Research Paper

Interferon-α Promotes the Expression of Cancer Stem Cell Markers in Oral Squamous Cell Carcinoma

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Abstract

Objectives: IFNα can stimulate an antitumor immune response and has a direct inhibition on cancer cells. This study is to test whether IFNα can activate dormant cancer stem cell (CSC) in oral squamous cell carcinoma (OSCC) to facilitate their elimination by chemotherapy.

Materials and methods: Nude mouse transplantation tumor model was established and administrated with IFNα and saline. The influence on CD44 and ALDH1A1 expression under IFNα treatment was detected by *in vivo* experiments. Flow cytometry, western blot, and immunofluorescence were used to detect the expression of CD44 and ALDH1A1 after INFa treatment in OSCC cell lines. Tumorsphere formation assay was conducted under incubation with IFNα for 2 weeks. Chromatin immunoprecipitation (ChIP) assays was used to examine the IFNα-induced transcriptional regulation of *CD44* and *ALDH1A1* expression. That IFNα-primed enhanced killing effect of chemotherapy was evaluated by MTT and western blot.

Results: IFNα transcriptionally activated the expression of *CD44* and *ALDH1A1* expression both *in vivo* and *in vitro*. IFNα-primed enhanced the cytotoxic inhibition effect of CDDP, erlotinib and nimotuzumab on OSCC cells.

Conclusion: These results suggest that IFNα could be administrated to patients prior to chemotherapeutic drugs, which will facilitate the killing of cancer stem cells in OSCC.

Key words: Interferon alpha; CD44; ALDH1A1; Oral squamous cell carcinoma

Introduction

Type I interferons (IFNs) mainly comprise IFNα and IFNβ proteins. IFNα has a major role not only in antiviral immune responses but also in the natural and the therapy-induced immunological control of virus-unrelated malignancies [1]. IFNα displays direct cytotoxicity on tumor cells in cell culture and preclinical studies [2]. IFNα has been reported to affect tumor cell proliferation, tumor lymph/ angiogenesis, and tumor metastasis [3]. IFNα induced by plasma dendritic cells (pDC) leads to tumor regression of melanoma, basal cell carcinoma, and T cell lymphoma [4]. Type I IFNs intervene all the

phases of cancer immunoediting including elimination, equilibrium and escape [5]. IFNα can activate proliferation of DC and NK cells, enhance the cytotoxicity and survival of NK cells and CD4+ T cell, and promote tumor antigen expression of tumor cells [6-9]. Trials of IFNα therapies in solid malignancies such as melanoma, renal cell carcinoma and Kaposi sarcoma have achieved varied success [10]. Given its pleiotropic actions, IFNα is expected to not only stimulate an antitumor immune response, but also directly impact on cancer cell proliferation and survival, which together can achieve maximum therapeutic effect on cancer cells.

CSC has emerged as a critical factor for cancer chemoresistance, relapse and metastasis which often lead to treatment failure. CSC can undergo self-renewal and differentiation to promote the initiation and maintenance of tumor. CD44 and ALDH1A1 were considered as classical surface markers of CSC in head and neck squamous cell carcinoma (HNSCC) [11]. CD44 is a type I transmembrane glycoprotein that mediates cell-cell and cell-matrix interaction through affinity for hyaluronic acid. It is the one of the first markers to identify HNSCC CSCs [12]. OSCC CSCs can be also isolated on the basis of functional activities of ALDH1A1, which contributes to chemotherapy resistance [13]. Recently, more and more therapies were investigated to target the CSC population. However, CSCs usually lie dormant to avoid killing by chemotherapeutic drugs [14]. So how to promote the exit of CSCs out of the dormant stage is particularly important. Pre-treated/primed with IFNα can awaken dormant haematopoietic stem cells *in vivo* [15]. Whether IFNα can also activate dormant CSCs in OSCC to facilitate CSC's elimination by chemotherapy remains unclear.

In our current study we demonstrated that IFNα promoted the expression of CSC markers CD44 and ALDH1A1 *in vitro* and *in vivo* models of OSCC. Moreover, we showed that primed-IFNα can enhance the tumor-killing effect of chemotherapy. Our results strongly suggest that IFNα could be given to patients prior to chemotherapy in order to activate maximal therapeutic effects on CSC.

