DR. BO WANG (Orcid ID : 0000-0001-9755-498X)

DR. JIE ZHENG (Orcid ID : 0000-0002-7961-6427)

Article type : Regular Article

Systemic chemotherapy promotes HIF-1α mediated glycolysis and IL-17F pathways in cutaneous T cell lymphoma

Bo Wang[1,2#], Kejia Li[3#], Honglin Wang[4], Xiaoyan Shen[1*], Jie Zheng[1]

Affiliations: [1]Department of Dermatology, Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200025, China

[2]Department of Dermatology, University of Michigan, Ann Arbor, MI 48109, USA

[3]Department of Dermatology, Ruijin Hospital North, School of Medicine, Shanghai Jiao Tong University, Shanghai 201800, China

[4]Shanghai Institute of Immunology, Institute of Medical Sciences, Shanghai Jiao Tong University, Shanghai 200025, China

Correspondence: Xiaoyan Shen

Address: 197 Ruijin Er Road, Shanghai, China 200025.

Telephone: 86-21-64370045

Fax: 86-21-64333548

email: shenxiaoyan2002cn@yahoo.com

This study was supported in part by research funding from the National Key Clinical Specialty (grant No. 2012649) to J.Z and National Natural Science Foundation of China (grant No. 30901293) to X.S.

Conflicts of interest: none declared

#These authors have equal contribution to this manuscript

Author contribution: K. Li & X. Shen performed the research. J. Zheng, X. Shen & H. Wang designed the research study. X. Shen， K. Li & B. Wang analyzed data. H. Wang contributed essential experimental guidance. B. Wang wrote the paper.

Keywords: cutaneous T cell lymphoma, mycosis fungoides, HIF-1α, T cell, pathogenesis, cancer.

Abstract

Background: Systemic chemotherapy is often the last resort of advanced cutaneous T cell lymphoma (CTCL). Tumor recurrence and adverse effects of systemic chemotherapy are the main limitations.

Objective: We aim to investigate the metabolic alterations in tumor cells after CHOP (cyclophosphamide,hydroxydaunorubicin, oncovin and prednisone) chemotherapy.

Methods and Results: In advanced CTCL, CHOP chemotherapy has no survival benefit and the duration of response is significantlyinferior to other canonical treatments. HIF-1α

is significantly elevated in lesions of advanced MF patients as well as tumor cell line Hut78 and tumor xenograft mice model. CHOP therapy also increased glycolytic activities in a HIF-1α-dependent manner. In CTCL xenograft tumor mice model, lesional cells showed a significant increase in IL-17F after chemotherapy, shifting towards a Th17 phenotype, which process is also regulated by HIF-1α. Echinomycin, HIF-1α inhibitor, was co-administered in xenograft tumor mouse models with CHOP and showed a significant reduction in tumor growth.

Conclusion: CHOP chemotherapy promotes glycolysis and IL-17 pathways in a HIF-1α-dependent fashion. Furthermore, HIF-1α blockade is promising as an accompanying agent in systemic chemotherapy for patients with advanced CTCL.

Introduction

Cutaneous T cell lymphoma (CTCL) is the most common primary cutaneous lymphoma, accounting for approximately 75% of all cutaneous lymphomas[1–3]. Various prognostic markers are important to estimate disease progression and guide therapeutics[4,5]. There are various treatment modalities available for mycosis fungoides(MF), the most common type of CTCL:skin-derived therapies and UV radiations are preferred for early stage MF. Systemic therapies are often reserved for patients with advanced MF and other types of CTCL. These include systemic multi-agent chemotherapies, interferon-α, bexarotene, HDAC inhibitors, immunotoxins and allogeneic hematopoietic stem cell transplantation.

Hughes et al. retrospectively analyzed the effectiveness of systemic chemotherapy in 144 MF and SS cases; median time to next treatment (TTNT) was 3.9 months, which is the shortest compared with other systemic therapies[6]. It has been shown that systemic chemotherapies did not improve prognosis of patients with early stage MF[7,8]. Systemic chemotherapy temporarily suppresses tumor growth, however, tumor recurs much faster compared to other treatment modalities. The molecular basis of limited efficacy of

aggressive chemotherapy in advanced CTCL is still not clear.

It is hypothesized that chemotherapy either alters oncogenic potential of tumor cells or it tunes microenvironment more favorable towards tumor growth. Reprogramming of metabolism is critical for cancer cells to gain extra energy for proliferation. Although oxidative phosphorylation generates more ATP, cancer cells prefer glycolysis as their main source of energy, aka Warburg effect[9–11]. Indeed, inhibition of glycolytic pathways have shown promising results as therapeutics for cancers, such as 2-deoxyglucose (2-DG) and etc[12,13].

