid stimulating hormone for hormone replacement therapy. Radioactive 131-iodine treatment plays a crucial role in the course of treatment, which can not only clear residue but also detect systemic metastasis[10]. However, there are about 15%-20% of patients with thyroid cancer suffer poor prognosis because their lesions or

metastasis lymph nodes have lost the ability of radioactive 131-iodine up-take, which is known as previous mentioned RR-DTC[11].

Cases meeting any one of criteria following are defined as RR-DTC: 1. metastatic lesions do not take radioactive 131-iodine after the first time of thyroidectomy; 2. metastatic lesions lose the ability of radioactive 131-iodine uptake gradually during treatment course; 3. some of the metastatic lesions with the ability of radioactive 131-iodine uptake while others not; 4. metastatic lesions take radioactive 131-iodine but patients' condition keep progressing. Because of disability of radioactive 131-iodine uptake, patients with RR-DTC usually have poor prognosis. It is reported that once being diagnosed with RR-DTC, median survival of these patients ranges from only 3 to 6 years[12]. Clinically, oral targeted drugs (such as sorafenib and lenvatinib) and radioactive 125-iodine seeds implantation are taking as palliative therapy for RR-DTC patients in progression. Nevertheless, lenvatinib and sorafenib help extent progression free survival (PFS) of RR-DTC patients but make no sense of improving overall survival (OS) of them. In addition, most patients have to interrupt targeted therapy because of intolerant side effects[13, 14]. Therefore, further studies are required to clarify pathogenesis of RR-DTC, which might provide new therapies for these patients.

Previous studies on RR-DTC suggest that occurrence of RR-DTC is related to activation of MAPK signal pathway and PI3K signal pathway, which facilitate tumor growth by changing cellular microenvironment, promoting angiogenesis, stimulating cell proliferation, and so on[15-17]. What is more, as is known to all, poor differentiation of RR-DTC cells leads to disability of radioactive 131-iodine uptake, which is major barrier for effective treatment. KEGG database demonstrates that activation of MAPK/ERK signal pathway regulates cell differentiation in kinds of diseases, such as rhabdomyosarcoma and breast cancer. Therefore, it reminds us that activation of MAPK/ERK signal pathway may result in poor cell differentiation in thyroid cancer.

Chitinase-3-like 1 (CHI3L1, Accession: P36222), which could regulate tumor growth by activating MAPK/ERK signal pathway, is reported to be over expressed in some malignant tumors, including prostate cancer, pancreatic cancer, and breast cancer[18, 19]. Luo D. et. found that CHI3L1 was highly expressed in DTC comparing to adjacent tissues[20]. Based on previous studies, our study assumes that CHI3L1 might express higher in RR-DTC than in DTC, which leads to poor differentiation of RR-DTC by activating MAPK/ERK signal pathway.

Sodium-iodide symporter (NIS) is a key protein for accumulating iodine in thyroid cells. In well differentiated thyroid cancer cells, expression of NIS is significantly higher than that in RR-DTC[21, 22]. Seung Hyun Son. et proposed that transfecting NIS gene to thyroid cancer cells could significantly increase radioactive 131-iodine uptake[23]. General speaking, expression level of NIS is considered to be positive related with cell differentiation in thyroid cancer.

We hypothesized that over expression of CHI3L1 is critical to poor cell differentiation, which would inhibit expression of NIS and decrease radioactive 131-iodine uptake in RR-DTC. In our study, DTC tissues were taken as control and the study found that CHI3L1 was significantly up-regulated in RR-DTC than in DTC.

Furthermore, the study found that expression of NIS was significantly inhibited by CHI3L1, which may be potential therapeutic target for RR-DTC.

