**DIXDC1 promotes tumor proliferation and cell adhesion mediated drug resistance (CAM-DR) via enhancing p-Akt in Non-Hodgkin’s lymphomas**

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DIX domain containing 1 (DIXDC1), is a human homolog of Ccd1, a DIX domain containing protein in zebrafish. The present study was undertaken to determine the expression and biologic function of DIXDC1 in Non-Hodgkin’s lymphoma (NHL). Clinically, we detected that the expression of DIXDC1 was significantly lower in the indolent lymphomas compared with the progressive lymphomas by immunohistochemistry analysis. Functionally, we found that DIXDC1 could promote cell proliferation via modulating cell cycle progression and PI3K/AKT signaling pathway in NHLs. Moreover, we confirmed that DIXDC1 was involved in the process of lymphoma cell adhesion mediated drug resistance (CAM-DR). Adhesion to fibronectin (FN) or HS-5 up-regulated DIXDC1 expression, and up-regulation of DIXDC1 resulted in an increased expression of p-AKT, which promoted CAM-DR. Our finding supports the role of DIXDC1 in cell proliferation, cell cycle and CAM-DR in NHLs. We propose that inhibition of DIXDC1 expression may be a novel therapeutic approach for NHLs patients, and it may be a target for drug resistance.

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**1. Introduction**

Non-Hodgkin’s lymphoma (NHL) constitute heterogeneous group of malignant lymphoproliferative disorders displaying a diverse range of biological phenotypes, clinical behaviours and prognoses [1]. The incidence of NHL is rising steadily over recent years and remains associated with significant mortality [2,3]. Despite intensive effort in developing new therapies, there has been little progress in improving survival for patients, and the emergence of clinical drug resistance remains a barrier to successful treatment of NHL [4–6]. Therefore, it is necessary to explore mechanisms of drug resistance in NHL.

Many investigators have shown that lymphoma tumor microenvironment provides sanctuary for lymphoma cells through interaction occurs between the lymphoma cell and its microenvironment (lymph node stroma) [7,8]. Previous studies have shown that adhesion to fibronectin (FN) or bone marrow stromal cells is a critical reason for mediating tumor resistance to cytotoxic therapy (cell adhesion mediated drug resistance (CAM-DR)) [9]. CAM-DR has been described mainly for hematopoietic malignancies, especially in NHL [10,11] and multiple myeloma (MM) [12], as well as other types of cancer [13,14]. However, how the lymphoma microenvironment influences lymphoma cell survival and response to therapy, as well as the molecular mechanisms involved, remains unclear.

DIX domain containing 1 (DIXDC1), is a human homolog of Ccd1 and a positive regulator in the Wnt signaling pathway [15–17]. The DIXDC1 is involved in a variety of processes, such as cell proliferation, cell cycle, protein folding, transcription, protein transport, signal transduction. In the field of cancer research, up-regulated expression of DIXDC1 was first found in colorectal cancer and over-expression of DIXDC1 might target p21 and cyclin D1 to promote colon cancer cell proliferation and tumorigenesis [18]. Moreover, DIXDC1 was reported to increase the invasion and migration ability of non-small-cell lung cancer [19]. Recently study revealed that DIXDC1 could promote gastric cancer invasion and metastasis through the activation of the Wnt pathway [20]. These observations all revealed that DIXDC1 might function as an oncogene promoting tumor progression. However, the functional role of DIXDC in non-
Hodgkin’s lymphoma (NHL) has never been elucidated. Considering the pivotal role DIXDC1 played in tumorigenicity control and the truth that DIXDC1 can regulate Akt activity, it would be interesting to verify whether DIXDC1 plays a key role in CAM-DR in NHL.

In this study, we aimed to investigate the expression and function of DIXDC1 in NHL and CAM-DR phenotype. Our study demonstrated for the first time that DIXDC1 is associated with clinical and pathologic factors in NHL. Furthermore, we also demonstrated that DIXDC1 plays a critical role in CAM-DR via enhancing p-Akt in NHL. Taken together, our study may provide a novel perspective for a better understanding of the role for DIXDC1 in NHL and CAM-DR.