Hypoxia induced factor-1α (HIF-1α) is one of the central regulators of cellular metabolism; HIF-1α intergrateswith hypoxia-response element (HRE) of target genesand is implicated in almost every aspect of cancer biology: tumor metastasis, angiogenesis, energy metabolism, apoptosis, etc. HIF-1α specificallyupregulates pathways involved in glycolysis and decreases substrates for aerobic metabolism. HIF-1α is well studied in various types of cancer, including hematopoietic malignancies[14,15]. Echinomycin is a quinoxaline antibiotic, which binds to HRE sequence and acts as a potent HIF-1α inhibitor[16].

In order to elucidate how systemic chemotherapy affects the metabolism and immune regulation of malignant T cells, we utilized several model systems and patient samples to show that CHOP chemotherapy promotes glycolysis and IL-17 pathways in a HIF-1α-dependent fashion.

Methods

Patient characteristics

Patients registry was from the cutaneous lymphoma clinic and general dermatology

clinic, Department of Dermatology, Ruijin Hospital, between 2003 and 2015. The diagnosis of CTCL was based on the criteria of the 2005 WHO-EORTC classifications[1] and staging of CTCL and MF was based on 2007 ISCL/EORTC recommendations[17].

Generation of tumor xenograft mouse model

We generatedex vivo mouse model of CTCLby inoculating $1*10^7$ HH cells subdermally to axillary skin of 4-week-old femaleBABL/c nude mice. Full-dose CHOP equivalent to human use resulted in significant lethality in mice. Dosage of CHOP regimen was optimized to cyclophosphamide 40 mg/kg, doxorubicin 3.3 mg/kg, vincristine 0.5 mg/kg, prednisone 0.2 mg/kg every week. Once tumor volume reaches $150mm^3$ (10-15 days after inoculation), CHOP regimen was delivered intravenously at weekly basis. In echinomycin treated group, 30ug/kg echinomycin was given by intraperitoneal injection every other day starting at same day of CHOP therapy.

Flow cytometry

Inoculated tumor cells were isolated from xenograft mouse model by passing through a 70 um nylon mesh filter into a 50ml conical tube. Cells were resuspended in PBS and layered over lympholyte M to isolate viable lymphocytes. All cell preparations were ACK lysed and washed before analysis. For surface staining, cells were suspended in PBS with 2% FCS containing Fc Block (50 ng/ml) prior to incubation with fluorochrome-conjugated Abs (anti–human CD4). The stained cells were analyzed with a FACSCanto II flow cytometer using FACSDiva software (v6.1.3, Becton Dickinson). Data were analyzed using FlowJo software (v9.3.2, TreeStar).

Cell Culture

HH cells (ATCC, CRL-2105) and Hut78 cells (Shanghai Institute of Biochemistry and

Cell Biology) were cultured per protocol. CHOP regimen were used at following concentration:cyclophosphamide 27.85 ug/ml, doxorubicin 1.858 ug/ml, vincristine 0.074ug/ml, prednisone 0.0325 ug/ml. Echinomycin was added to culture media at final concentration of 1.5nM.

RT-PCR

RNA was extracted either from cells or skin biopsy samples using TRIzol® (Life Technologies) and further purified using the RNeasy kit with on-column DNase I digestion (Qiagen). For qPCR, 1 μg of RNA was reverse-transcribed using iScript™. 0.5 μl was used with the SYBR® GreenMasterMix and each reaction was performed in triplicate. qRT-PCR was performed on the Bio-Rad iCycler with MyIQ single color real-time PCR detection system module with the following parameters: 95°C at 3 min, (95°C 10 s, 55°C 30 s) Å~ 40, 95°C 1 min, 55°C 1 min. The fold change in transcript levels was calculated using the ΔΔCt method. β-actin was used as controls.

Primers (5' to 3')

HIF1A: CATCTCCATCTCCTACCCACA and CTTTTCCTGCTCTGTTTGGTG

IL17F: GAAGGTGCTGGTGACTGTTG and CCTGTGAAGTGGAGGGAATT

STAT3: GGCACCTTCCTGCTAAGATTC and GGTCTTACCGCTGATGTCCTT

GLUT1: AGGAGATGAAGGAAGAGAGTCG and TGCTCGTGGAGTAATAGAAGACA

HK2: CATCTGCTTGCCTACTTCTTCA and ATCTGGAGTGGACCTCACAAAG

GPI: ATACCCTGTGACTTCCTCATCC and AATAGAGTTGGTTGGGCGATTT

PKM: AGTGTGACGAGAACATCCTGTG and AGAGAAATAAGCCCATCATCCA

ENO1: AAGGGTGTCTCAAAGGCTGTT and TTTTCTGTTCCATCCATCTCG

LDH: TATGGAGTGGAATGAATGTTGC and CCAGGATGTGTAGCCTTTGAGT

Results

Limited therapeutic effect of CHOP chemotherapy

We retrospectively analyzed 18patientswith advanced CTCL (including advanced MF, PTCL and c-ALCL) from registry in Cutaneous Lymphoma Clinic, Department of Dermatology, Ruijin Hospital. We compared the overall survival and duration of response in9patients received CHOP chemotherapy and 9 patients received non-chemotherapy (which includes combination of topical corticosteroids, narrow-band UVB and bexarotene). No statistical difference was observed in the distribution of patients between to two groupsin age and gender.There is no significant difference in overall survival between CHOP and non-chemotherapy groups (log-rank p=0.75, Fig 1A). However, compared with patient received non-chemotherapy, patient in CHOP group has a significantly decreased duration of response (time in remission, p=0.0034, log-rank Hazard ratio=3.54, Fig 1B).