Materials And Methods

1.sample collection

Following the inclusion criteria, our study collected 3 metastatic lymph nodes from 3 patients with RR-DTC and 3 metastatic lymph nodes from 3 patients with DTC, which had been confirmed by histopathologic examination and the whole-body scan of radioactive ¹³¹-iodine. All of the lymph nodes were rinsed by PBS for 3 times, after which each lymph node was incised to 2 parts, one part was formalin-fixed paraffin-embedded for immunohistochemical staining, another part was stored at -80°C for proteomics research. In addition, to enlarge the sample size, our study included 3 metastatic lymph nodes from patients with DTC and 3 metastatic lymph nodes with RR-DTC besides the patients mentioned previously. The study was approved by the Ethics Committee of Chongqing Medical University (CSTC2019jcyj-msxmX0327) and all patients involved provided the informed consents.

2.Proteomics technology and bioinformation analysis

According to the manufacturer, all samples were performed proteomics researches by iTRAQ technology coupled with Mass Spectrometry (MS). The raw data of MS were loaded input PD software (Proteome Discoverer 1.4, Thermo), which would select mass spectrum following preset criteria. The selected mass spectra were searched by Mascot (version 2.3.0). After that, PD software would perform quantitative analysis based on searching results and selected mass spectrum. According to the outcome of quantitative analysis, annotation of all identified protein was performed using GO database and KEGG pathway database.

3.Immunohistochemical staining

Paraffin embedded formalin fixed pathological tissue sections for immunohistochemical assay were kindly provided by pathology department of the first affiliated hospital of Chongqing Medical University. The expression of CHI3L1 was examined by immunohistochemical kits (SP9000, ZSGB-Bio, China) following the standard protocol. Antibody CHI3L1(ab77528, Abcam, US) was diluted to optimal concentration (1:1000). Observing the section under microscope after being processed with DAB solution, counterstained by hematoxylin, dehydrated and transparented using ethanol and xylene. Image J software was used to assess immunostaining intensity.

4. Cell culture and lentiviral transfection

PTC-K1 cells, which were purchased from Otwo Biotech, were cultured in complete DMEM medium (supplemented with 89% DMEM+ 10% FBS+1% penicillin-streptomycin, V/V/V), and maintained in 5% carbon dioxide, 37.0 °C and 95% humidity incubator. CHI3L1 overexpression lentiviral vectors and control vectors, obtained from SANGON Biotech, were used to infect PTC-K1 cells following the manufacturer's protocol. MOI was set at 30. Cells were cultured in 2ug/ml puromycin for two weeks until CHI3L1 overexpression stable transfectant and control stable transfectant were conducted.

5. Western blotting

Total protein of each sample was extracted by RIPA buffer (P0013B, Beyotime, China) containing protease inhibitor (100:1, V/V), which was followed by measuring concentration of total protein by BCA protein Assay kits. Equal amount of 40ug protein sample per lane was loaded on 10% SDS-PAGE gel (P0012A, Beyotime, China). The antibodies of CHI3L1, NIS (bs-0048R, Bioss Antibodies, China), MEK1 (ab32091, ABCAM, US), ERK1/2 (ab184699, ABCAM, US) and GAPDH (AB-P-R001, Good HERE, China) were used in dilution of 1:1000, 1:500, 1:1000, 1:10000, and 1:500, respectively. Band density was measured by Image J software.

6. Statistical analysis

Statistical analysis were performed by Student's t-test and one-way analysis of variance (one-way ANOVA) with the use of SPSS 22.0 software and GraphPad Prism 7.00 software. All data were presented as mean±SD. For proteomics research, fold change >1.2 was regarded as differential proteins between two group. P value were judged significant if they were less than 0.05.

Results

1. Differential proteins between RR-DTC tissue and DTC tissue

All the patients involved for proteomics research were eligible for inclusive criteria based on radioactive ¹³¹-iodine whole body imaging and pathological sections. CT imaging demonstrated the metastatic lymph nodes were located in cervical while no ¹³¹-iodine up-take was found in thyroid retention imaging. After cervical lymph nodes dissection, the metastatic lymph nodes were identified to be papillary thyroid cancer (Figure 1). For proteomics study, 665 differential proteins were found between two groups in total. Compared with DTC group, 327 proteins, including CHI3L1, extracellular matrix protein (ECM), 14-3-3 epsilon, 14-3-3 sigma, were up-regulated in RR-DTC while 338 proteins were down-regulated in RR-DTC, including myosin-9, cell division control protein 42 (CDC42), and Ras-related C3 botulinum toxin substrate

(RAC2). Notably, some cancer stem cell marks, such as ALDH and CD44, were found to be up-regulated in RR-DTC comparing to DTC, which indicated poor dedifferentiation of RR-DTC.