Materials and methods

Materials

Human recombinant IFNα was purchased from PeproTech company (Rocky Hill, NJ). A mouse monoclonal antibody against CD44 (CST) and a rabbit monoclonal antibody against ALDH1A1 (Abcam) were applied in this study. GAPDH and FITC-labeled secondary antibody were purchased from Proteintech (Rosemont, IL).

In vivo experiments

The nude mouse transplantation tumors were established with Cal27, a OSCC cell line, at 1×10^6 cells/point. IFNα was administrated 20 000 IU/day by subcutaneous injection after tumor formation, while the controls with normal saline (5 mice/group). Three weeks after administration, all the mice were sacrificed and the tumor tissues were preserved in liquid nitrogen and embedded in paraffin. Protein was extracted from the fresh tissue. Paraffin-cut sections were prepared for immunohistochemistry. The animal experiment was approved by Ethics Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine.

RNA isolation and qPCR analysis

Total RNA was extracted by TRIzol reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions, and cDNA was generated using a Reverse Transcriptase Kit. Real-time quantitative PCR was performed in triplicate with Power PCR SYBR Green Master Mix (Takara Biotechnology, Dalian, China) using the ABI PRIAM Step-One Real-time PCR System (Applied Biosystems, Carlsbad, CA, USA) with results normalized to GAPDH expression. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. CD44 primers: Forward: 5'- GGACCTTCATCCCAGTGACC -3', Reverse: 5'- GCTCCACCTTCTTGACTCCC - 3'. ALDH1A1 primers: Forward: 5'- ATCAAAGAAGCTGCCGGGAA -3', Reverse: 5'- GCATTGTCCAAGTCGGCATC -3'. GAPDH primers: Forward: 5'-CCTCTGACTTCAACAGCGAC -3', Reverse: 5'-TCCTCTTGTGCTCTTGCTGG -3'.

Immunohistochemistry and immunofluorescence

Xenograft tumors of nude mice were formalin-fixed and paraffin-embedded. Sections were heated by water bath at 100 °C with citrate buffer solution (pH 6.0) for 20 min to retrieve antigen. CD44 at 1:50 dilution and ALDH1A1 at 1:400 dilution were incubated overnight at 4 ℃. Goat secondary antibody against mouse/rabbit immunoglobulin was incubated for 1 hour at room temperature. 3, 3`-diaminobenzidine (DAB) detection was developed under light microscope. Every step of the wash was used phosphate buffered saline solution (PBS) for 5 min three times. The immunoreaction staining score was calculated by multiplying the percentage of positive cells and the staining intensity. For immunofluorescence, FITC-labeled secondary antibodies against mouse and rabbit were incubated for 1 hour in the cassette. The percentage of positive cells was counted under fluorescence microscope. Image-pro plus 6.0

software was used to measure mean optical density for immunohistochemistry analysis.

Cell cultures

The OSCC cell lines HN4 and HN30 were kindly presented by the by the University of Maryland Dental School (USA). These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin-streptomycin. All cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C.

Flow cytometry analysis of CSC markers

Flow cytometry was performed as described previously [16]. Briefly, HN4 and HN30 were treated with 0, 2, 20 ng/ml IFNα in 12 well-plate for 72 h. The cells were collected, washed and incubated with CD44 and ALDH1 antibodies at 1:100 dilution. After 1 hour incubation, cells were washed and incubated with FITC-labeled secondary antibody for 30 min. Then cells were washed twice and resuspended in 100 µl of FACS buffer. The stained cells were analyzed on BD FORTASA flow cytometer. The final results were operated using FlowJo software (Tree Star).

Western blot

Western blot assay was performed as briefly described [17]. Cells were lysed and quantified. Protein samples (30 µg/lane) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then electrophoretically transferred onto PVDF membranes using a wet transfer system (Bio-Rad, USA). The membranes were incubated overnight with the primary antibody of rabbit anti-ALDH1A1 monoclonal antibody (1:1 000) and mouse anti-CD44 monoclonal antibody (1:1 000). Antibodies against PARP, p-STAT1 (Tyr701), p-Akt (Ser473), p53, p-ERK1/2 (Thr202/Tyr204), and p-P38 (Thr180/Tyr182) were purchased from CST company and incubated at 1:1 000 dilution overnight. After incubating with fluorescent-based anti-rabbit/mouse IgG second antibody (Fermentas, Vilnius, Lithuania) at 1:10 000 dilution for 1 h in a dark place, immunoreactive bands were scanned and analyzed using Odyssey Infrared Imaging System (LICOR Biosciences, Lincoln, NE). GAPDH, β-actin, α-tubulin was used as internal control.