HIF-1α is upregulated in advanced MF and CHOP chemotherapy

As the initial step to address whether tumor microenvironment is altered by CHOP chemotherapy, we focused on HIF-1α, a master regulator of cellular metabolism. We first measured lesional *HIF1A* expression in early and advanced MF patients. HIF-1α expression was elevated by 2.3 fold in advanced MF (Fig 2A). Mirroring that, HIF-1α upregulation was also observed in HuT78 cells after CHOP treatment, indicating CHOP therapy tunes tumor cells more hypoxic as found in advanced MF (Fig 2B). To examine whether this effect holds true in a more physiological model, we generatedtumor xenograft mouse mode: HH cells inoculated in BABL/c nude mice (see Method Section). Indeed, HIF-1α expression is significantly increased when xenograft were treated with CHOP. HIF-1α upregulation is reversible with intraperitoneal injection of echinomycin, a

blocking agent of HIF-1α (Fig 2C).

CHOP chemotherapy upregulates glycolysis via HIF-1α

Since HIF-1αis a key regulator in cellular metabolism, we sought to investigate the glycolysis pathway by measuring the expression of key enzymes in glycolysis pathway in Hut-78 cell line. Expression of *GLUT1*, *HK2*, *GPI*, *ENO1* and *PKM* (transcripts encode for glucose transporter 1, hexokinase 2, glucose-6-phosphate isomerase, enolase 1 and pyruvate kinase) were significantly upregulated by CHOP in Hut78 cells (Fig 2D). Expression of LDH-α remained unchanged by CHOP. The same upregulationin glycolytic pathways was observed in the xenograft tumor (Fig 2E). Addition of echinomycin completely reversed the increase of glycolytic enzymes in both models, indicating a mediating role of HIF-1α(Fig 2D and E).

Increased lesional IL-17F after CHOP treatment

Differentiation of either Treg cells or Th17 cells is dependent of glycolysis and local oxygen concentrations. IL-17F expressing cells were quantified by flowcytometry in inoculated tumors. Single dose of CHOP infusion significantly increased lesional IL-17F positive cells (Fig 3A-C). Serum IL-17F was measured in 50 MF patients including 20 early MF (stage I and IIa) and 30 advanced MF (stage IIb, III and IV). A large number of patients with advanced MF had elevated circulating IL-17F (Fig 3D).

In Hut78 cell line and xenograft tumor model, IL-17F transcript level was upregulated by CHOP, recapitulating the in vivo findings. Expressions of HIF-1α, Stat3 and RORγT were also increased by CHOP, further elucidating the mechanism of IL-17F upregulation. The upregulation was blocked by echinomycin, thus suggesting a key regulatory role of HIF-1α (Fig 4A and B).

Therapeutic effect of HIF-1α inhibition in CTCLxenograft model

In order to investigate whether HIF-1α inhibition has therapeutic effect towards advanced CTCL, we tested in tumor xenograftmouse model. In this model, weekly CHOP infusion was administered when tumor volume reached 150mm^3, after 20 days of weekly infusion, tumor size was significantly reduced (Fig 4C). It is worth mentioning that such significant difference was no longer observed after 40 days, mirroring the limited long-term therapeutic effect of chemotherapy in advanced CTCL.During CHOP therapy, 30ug/kg echinomycin was delivered intraperitoneally once every two days to block HIF-1α. Compared with CHOP regimen, combination of echinomycin and CHOP showed a more significant reduction of tumor sizes starting at the third week. Such reduction is persistent towards the 53rd day (Fig 4C).

Discussion

In summary, our study suggests that CHOP chemotherapy alters tumor cell glycolysis and IL-17 pathways via HIF-1α. We provided potential mechanisms of how systemic chemotherapy failed to achieve satisfactory efficacy in advanced CTCL and often leads to tumor recurrence.

In patients with advanced CTCL, CHOP chemotherapy leads to rapid tumor regression, however, the duration of response is significantlyinferior to other canonical treatments. Consistent with published studies, CHOP chemotherapy resulted in similar overall survival with non-chemotherapy treatments. By using two different model systems: HuT78 and tumor xenograft mouse models, we confirmed upregulation of HIF-1α after CHOP treatment, which is the same change in advanced CTCL. It suggested that CHOP chemotherapy tunes the tumor in the same manner asin aggressive CTCL, altering gene expressions by upregulating HIF-1α. Hence provided rationale of limited efficacy and increased tumor recurrence of CHOP chemotherapy in CTCL.