2. Materials and methods

2.1. Patients and specimens

A total of 46 indolent lymphoma (including 18 follicular lymphoma (FL), 28 extranodal-associated lymphoid tissue (MALT)), 66 progressive lymphoma (including 39 diffuse large B-cell lymphomas (DLBCL), 19 Burkitt lymphomas) and 20 reactive lymphoid (RL) tissues samples were obtained from the Department of Pathology, Affiliated Cancer Hospital of Nantong University (Nantong, China) from 1993 to 2005. The histological types of NHL cases were classified according to the World Health Organization (WHO) guidelines. The collection of NHL tissues was approved by the research ethics committee of the institute, and written informed consent was obtained from every patient. Tissues were fixed in 10% buffered formalin and embedded in paraffin for immunohistochemical study. Then, 2 reactive lymphoid and 6 pathologically confirmed DLBCLs fresh-frozen tissues from Affiliated Cancer Hospital of Nantong University were used for Western blot analysis.

2.2. Immunohistochemical staining

Immunoprecipitation was performed as described previously [21]. In short, slides were dewaxed by three 15 min and washed with xylene. Rehydration of tissues was performed by 5 min washes in 100%, 95%, and 80% ethanol and distilled water. Antigen retrieval was performed by heating the samples at 120 °C for 3 min in 10 mmol/L sodium citrate (pH 6.0). The tissue sections were incubated with anti-DIXDC1 (diluted 1:100; Santa Cruz Biotechnology) or Ki-67 (diluted 1:100; Santa Cruz Biotechnology) or DIXDC1 (diluted 1:10; Santa Cruz Biotechnology) for 4 h at room temperature. Antigen antibody reactions were visualized with DAB (Dako, Glostrup, Denmark). Tissue sections were then counterstained with hematoxylin, dehydrated and mounted.

2.3. Cell culture and preparation of transient transfection and siRNA

Burkitt lymphoma cell-line Daudi and diffuse large B cell lymphoma (DLBCL) cell lines OCI-Ly8 obtained from Fudan University (Shanghai, China). Bone marrow stromal cell line HS-5 obtained from Cell Library, China Academy of Science. The human lymphoma cell lines were grown in suspension in RPMI 1640 (Sigma-Aldrich, Rehovot) and the HS-5 was grown in DMEM (Sigma–Aldrich, Rehovot), supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) at 37 °C and 5% CO2.

Full-length DIXDC1 cDNA were amplified using PCR and sub cloned into pCMV-HA and pcDNA3.1 constructs [22]. The following primers were used: DIXDC1 forward, 5′-GCT GAT CCT CAT TCC AGT TTC CA-3′, DIXDC1 reverse, 5′-AAT GCC ACC AGG CGA CAA TAC TA-3′. The control-siRNA and DIXDC1-siRNA were obtained from Qiagen (cat. no. 1027020). The sequences of siRNA duplex targeting DIXDC1 are: sense, 5′-AGC UGG AGC AGG GAU dTdT-3′; antisense, 5′-AUC CUC CUC CCA AGG CAU dCdC-3′. Transfection was performed using lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions and transfected cells were used for subsequent experiments 48 h after transfection.

2.4. Cell co-culture

At first, dishes were coated with 40 mg/ml human FN (Sigma-Aldrich) or HS-5 cells at 37 °C. Then, lymphoma cells (102 cells/ml) were allowed to adhere to preincubated FN or HS-5 for 6 h and non-adherent cells were removed by five washes using washing buffer (DMEM + 0.1% BSA). Attached cells were incubated for 24 h at 37 °C. Finally, attached cells were carefully removed by washing buffer (PBS) for next experiments, with the HS-5 or FN monolayer kept intact.

2.5. Western blot analysis and antibodies

Western blot analysis was performed as described previously [23]. The following monoclonal antibodies were purchased from Santa Cruz Biotechnology: anti-DIXDC1 (1:1000); anti-p21 (1:500); anti-Cyclin D1 (1:500); anti-Akt (1:1000), anti-Gsk-3β (1:1000), anti-p-Akt (1:500) and anti-Gsk-3β (1:500); anti-β-actin (1:1000 Sigma); anti-myc (1:1000 Sigma); Protein was run on a 10% PAGE and transferred to polyvinylidine difluoride filter (PVDF) membranes. The membranes were blocked with PBS containing 5% skim milk and 0.1% Tween-20 and then incubated with primary antibody overnight at 4 °C. After washing with PBS containing 0.1% Tween-20 three times, each for 5 min, filters were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Values were responsible for at least three independent experiments.

2.6. Cell viability assay

The cells were seeded on a 96-well plate (Corning Inc., Corn ing, NY, USA) with a density of 1×104/well in volumes of 100 μL and grew 12 h with or without the addition of doxorubicin (Sigma–Aldrich, St. Louis, MO, USA). Then, cell proliferation was measured by Cell Counting Kit (CCK)-8 (Dojin, Kumamoto, Japan). The absorbance was read using a fluorometer (CytoFluor; Applied Biosystems, Foster City, CA, USA) at 450 nm with a reference wavelength of 630 nm.