Tumorsphere formation

The sphere formation analysis was performed as described elsewhere [18]. Briefly, HN4 and HN30 cells were cultivated in the serum free RPMI-1640 medium containing 20 ng/ml of fibroblast growth factor, 20 ng/ml epithelial growth factor, and 1% B27, with 0, 2, 20 ng/ml of IFNα in low adhesion plate. After two weeks, all the spheres were filtered through a 70 µm-filter membrane. Only the spheres greater than 70 µm were counted. The sphere formation efficiency was calculated.

Chromatin Immunoprecipitation (ChIP)

ChIP was strictly operated according the protocol of SimpleChIP Enzymatic Chromatin IP kit (purchased from CST). After incubation with 20 ng/ml IFNα for 48 h, HN4 was fixed with 1% formaldehyde for 10 min and quenched with glycine for 5 min. Cells were washed twice in PBS and re-suspended in lysis buffer. Then the lysis was digested by micrococcal nuclease at 37°C for 20 min to length of approximately 150-900 bp. Digestion was terminated by the addition of 0.5 M EDTA. Chromatin was sonicated at 30% output for 6×10 sec. Clarify lysates by centrifugation at 10 000 rpm for 10 min at 4 °C. The supernatant was diluted with ChIP buffer (1:9). Add the immunoprecipitating antibodies into 500 µl of the diluted chromatin. For the positive control Histone H3 rabbit mAb, add 10 µl to the IP sample. For the negative control normal rabbit IgG, add 5 µl to the IP sample. 5 µl p-STAT1 (Try 701) (CST) was added into the sample. Incubate IP samples overnight at 4 °C with rotation. Add 30 µl of ChIP Grade Protein G Magnetic beads and incubate for 2 h at 4 °C with rotation. Wash the immunoprecipitated chromatin with three times low salt wash and once high salt wash. Eluted chromatin was reversed cross-links by adding 6 µl 5M NaCl and 2 µl proteinase K into every samples overnight at 65 °C. Reverse cross-linked DNA was purified by spin columns. Quantitative polymerase chain reaction analysis of purified ChIP DNA (ChIP-qPCR) was performed to calculate the enrichment percentage of promoter region using $2^{-\Delta \Delta CT}$ formula. The CD44 promoter region primers: Forward: 5'- ATCTAGGTG TTCTAGCTCCTGAATC -3', Reverse: 5'- CTACCATT CCTAGAGAAGGGAGTC -3'. The ALDH1A1 promoter region primers: Forward: 5'- CTTGGTCCCTAG GTTCTCACAA -3', Reverse: 5'- CACTTAAGCAGAT CTTTTCTGCC -3'.

Cell viability assay

HN4 and HN30 was incubated with 0, 2 ng/ml IFNα for 48 h. After INFα primed, 3 000 cells per well were seeded in the 96-well plates. They were then treated with the indicated concentration of drugs in 10% FBS DMEM containing CDDP, erlotinib, nimotuzumab for 72 h. The total cell numbers were assessed after incubation with 20 µl of MTT for 4 h. The optical density (OD) of each well was measured using a microplate reader at 490 nm, and the OD values are presented as the means $\pm$ SD.

Statistical analysis

Statistical analysis was performed using the software of GraphPad Prism version 6 for Windows (GraphPad Software, San Diego, CA, USA). All values are expressed as mean $\pm$ SD, and Student's t test was performed to assess the statistical significance of difference. P < 0.05 was considered statistically significant.

Results

IFNα induces the expression of CSC markers CD44 and ALDH1A1 *in vivo*

From *in vivo* experiment, we have confirmed the tumor inhibitory effect of IFNα in OSCC xenograft in nude mice. The tumor volume in IFNα group was much smaller than control (104.9 $\pm$ 22.1 vs 60.84 $\pm$ 7.88, P < 0.01. **Figure 1A**). The tumor weight in treatment group also decreased compared to control (227.5 $\pm$ 36.4 vs 122.9 $\pm$ 12.5, P = 0.02. **Figure 1B**). PCR assay demonstrated that mRNA expression of CD44 and ALDH1A1 was also significantly elevated in treatment group (**Figure 1C**). Immunohistochemistry (IHC) assay indicated that CD44 localizes on cell membrane, while ALDH1A1 is in the cytoplasm (**Figure 1D**). The relative mean optical density (MOD) of CD44 that represents the IHC score for CD44 expression was significantly higher in IFNα-treatment tumors than that in the control (5.667 $\pm$ 0.409 vs 1.100 $\pm$ 0.208, *P* < 0.01). Similarly, MOD of ALDH-1A1 was also much higher in IFNα treatment group. Our western blot showed that CD44 and ALDH1A1 proteins was elevated in tumors of random three mice of the treatment group compared to the untreated control (**Figure 1E**).