After CHOP chemotherapy and in advanced CTCL, a vicious cycle is created where CTCL microenvironment becomes more hypoxic, continuous HIF-1α activation drives malignant T cells towards Th17 phenotype.Previous studies revealed that malignant T cells can have a Th17 phenotype and induce expression of angiogenic factor and MMPs to promote tumor growth[18,19]. IL-17F expression correlates with progressive diseases and worse clinical outcomes. The increase of IL-17F during CHOP chemotherapy is HIF-1α dependent; supporting that Th17 phenotype is a downstream effect ofhypoxia. In our study, IL-17 expression is induced by Stat3 and RORγT, the same mechanism with other published studies[18,20–22]: Stat3 inhibition decreases proliferation and increased survival of CTCLtumor cells in vitro. Mouse model carrying activated form of Stat3 showed significant increase of IL-17 and IL-22 producing T cells. Since all the above downstream effect of HIF-1α activation promotes tumor growth and invasion, we utilized xenograft model to reveal the therapeutic effect of HIF-1α inhibitor in CTCL mouse model.

Echinomycin is a quinoxaline antibiotic, which has showed its therapeutic potentials in a plethora ofcancers such as hematological malignancies, lung cancer and soft tissue sarcomas[23,24].Besides echinomycin, many anti-cancer medications including digoxin and bortezomib showed anti- HIF-1α activities[25].

In conclusion, CHOP chemotherapy alters tumor cell metabolism through HIF-1α, same mechanism in aggressive CTCL; altering glycolysis and IL-17 pathways. Our data provided evidence for the limited efficacy of CHOP chemotherapy in CTCL and showedHIF-1α blockade is promising as an synergic agent with systemic chemotherapy for patients with advanced CTCL.

Acknowledgment

This study was supported in part by research funding from the National Key Clinical Specialty (grant No. 2012649) to J.Z and National Natural Science Foundation of China (grant No. 30901293) to X.S.

Reference:

1 Willemze R, Jaffe ES, Burg G, *et al.* WHO-EORTC classification for cutaneous lymphomas. *Blood* 2005; **105**:3768–85.

2 Criscione VD, Weinstock MA. Incidence of cutaneous T-cell lymphoma in the United States, 1973-2002. *Arch Dermatol* 2007; **143**:854–9.

3 Bradford PT, Devesa SS, Anderson WF, Toro JR. Cutaneous lymphoma incidence patterns in the United States: a population-based study of 3884 cases. *Blood* 2009; **113**:5064–73.

4 Scarisbrick JJ, Prince HM, Vermeer MH, *et al.* Cutaneous Lymphoma International Consortium Study of Outcome in Advanced Stages of Mycosis Fungoides and Sézary Syndrome: Effect of Specific Prognostic Markers on Survival and Development of a Prognostic Model. *J Clin Oncol* 2015; **33**:3766–73.

5 Shen X, Wang B, Li K, *et al.* microRNA signatures in diagnosis and prognosis of cutaneous T cell lymphoma. *J Invest Dermatol* 2018. doi:10.1016/j.jid.2018.03.1500.

6 Hughes CFM, Khot A, McCormack C, *et al.* Lack of durable disease control with chemotherapy for mycosis fungoides and Sï¿½zary syndrome: A comparative study of systemic therapy. *Blood* 2015; **125**:71–81.

7 Kaye FJ, Bunn PA, Steinberg SM, *et al.* A Randomized Trial Comparing Combination Electron-Beam Radiation and Chemotherapy with Topical Therapy in the Initial Treatment of Mycosis Fungoides. *N Engl J Med* 1989; **321**:1784–90.

8 Axelrod PI, Lorber B, Vonderheid EC. Infections complicating mycosis fungoides and Sézary syndrome. *JAMA* 1992; **267**:1354–8.

9 Soga T. Cancer metabolism: key players in metabolic reprogramming. *Cancer Sci* 2013; **104**:275–81.

10 Ward PS, Thompson CB. Metabolic reprogramming: a cancer hallmark even

warburg did not anticipate. *Cancer Cell* 2012; **21**:297–308.

11 Renner K, Singer K, Koehl GE, *et al.* Metabolic Hallmarks of Tumor and Immune Cells in the Tumor Microenvironment. *Front Immunol* 2017; **8**:248.

12 Le A, Cooper CR, Gouw AM, *et al.* Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. *Proc Natl Acad Sci U S A* 2010; **107**:2037–42.

13 Bénéteau M, Zunino B, Jacquin MA, *et al.* Combination of glycolysis inhibition with chemotherapy results in an antitumor immune response. *Proc Natl Acad Sci U S A* 2012; **109**:20071–6.

14 Faubert B, Boily G, Izreig S, *et al.* AMPK is a negative regulator of the Warburg effect and suppresses tumor growth in vivo. *Cell Metab* 2013; **17**:113–24.