2. CHI3L1 was up-regulated in RR-DTC tissues than in DTC tissues.

The Expression of CHI3L1 in RR-DTC tissue is about twice higher than that in DTC tissue according to the results of proteomics research, which was detected by iTRAQ technology. To enlarge sample size and verify the reliability of proteomics research results, immunohistochemical staining for CHI3L1 was performed with 12 paraffin embedded formalin fixed pathological tissue sections collecting from different patients (6 from RR-DTC patients, 6 from DTC patients as control). Immunohistochemical staining demonstrated that CHI3L1 was located in both cytoplasm and cytomembrane. The staining intensity was significantly stronger in RR-DTC tissues than in DTC tissues (Figure 2), which is in accord with proteomics research results.

3. Over expression of CHI3L1 would decrease expression of NIS in thyroid cancer cells.

To explore whether CHI3L1 inhibits expression of NIS, CHI3L1 over-expressed stable transfectant, control stable transfectant, and PTC-K1 cells were cultured for western blotting. The results demonstrated that expression of NIS was significantly decreased in CHI3L1 over-expressed stable transfectant than in control stable transfectant and PTC-K1 cells. There was no significant difference in expression of NIS between control stable transfectant and PTC-K1 cells (Figure 3). It demonstrated that high expression level of CHI3L1 would significantly inhibit expression of NIS ($P < 0.05$).

4. CHI3L1 could activate MEK/ERK1/2 signal pathway in thyroid cancer cells.

Western blotting was performed to detect whether MEK/ERK1/2 signal pathway was activated by CHI3L1 in thyroid cancer cells. As shown in Fig.3, MEK1 and ERK1/2 in CHI3L1 over-expressed stable transfectant were significantly higher than that in control stable transfectant and PTC-K1 cells ($P < 0.05$). No significant difference of MEK1 and ERK1/2 was found between control stable transfectant and PTC-K1 cells, which demonstrates that CHI3L1 could activate MEK/ERK1/2 signal pathway.

Discussion

Proper radiotherapy would extend overall survive rate and improves prognosis in DTC patients. However, occurrence of RR-DTC, which results from cell dedifferentiation, brings great challenges to clinical

physicians because such patients have lost ability of iodine accumulation and become resistant to existing radiotherapy. It has been proved that RR-DTC would significantly decrease overall survival of patients even though they received timely systemic treatments except radioactive ¹³¹-iodine therapy, which presents necessity of radiotherapy in standard treatment course[24]. Nevertheless, there are some studies demonstrate that unnecessary repeated radioactive ¹³¹-iodine therapy would lead to severe complications, including early complication (such as acute sialoadenitis, nausea, and stomatitis or ulcers) and late complication (such as pulmonary fibrosis, neutropenia, and second primary malignancies)[25]. According to the 2015 American Thyroid Carcinoma guidelines, radioactive ¹³¹-iodine therapy should be interrupted once patients were diagnosed with RR-DTC[21]. Given the circumstance, we performed proteomics studies hoping to find new molecular targeting for radiotherapy strategy to replace radioactive ¹³¹-iodine therapy.

There are many genomics studies on RR-DTC, which have revealed some related genes involving in occurrence of RR-DTC, for instance TERT, BRAF^{V600E}, and paired box gene-8(PAX-8)[22, 26, 27]. However, studies on genetic level could not illustrate pathogenesis of RR-DTC completely since genes could function after being translated to proteins. The process including posttranslational modification (such as phosphorylation and acetylation) would vary gene functions to some extent. As is known to all, protein is the direct performer of vital movement. Studies on proteomic level would explain mechanism of functional change in RR-DTC better. Notably, in our study, DTC tissues were collected as control for proteomics study while most studies on tumors collect tumor adjacent normal tissue as control. Both control group and experimental group are thyroid cancer tissues at different differentiated levels. Therefore, identified differential proteins in our study could reflect the influence of protein expression level on cell function. In addition, ALDH and CD44, markers for cell dedifferentiation, were found to be over expressed in RR-DTC, which is accord with the broad recognized idea about poor differentiation in RR-DTC.