2.7. Apoptosis measurements

Drug-induced apoptosis following exposure to doxorubicin (Sigma) was detected in NHL cells. Then, apoptotic cells were detected using Annexin-V–FLUOS Staining Kit (Roche) according to the manufacturer’s protocol. Cells were washed three times and resuspended in 100L of Annexin-V–FLUOS labeling solution at a concentration of 1×106 cells/ml incubate in the dark for 15 min. Flow cytometry (BD FACSAriaII) was performed to analyze the labeled cells.

2.8. Soft agar colony assays

In short, cells were suspended at 1×103 cells in 0.5 mL of 0.35% agar solution (Sigma–Aldrich, St. Louis, MO, USA) containing RPMI 1640 cell culture medium and layered on top of a 0.8% agar layer in 24-well plates. Plates were then maintained for 14 days at 37 °C with 5% CO2. Cell colonies were stained with 0.5% crystal violet and visualized by microscopy.
3.2. DIXDC1 knockdown inhibited tumor proliferation

Since DIXDC1 was correlated with Ki-67 expression (Fig1), a proliferation marker, we speculated that DIXDC1 was associated with cell proliferation. To confirm our assumption, OCI-Ly8 and Daudi cells were transfected with DIXDC1-siRNA or control-siRNA. The interference efficiency was confirmed by western blot analyses (Fig. 2A). Subsequently, CCK-8 assays and soft agar colony
Fig. 2. DIXDC1 promoted the proliferation of NHL cells. (A) OCI-Ly8 and Daudi cells were transfected with DIXDC1-siRNA or control-siRNA. The interference efficiencies were confirmed by western blot. (B) OCI-Ly8 and Daudi cells were transfected with DIXDC1-siRNA or control-siRNA. CCK-8 assays were performed using myc-DIXDC1 or myc-control to evaluate the cell growth at the indicated time. The data were means ± SEM of three independent experiments (*,#P < 0.05). (C) Soft agar colony formation assays were performed to evaluate the effect of DIXDC1 on cell proliferation. The data were means ± SEM of three independent experiments (*,#P < 0.05). (D) OCI-Ly8 and Daudi cells were transfected with myc-DIXDC1 or myc-control. The interference efficiencies were confirmed by western blot. (E) OCI-Ly8 and Daudi cells were transfected with myc-DIXDC1 or myc-control. CCK-8 assays were performed to evaluate the cell growth at the indicated time. The data were means ± SEM of three independent experiments (*,#P < 0.05). (F) Soft agar colony assays were performed using myc-DIXDC1 or myc-control. Colony numbers were normalized to the number of colonies formed by cells expressing myc-control. The data were means ± SEM of three independent experiments (*,#P < 0.05).

Fig. 3. DIXDC1 inhibited cell cycle progression. (A and B) OCI-Ly8 and Daudi cells were transfected with either DIXDC1-siRNA or myc-DIXDC1 or their respective control. Flow cytometric analysis was performed to analyze the cell cycle distribution. Data are presented as mean ± SEM of three independent measurements (*,#P < 0.05). (C) OCI-Ly8 and Daudi cells were transfected with either DIXDC1-siRNA or control-siRNA. Western blot assays were performed to analyze the expression of DIXDC1, p21, Cyclin D1 and PCNA. (D) A bar chart demonstrated the expression ratio of DIXDC1, p21, Cyclin D1 and PCNA relative to β-Actin by densitometry. Data are presented as mean ± SEM of three independent measurements (*,#P < 0.05).
assays were used to investigate the effect of DIXDC1 knockdown on cells growth, respectively. The results revealed that knockdown of DIXDC1 led to a significant inhibition of cell growth rate and colony formation ability in OCI-Ly8 and Daudi cells (Fig. 2B and C). In addition, myc-tagged DIXDC1 (myc-DIXDC1) was transfected into OCI-Ly8 and Daudi cells. The interference efficiency was then confirmed by western blot analyses (Fig. 2D). To further confirm its role in cell proliferation, CCK-8 assays and soft agar colony assays demonstrated that overexpression of DIXDC1 led to a significant increase of cell growth rate and colony formation ability in OCI-Ly8 and Daudi cells (Fig. 2E and F). All the data suggested the possibility that DIXDC1 might play a role in regulating cell proliferation.