15 Pang Y-Y, Wang T, Chen F-Y, *et al.* Glycolytic inhibitor 2-deoxy-d-glucose suppresses cell proliferation and enhances methylprednisolone sensitivity in non-Hodgkin lymphoma cells through down-regulation of HIF-1α and c-MYC. *Leuk Lymphoma* 2015; **56**:1821–30.

16 Kong D, Park EJ, Stephen AG, *et al.* Echinomycin, a Small-Molecule Inhibitor of Hypoxia-Inducible Factor-1 DNA-Binding Activity. *Cancer Res* 2005; **65**:9047–55.

17 Olsen E, Vonderheid E, Pimpinelli N, *et al.* Revisions to the staging and classification of mycosis fungoides and Sezary syndrome: a proposal of the International Society for Cutaneous Lymphomas (ISCL) and the cutaneous lymphoma task force of the European Organization of Research and Treatment of Cancer (EORTC). *Blood* 2007; **110**:1713–22.

18 Krejsgaard T, Ralfkiaer U, Clasen-Linde E, *et al.* Malignant Cutaneous T-Cell Lymphoma Cells Express IL-17 Utilizing the Jak3/Stat3 Signaling Pathway. *J Invest Dermatol* 2011; **131**:1331–8.

19 Lauenborg B, Litvinov I V, Zhou Y, *et al.* Malignant T cells activate endothelial cells via IL-17 F. *Blood Cancer J* 2017; **7**:e586.

20 Fanok MH, Sun A, Fogli LK, *et al.* Role of Dysregulated Cytokine Signaling and Bacterial Triggers in the Pathogenesis of Cutaneous T-Cell Lymphoma. *J Invest Dermatol* 2018; **138**:1116–25.

21 Yang XO, Pappu BP, Nurieva R, *et al.* T Helper 17 Lineage Differentiation Is Programmed by Orphan Nuclear Receptors RORα and RORγ. *Immunity* 2008; **28**:29–39.

22 Krejsgaard T, Litvinov I V, Wang Y, *et al.* Elucidating the role of interleukin-17F in cutaneous T-cell lymphoma. *Blood* 2013; **122**:943–50.

23 Chang AY, Kim K, Bonomi P, *et al.* A Randomized Phase II Trial of Echinomycin , Trimetrexate , and Cisplatin plus Etoposide in Patients with Metastatic Nonsmall Cell Lung Carcinoma An Eastern Cooperative Oncology Group Study (E1587). *Cancer* 1996; **82**:292–300.

24 Wang Y, Liu Y, Malek SN, *et al.* Targeting HIF1a Eliminates Cancer Stem Cells in Hematological Malignancies. *Cell Stem Cell* 2011; **8**:399–411.

25 Aziz N, Stanbridge EJ, Shafee N. Bortezomib attenuates HIF-1- but not HIF-2 - mediated transcriptional activation. *Oncol Lett* 2015; **10**:2192–6.

Figure Legends

Fig 1. Limited therapeutic effect of CHOP chemotherapy in advanced CTCL

Kaplan-Meier plot of patients overall survival (A) and duration of response (B) between CHOP chemotherapy and non-chemotherapy groups. 9 advanced CTCL patients were included in each group.

Fig 2. CHOP chemotherapy promotes tumor cell glycolysis via HIF-1α

*HIF1A*mRNA levelswere measured by realtimePCR in skin biopsy samples from lesional skin of early MF (n=7) and advanced MF (n=10)(A), cell lysates of HuT78 cells with treatment of CHOP and echinomycin (n=5) (B) and xenograft tumor with treatment of CHOP and echinomycin (n=4)(C).

(D, E) After treatment with CHOP or CHOP+echinomycin, RNA was isolated from Hut78 cellsor xenograft tumorsand used for real time PCR analyses of glycolytic enzymes. Expression levels in sham group were set to 1. Results are normalized to β-actin transcript levels. n=4.

All graphs show means +/- SEM;*,$p<0.05$;**, $p<0.01$.

Fig 3. Increased lesional IL-17F after CHOP treatment

(A, B)Sorted Th17 cells ($CD4^+$, $CD17F^+$) from xenograft mouse models after treatment with Sham or CHOP regimen.

(C)Quantification of IL-17F positive cell numbers in panel A and B, n=3.

(D) Serum IL-17F protein level was quantified by ELISA in patients with early stage MF and advanced stage MF. All graphs show means +/- SEM;*,$p<0.05$. n=5.

Fig 4. Therapeutic effect of HIF-1α inhibition in CTCLxenograft model

(A, B) mRNA levels of *IL17F* and its regulatory genes: HIF1A, STAT3 and RORγTwere measured by qRT-PCR in HuT78 cells and xenograft tumor with treatment of sham, CHOP or CHOP+echinomycin. Results are normalized to β-actin transcript levels. n=5.

(C) Xenograft tumor volume (mm^3) of mice receiving intraperitoneal injections of Sham treatment (black), CHOP regimen (blue) and CHOP plus echinomycin treatment (red).