IFNα upregulates the expression of CD44 and ALDH1A1 and promotes tumorsphere formation *in vitro*

To investigate the impact of INFα on expression of markers in HNSCC cells, we treated HN4 and HN30 cells with IFNα and examined the expression CD44 and ALDH1A1 using flow cytometry. As shown in **Figures 2A-B**, after 72 h of treatment the mean fluorescence intensity (MFI) of CD44 and ALDH1A1 were increased in an INFα–dependent manner. Similarly, the percentage of CD44 and ALDH1A1 positive cells were significantly elevated after treatment (**Figures 2C-D**), suggesting that IFNα upregulated CD44 and ALDH1A1 expression in both HN4 and HN30 cells.
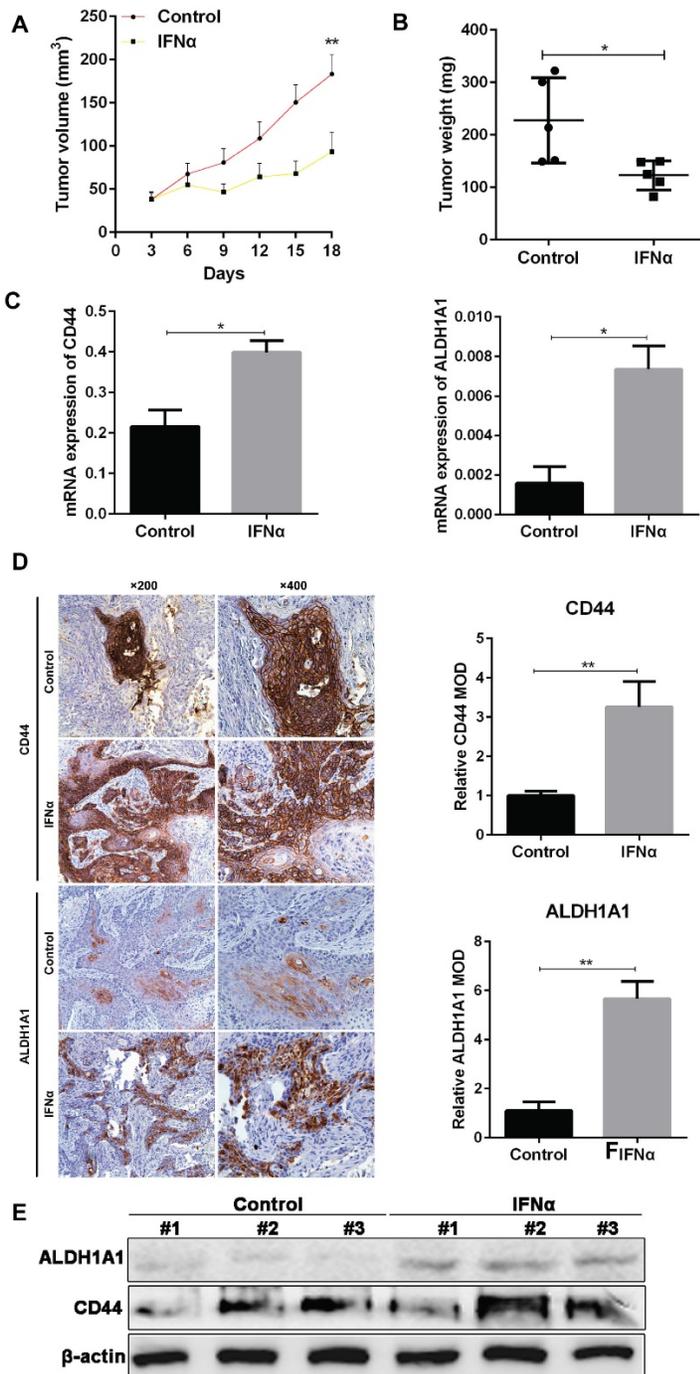
Figure 1. IFNα induces the expression of CSC markers CD44 and ALDH1A1 *in vivo*. (A) The tumor volume was measured every three days with vernier caliper and calculated as a×b^2/2 (a: length, b: width). (B) The tumor weight in each group was measured after