By iTRAQ technology and immunohistochemical staining, CHI3L1 was found to be significantly up-regulated in RR-DTC comparing to DTC. Previous studies on CHI3L1 (also known as YKL-40) manifest that CHI3L1 is related to many diseases, involving immune response, amyotrophic lateral sclerosis, and axonal damage[18, 28, 29]. On top of that, elevated CHI3L1 is found in malignant tumors (such as pancreatic cancer, osteosarcoma, lung cancer, cervical cancer) and positive related with tumor staging and poor survival[29, 30]. Our study found that after over expressing CHI3L1 in DTC, expression of NIS would be inhibited, which is closely related to iodine accumulation and indicates grade of thyroid cancer cell differentiation. Meanwhile, MEK/ERK1/2 signal pathway, a signal pathway regulating cell differentiation, was activated after CHI3L1 over expression. Hence, we believe that CHI3L1 exerts important impacts on thyroid cancer cell dedifferentiation via MEK/ERK1/2 signal pathway.

Predicted protein structure of CHI3L1 by SWISS-MODEL shows that it owns more than one transmembrane domain. The result of immunohistochemical staining reveals that CHI3L1 is located in cytoplasm and membrane, which is in accord with the predicted structure. A study on osteosarcoma manifests that a specific ligand interacting with CHI3L1 could inhibit cell migration and invasion, among

which the therapeutic effect is related with CHI3L1 expression positively. It indicates that CHI3L1 might be a potential therapeutic targeting for osteosarcoma[31]. Since CHI3L1 locates on membrane, radionuclide labeled ligand could bind with it easily. Given the results of previous studies and over expression of CHI3L1 in RR-DTC, we suppose that specific ligand labeled by radionuclide (like ¹⁷⁷-Lutecium) of CHI3L1 might perform radiotherapy among RR-DTC patients, whose primary focus and metastasis have lost the ability of iodine accumulation. Based on the results of our study, a novel agent might be developed and a feasible therapeutic strategy could be applied to such patients to ameliorate their anxiety and improve prognosis.

Our study revealed that up-regulated CHI3L1 would inhibit the expression of NIS, which was widely accepted to be essential for iodine accumulation, by activating MEK/ERK1/2 signal pathway in vitro. Based on its high expression in RR-DTC and appropriate location, our further study would focus on detecting CHI3L1 to be a potential molecular therapeutic targeting for RR-DTC.

Declarations

Funding

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Code availability

Not applicable

Authors' contributions

Dong Duan and Fengqiong Hu made substantial contributions to the conception and design of the work; Yunjie Li, Yifan Wu, Jie Deng and Xin Huang carried out the experiment, Chunyan Zhou and Mengxue wu analyzed the data, Yunjie Li drafted the work and Dong Duan revised it. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Our study was approved by the ethics committee of Chongqing Medical University and all patients involved provide informed consents.

Consent for publication

The authors declare that consent for publication of the individual images has been obtained from the patients.