All graphs show means +/- SEM; ns, not significant; *,$p<0.05$;**, $p<0.01$; ***, $p<0.001$. $n\geq8$.

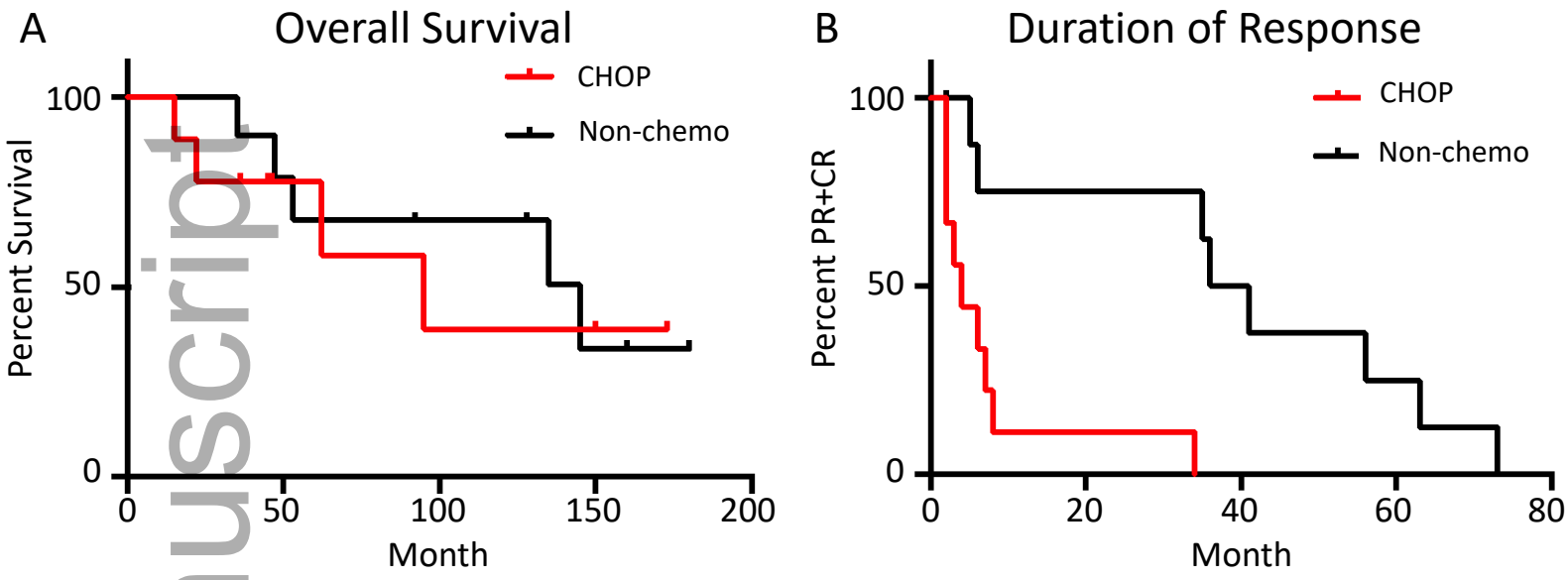

Fig 1. Limited therapeutic effect