administration. (C) Real-time PCR analyses of *CD44* and *ALDH1A1* mRNA in tumor samples from OSCC mouse xenograft with and without IFNα treatment. (D) IHC staining of CSC markers in OSCC xenograft tumor samples with and without IFNα treatment. Relative MOD of CD44 and ALDH1A1 in IHC staining was analyzed. (E) Western blot of tumor samples from OSCC mouse xenograft with and without IFNα treatment. Magnification: ×200, ×400.
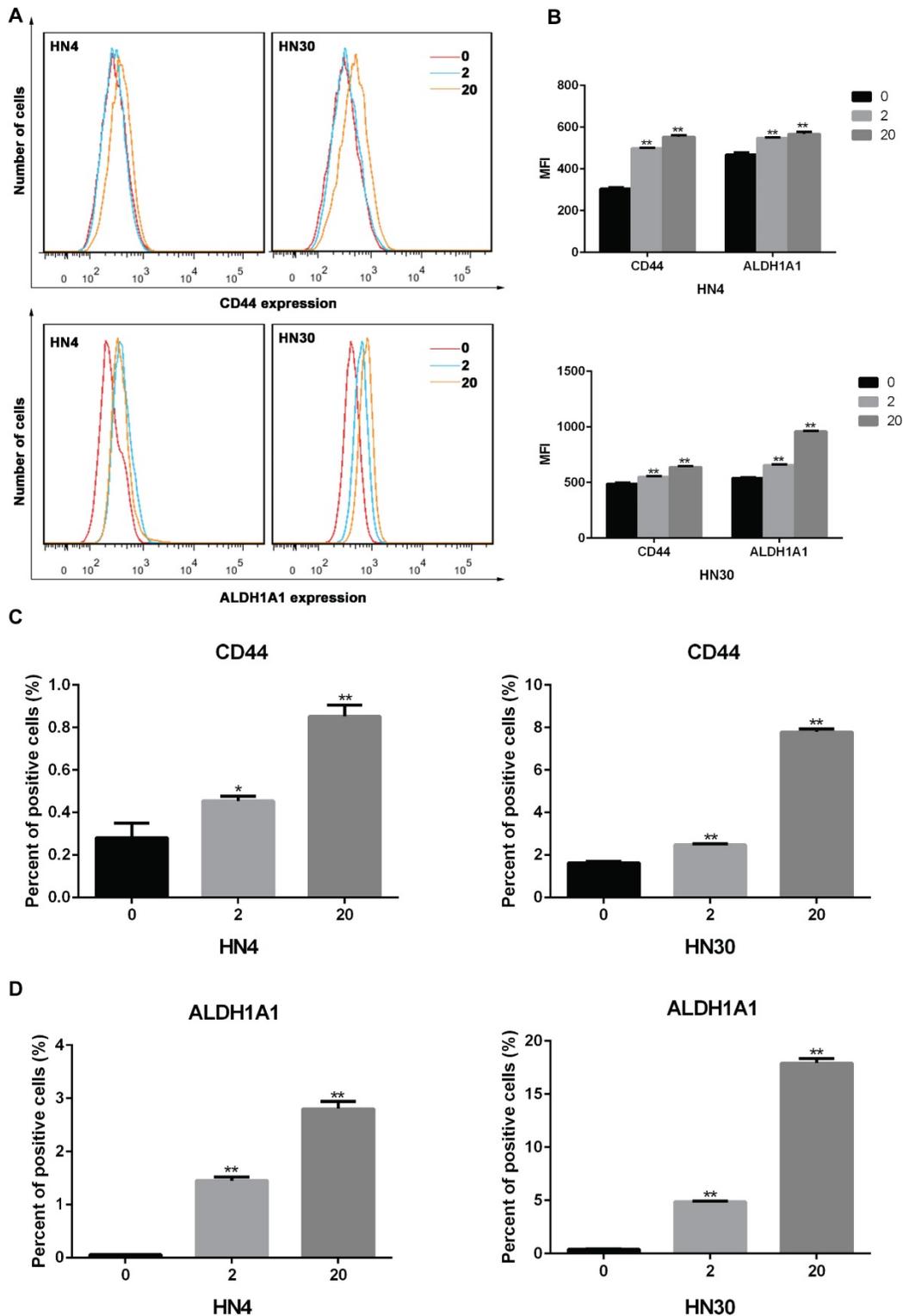
Figure 2. IFNα upregulates the expression of CD44 and ALDH1A1 *in vitro*. (A) Flow cytometry analyses of CD44 and ALDH1A1 expression in HN4 and HN30 cells in the absence (0) and presence of INFα treatment (2ng/ml; 20ng/ml) for 48h. (B) Mean fluorescence intensity (MFI) of CD44 and ALDH1A1 in the absence (0) and presence of INFα treatment (2ng/ml; 20ng/ml) for 48 h. (C-D) Percent of positive CD44 and (C) ALDH1A1 (D) cells in HN4 and HN30 before and after INFα treatment.