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Figures

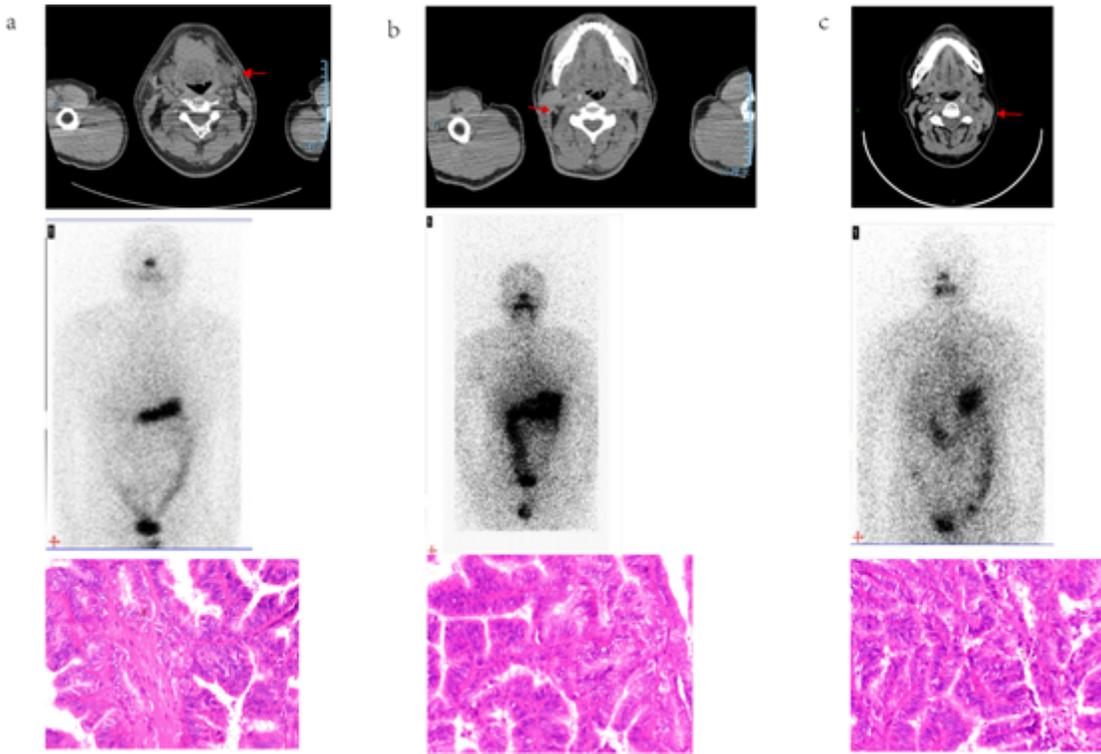


Figure 1

Clinical imaging and pathological sections of three patients with RR-DTC. Metastatic lymph nodes were found on the left/right side of the neck (red arrows) by CT imaging (the first line). There was no abnormal radioactive ¹³¹-iodine uptake in thyroid retention imaging (the second line). Papillary thyroid cancer was verified by pathological sections (the third line).

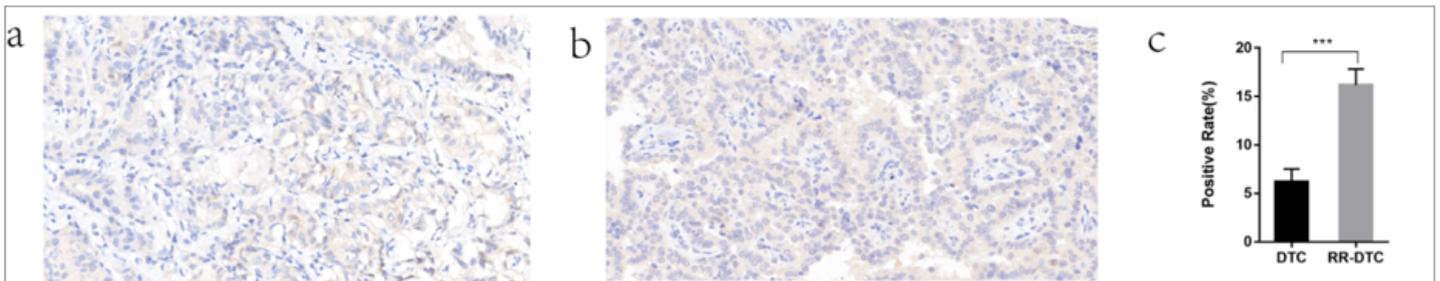


Figure 2

Immunohistochemical staining of CHI3L in DTC patients (a) and RR-DTC patients (b). The positive rate of RR-DTC was significantly stronger than DTC (c). ***P<0.001.