of CHOP chemotherapy in advanced CTCL

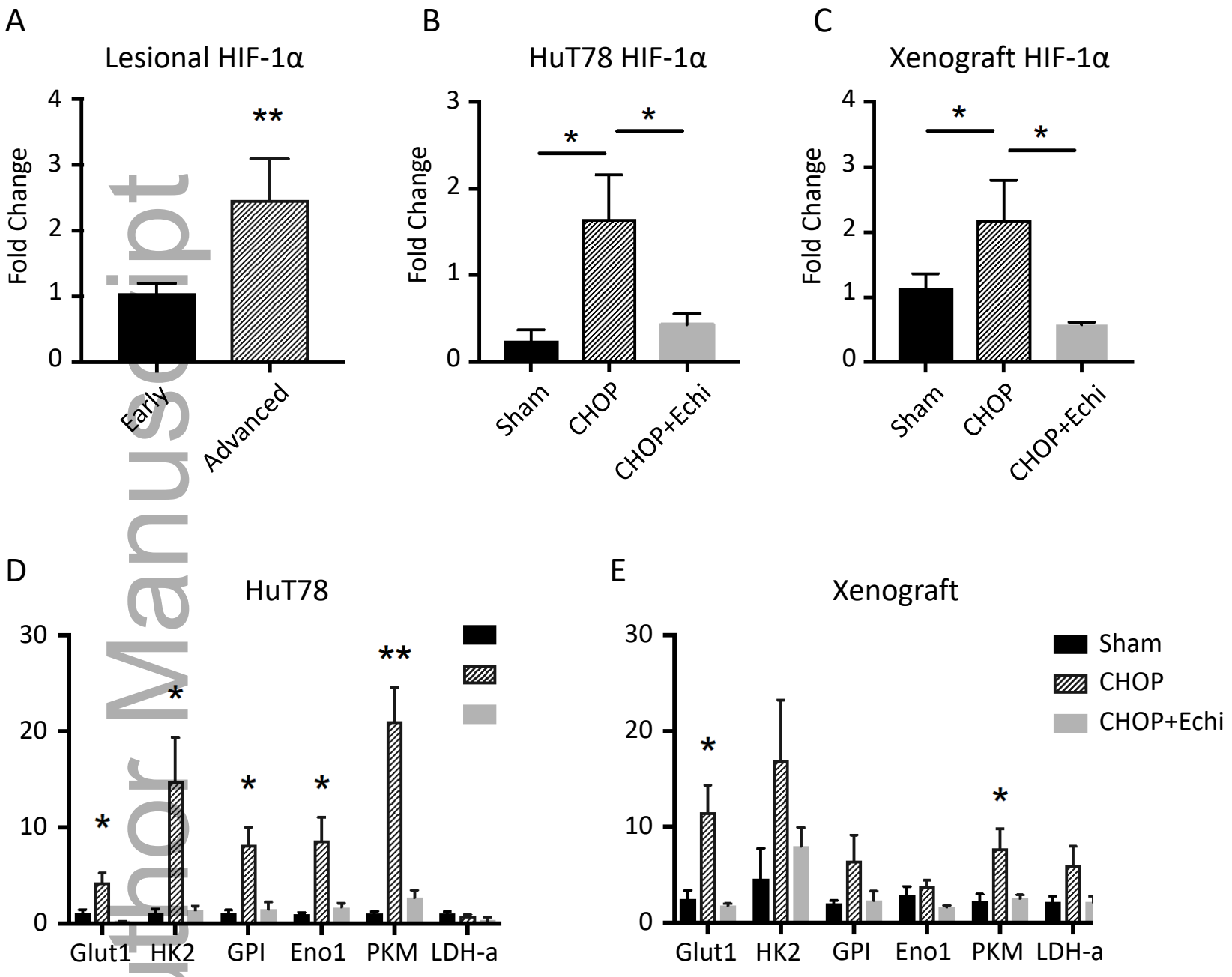

Fig 2. CHOP chemotherapy promotes tumor cell glycolysis via HIF-1α

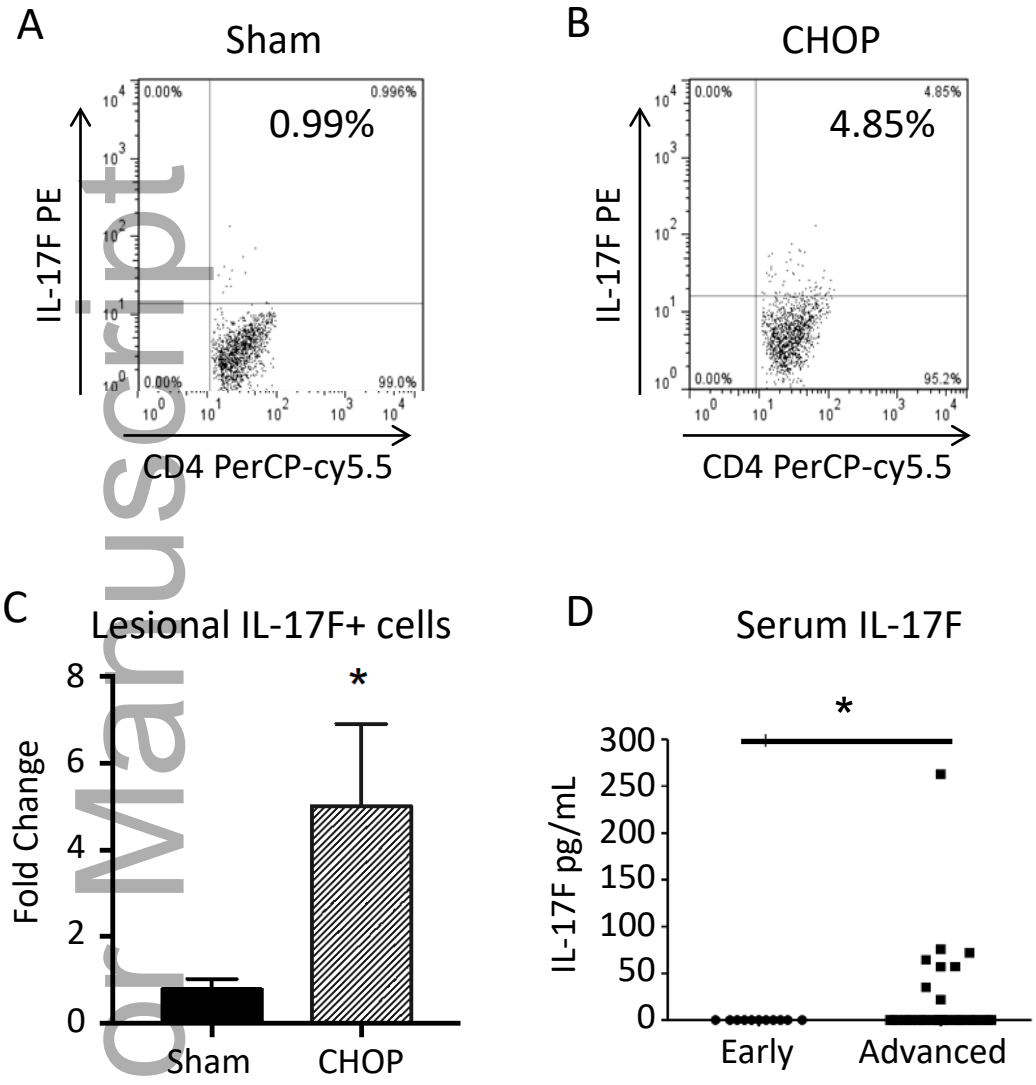