Consistent with the results from flow cytometry, western blot also indicated that the CD44 and ALDH1A1 expression were increased after IFNα incubation (**Figure 3A**), while immunofluorescence assay showed that CD44 and ALDH1A1 positive cells were enriched after 20 ng/ml IFNα treatment (**Figure 3B**). CD44 located on the cell membrane (**Figure 3C**), whereas ALDH1A1 located in the cytoplasm of HN4

and HN30 cells (**Supplementary Figure 1**). Together, our results demonstrated that IFNα upregulated CD44 and ALDH1A1 expression in both HN4 and HN30 cells in a dose-dependent manner.

Sphere-forming capacity of tumor cells correlates directly with CSC phenotype and thus tumorigenic efficiency of the cancer cells can be determined based on the number of spheres that originate from specific number of seeded cells [19]. HN4 and HN30 were examined for their ability to form tumorspheres *in vitro* (**Figure 4A**). The number of tumorsphere increased after IFNα stimulation for two weeks (**Figure 4B**). Furthermore, sphere forming efficiency also increased in HN4 and HN30 with the treatment of IFNα (**Figure 4C**), suggesting that IFNα promoted tumorsphere formation of HN4 and HN30 cells.

IFNα transcriptionally activates *CD44 and ALDH1A1* expression through inducing the binding of phosphorylated (activated) signal transducer and activator of transcription 1 (STAT1) to *CD44 and ALDH1A1* promoters

In response to IFNα stimulation, STAT1 forms hetero-dimers with STAT2 that bind the Interferon-Stimulated Response Element (IRSE) promoter element [20]. So we speculated that IFNα transcriptionally activated *CD44* and *ALDH1A1* expression through similar mechanism. In support of this, ChIP-PCR assay indicated that Tyr-701 phosphorylated STAT1 bound to the promoter region of *CD44* and *ALDH1A1* under IFNα stimulation in HN4 cells (**Figure 5A**), whereas no obvious binding was detected in normal IgG control (**Figure 5B**). This result strongly suggests that IFNα can induce phosphorylation (activation) of STAT1, leading its binding to the upstream promoters of *CD44* and *ALDH1A1* and transcriptionally activate the expression of *CD44* and *ALDH1A1* in HN4 cells.

Primed-IFNα can enhance the tumor-killing effect of CDDP, Erlotinib and Nimotuzumab