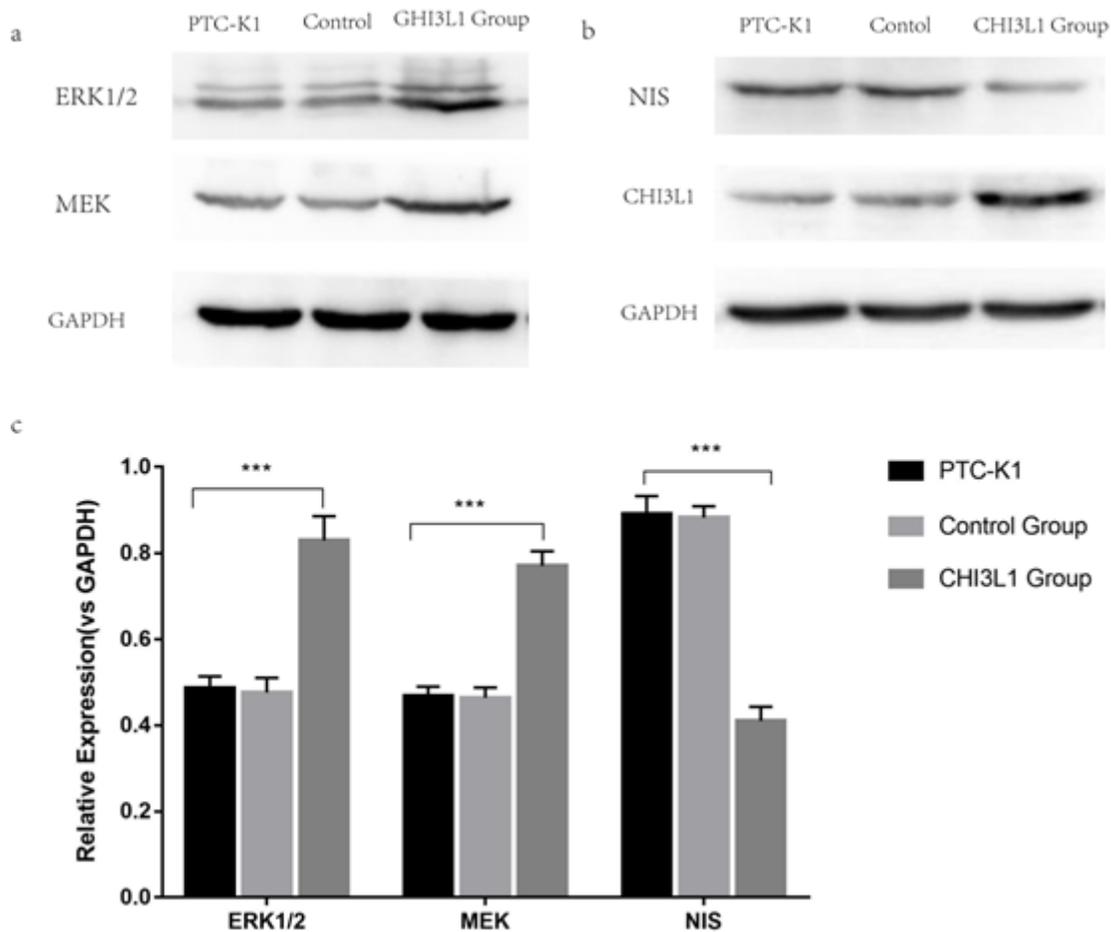


Figure 3

Western blotting of three groups. (a). Expression of ERK1/2 and MEK in CHI3L1 over expression group is significantly up-regulated than that in PTC-K1 and control group, which indicated activation of MEK/ERK1/2 signal pathway by CHI3L1 over expression. (b). Expression of NIS is significantly lower in CHI3L1 over expression group than in PTC-K1 and control group, which demonstrated that over expression of NIS would inhibit the expression of NIS and decrease the radioactive ¹³¹-iodine uptake in RR-DTC. (c). Statistical analysis of three groups. The expression of ERK1/2, MEK, and NIS in CHI3L1 over expression group was significantly different comparing to PTC-K1 and control group. *** P<0.001.