3.3. DIXDC1 knockdown inhibited cell cycle progression

To further confirm the effect of DIXDC1 on cell cycle regulation. Flow cytometric was conducted and showed that down-regulated DIXDC1 expression generate an increase in the percentage of G1-
phase cells and decrease in the percentage of S-phase cells, whereas overexpressed DIXDC1 resulted in a significant increase in the percentage of G1-phase cells (Fig. 3A and B). Previous study has been reported that overexpressed DIXDC1 promoted G0/G1 to S phase transition concomitantly with up-regulation of cyclin D1 and down-regulation of p21 [18]. Based on this, we evaluated the expression of Cyclin D1 and p21 in OCI-Ly8 and Daudi cells. Western blot showed that knockdown of DIXDC1 led to a significant decrease of cyclin D1 and PCNA expression and an increase of p21 (Fig. 3C and D). All the data indicated that knockdown of DIXDC1 in NHL cells inhibits cell cycle progression.

3.4. DIXDC1 promoted cell proliferation and inhibited cell cycle by enhancing phospho-AKT expression

It has previously been reported that DIXDC1 regulate proliferation of non-small-cell lung cancer (NSCLC) via the PI3K/Akt pathway [19]. Therefore, we asked whether DIXDC1 could also promote cell proliferation by regulating PI3K/Akt signaling pathway in NHL. To confirm our assumption, OCI-Ly8 and Daudi cells were transfected with DIXDC1-siRNA or myc-tagged DIXDC1 (myc-DIXDC1) or their respective control. Western blot assays showed that knockdown of DIXDC1 led to a reduced expression of p-AKT and p-GSK-3β, but not in total AKT and GSK-3β. On the contrary, overexpression of DIXDC1 led to an increased expression of p-AKT and p-GSK-3β (Fig. 4A and B). Then, OCI-Ly8 and Daudi cells were transfected with AKT inhibitor (MK2206) followed by cell cycle distribution. Flow cytometric analysis showed that MK2206 significantly increased the percentage of G1-phase cells compared to the DMSO treated cells (Fig. 4C). Moreover, we demonstrated that whether the PI3K/Akt pathway was involved in the proliferative effect of DIXDC1 in NHL. Intriguingly, DIXDC1-mediated promotion of cell proliferation was abrogated by MK2206 in both OCI-Ly8 and Daudi cells (Fig. 4D). In addition, flow cytometric assays were used to investigate the effect of DIXDC1 on apoptosis. We found that knockdown of DIXDC1 increased apoptosis, and the anti-apoptotic effects of DIXDC1 were augmented in cells treated with MK2206. As expected, overexpression of DIXDC1 reduced apoptosis; however, anti-apoptotic effects of DIXDC1 were abrogated in cells treated with MK2206 (Fig. 4E).

3.5. DIXDC1 promoted cell adhesion-mediated drug resistance via the AKT pathway in NHL

As PI3K/AKT signaling played an important role in cell adhesion mediated drug resistant, we speculated that DIXDC1 might be
involved in the process of lymphoma cell adhesion mediated drug resistance. Cell adhesion assays were first performed to assess the expression of DIXDC1. Western blot showed that DIXDC1 expression was more obvious when cells adhered to FN or HS-5 cells compared with cells cultured in suspension (Fig. 5A and B). Our previous studies showed that the proper doxorubicin concentration was 1 μM, at which these two cells were sensitive to drug induced apoptosis [27]. Thus, OCI-Ly8 and Daudi cells were treated with 1 μM doxorubicin (Doxo). CCK-8 assays showed that cells adhered to FN or HS-5 cells significantly protected it from cytotoxicity compared to cells cultured in suspension. And we found that this protective effect was decreased in cells transfected with DIXDC1-siRNA. As expect, flow cytometric analysis also showed that knockdown of DIXDC1 in OCI-Ly8 and Daudi cells adhesion to FN or HS-5 cells significantly increased cell apoptosis (Fig. 5C and D). Subsequently, to determine whether DIXDC1 regulated CAM-DR phenotype via the AKT pathway, OCI-Ly8 and Daudi cells were treated with MK2206 (Akt inhibitor) or DMSO (control). CCK-8 assays showed that overexpression of DIXDC1 in OCI-Ly8 and Daudi cells adhesion to FN or HS-5 cells significantly increased cell viability, but the protective effect mediated by DIXDC1 was clearly reversed in MK2206-treated cells (Fig. 5E and F). All the data indicated that DIXDC1 promoted CAM-DR was PI3K/Akt dependent.