Fig 3. Increased lesional IL-17F after CHOP treatment

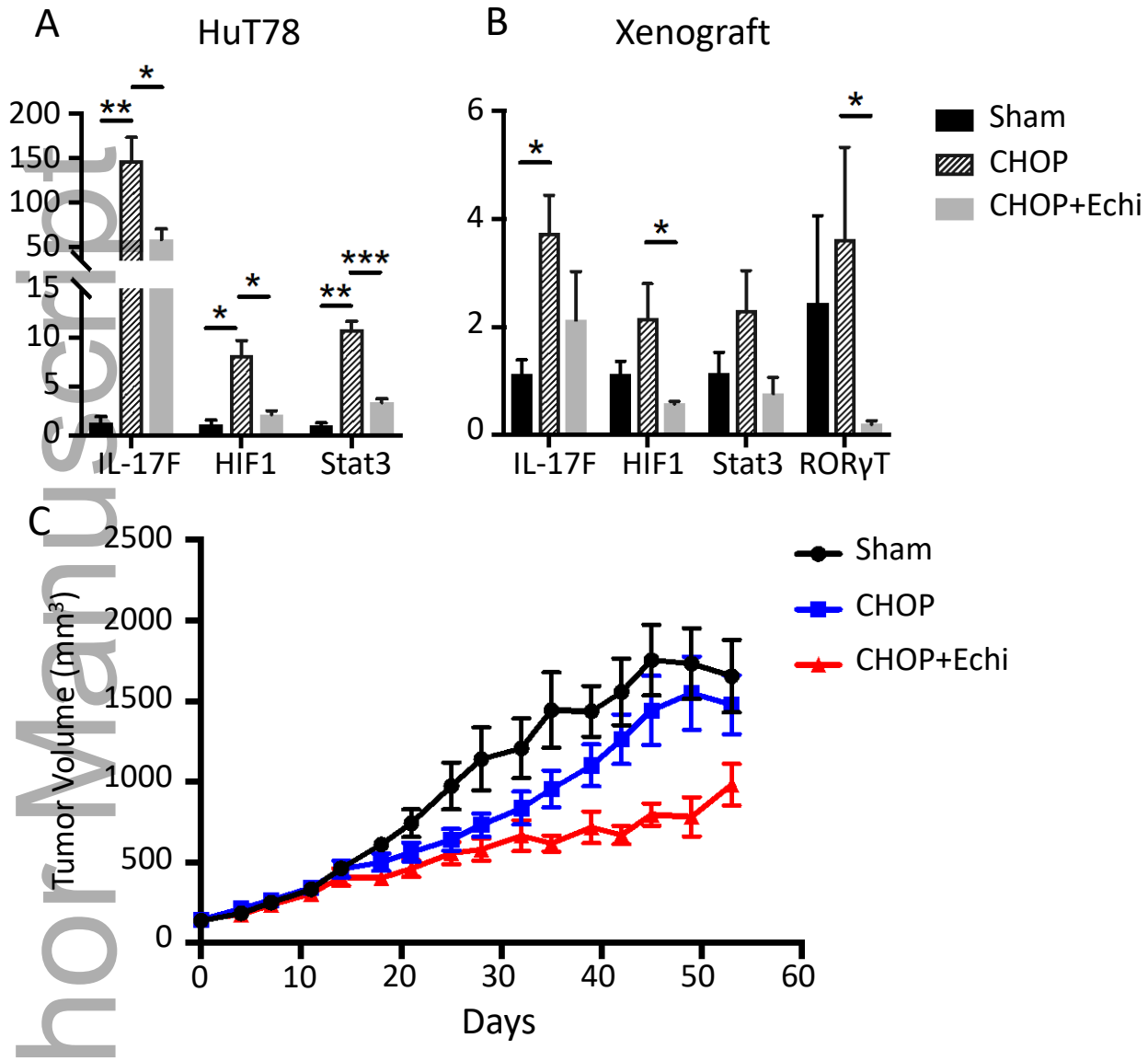

Fig 4. Therapeutic effect of HIF-1α inhibition in CTCL xenograft model