4. Discussion

Non-Hodgkin’s lymphoma (NHL), a heterogeneous group of malignancies derive from B- or T-cell, is the most common hematopoietic and lymphoid malignancies [24]. The incidence of NHL is increasing over recent years and is still associated with significant mortality. Despite intensive effort in developing new therapies, there has been significant relapse rate of patients with aggressive and indolent lymphomas [25]. Drug resistance is a common reason that contributes to short overall survival in NHL [26]. Previous studies have shown that adhesion to FN or HS-5 cells confers a multidrug-resistant phenotype and this disruption of cell adhesion-mediated signaling may improve the efficacy of chemotherapy drugs [27]. DIXDC1, DIX domain containing 1, is a human homolog of Cdc1 and a positive regulator in the Wnt and PI3K/Akt signaling pathway. The DIXDC1 is involved in a variety of processes, such as cell proliferation, cell cycle, protein folding, transcription, protein transport, signal transduction [28]. In the field of cancer research, up-regulated expression of DIXDC1 was found in many human cancers, such as colorectal cancer [18], non-small-cell lung cancer [19] and gastric cancer [20], indicating that DIXDC1 might act as an oncogene promoting tumor progression. However, it is largely unknown the biological role of DIXDC1 in the formation and progression of NHL. In this study, we found that DIXDC1 act as an oncogene in NHL. High DIXDC1 staining was found in progressive lymphoma by immunohistochemical analysis. As except, CCK-8 assays and soft agar colony assays also showed that knockdown of DIXDC1 could decrease the proliferation of NHL cells.

Previous study demonstrated that DIXDC1 promoted cell proliferation and inhibited apoptosis by enhancing phospho-AKT expression in colorectal cancer [18]. Consistent with the findings, our study indicated that knockdown of DIXDC1 led to a reduced expression of p-AKT, and overexpression of DIXDC1 led to an increased expression of p-AKT. Mounting evidence demonstrated that the PI3K/Akt pathway played an important mediator in cell adhesion mediated drug resistance (CAM-DR) [27]. Therefore, we speculated that DIXDC1 might be involved in the process of CAM-DR in NHL. Our findings showed that adhesion to FN or HS-5 cells increased the expression of DIXDC1, and the elevated levels of DIXDC1 significantly up-regulated p-Akt expression. Subsequently, CCK-8 assays showed that overexpression of DIXDC1 in OCI-Ly8 and Daudi cells adhesion to FN or HS-5 cells significantly increased cell viability, but the protective effect mediated by DIXDC1 was clearly reversed in MK2206(Akt inhibitor)-treated cells. However, how adhesion to FN or HS-5 cells influences DIXDC1 expression is still unknown and needs to be further elucidated.

It is reported that promoted G1/S cell cycle progression in DIXDC1-overexpression colon cancer cells might be facilitated by DIXDC1-mediated p21 down-regulation and Cyclin D1 up-regulation [18]. We assessed cell cycle distribution and found that DIXDC1 silencing induced cell cycle arrest in G0/G1 phase. Subsequently, we evaluated the expression of Cyclin D1 and p21 in OCI-Ly8 and Daudi cells. The Cyclin D1 is a key regulator of the G1/S transition [29], and the p21 protein is a CDK inhibitor and a negative regulator in the G1/S transition [30]. We found that knockdown of DIXDC1 led to decreased expressions of Cyclin D1 and increased expression of p21. Moreover, the cell proliferation promoting effect of DIXDC1 by targeting p21 and Cyclin D1 was confirmed in colon cancer. Consistent with this results, we found that knockdown of DIXDC1 could decrease cell proliferation via modulating cell cycle progression. Western blot showed that knockdown of DIXDC1 led to a significant decrease of Cyclin D1 and PCNA expression and an increase of p21.

In conclusion, our study showed that DIXDC1 is a potential tumor oncogene in NHLs. Up-regulation of DIXDC1 could accelerate the proliferation of NHL cells and promote CAM-DR phenotype in NHL. In the future, this may be an effective strategy for treatment of NHL.

Conflict of interest statement

We declare that we have no conflict of interest.

Authors contributions

Chun Cheng, Yu Ouyang designed and performed the experiments. Fei Zhong and Qiru Wang participated in the statistical analysis. Linlin Ding performed the flow cytometric experiments. Yu Ouyang and Fei Zhong participated in the Cell viability assay and soft agar colony assay. Lingling Chen assisted with immunohistochemical staining analysis and helped to select suitable cases. Peipei Zhang participated in the cell line culture. Yu Ouyang and Yuchan Wang searched the related literature and prepared the manuscript. All authors were involved in editing the paper and have approved the submitted version.

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References

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