From the above results, we confirmed the activation of CSC markers by IFNα in OSCC cells. We hypothesized that the tumor cells would be more sensitive to chemotherapy after activation of CSC. To test this, HN4 and HN30 were treated with 20 ng/ml IFNα for 48 hour, and then seeded in 96-well plates and further treated with CDDP (a classic cytotoxic drug for OSCC), Erlotinib and Nimotuzumab (epidermal growth factor receptor inhibitors), respectively. Nimotuzumab binds to the epidermal growth factor receptor (EGFR) and Erlotinib is a tyrosine
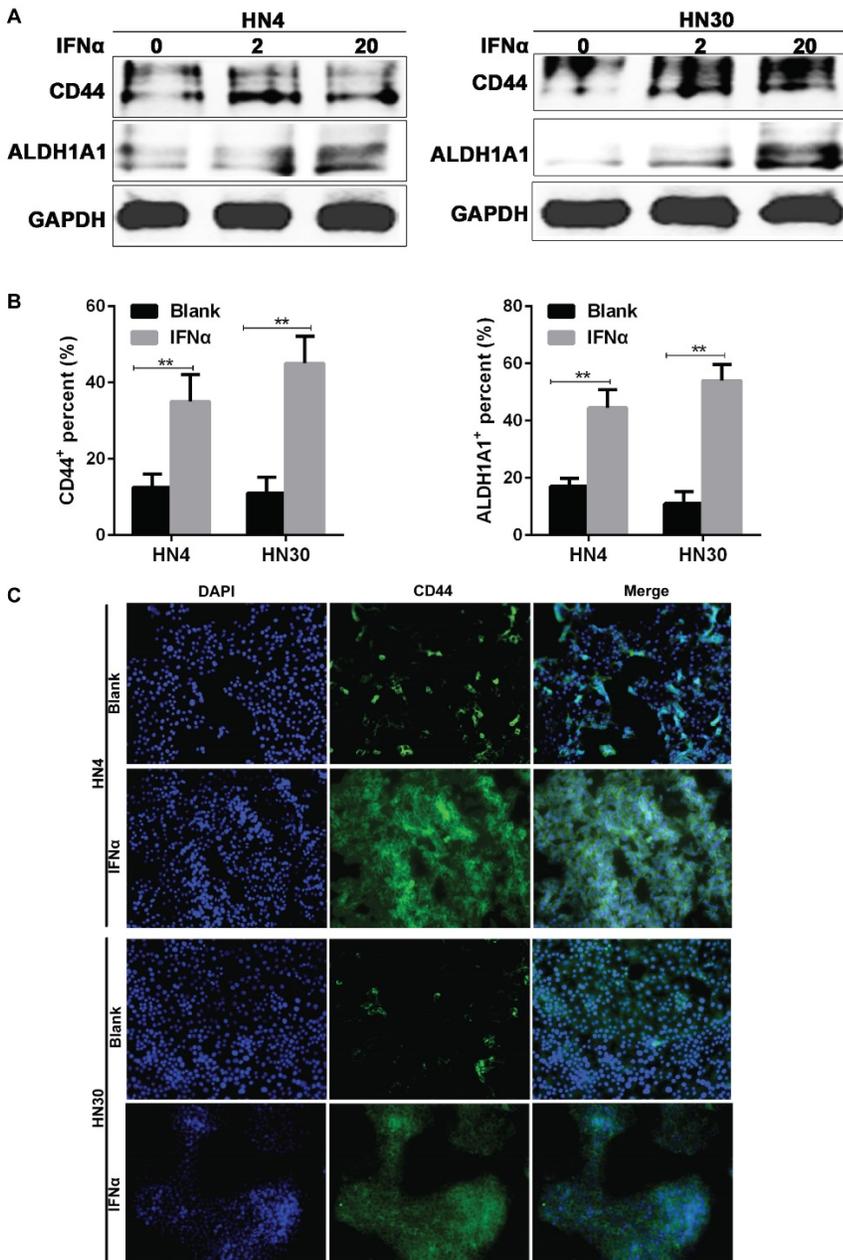
Figure 3. IFNα upregulates the expression of CD44 and ALDH1A1 in OSCC cells. (A) Western blot of CD44 and ALDH1A1 in HN4 and HN30 cells before and after IFNα treatment (2ng/ml; 20ng/ml) for 48h. (B) Immunofluorescent (IF) assay of CSC markers in HN4 and HN30 cells before and after IFNα treatment. (C) Representative images of IF staining of CD44 in HN4 and HN30 in the presence and absence of IFNα treatment. Magnification: ×200.

kinase inhibitor of EGFR in HNSCC [21]. Our result showed that IFNα sensitized the cells to the tumor inhibitory effect of CDDP (**Figure 6A**), Erlotinib (**Figure 6C**), and Nimotuzumab (**Figure 6E**), respectively. Combination of primed-INFα treatment with 1 µg/ml CDDP, PARP, p-ERK and p-P38 were elevated while p-Akt and p53 decreased (**Figure 6B**). Combination of primed-INFα treatment with 5 µM Erlotinib, p53 decreased while p-STAT1, p-ERK and p-P38

increased (**Figure 6D**). In addition, PARP, p-Akt, p-P38 and p-ERK were increased and only p53 decreased under 200 µg/ml Nimotuzumab (**Figure 6F**). Taken together, our results demonstrated that OSCC cells primed with IFNα can be efficiently eliminated by treatment of CDDP, Erlotinib and Nimotuzumab *in vitro*.
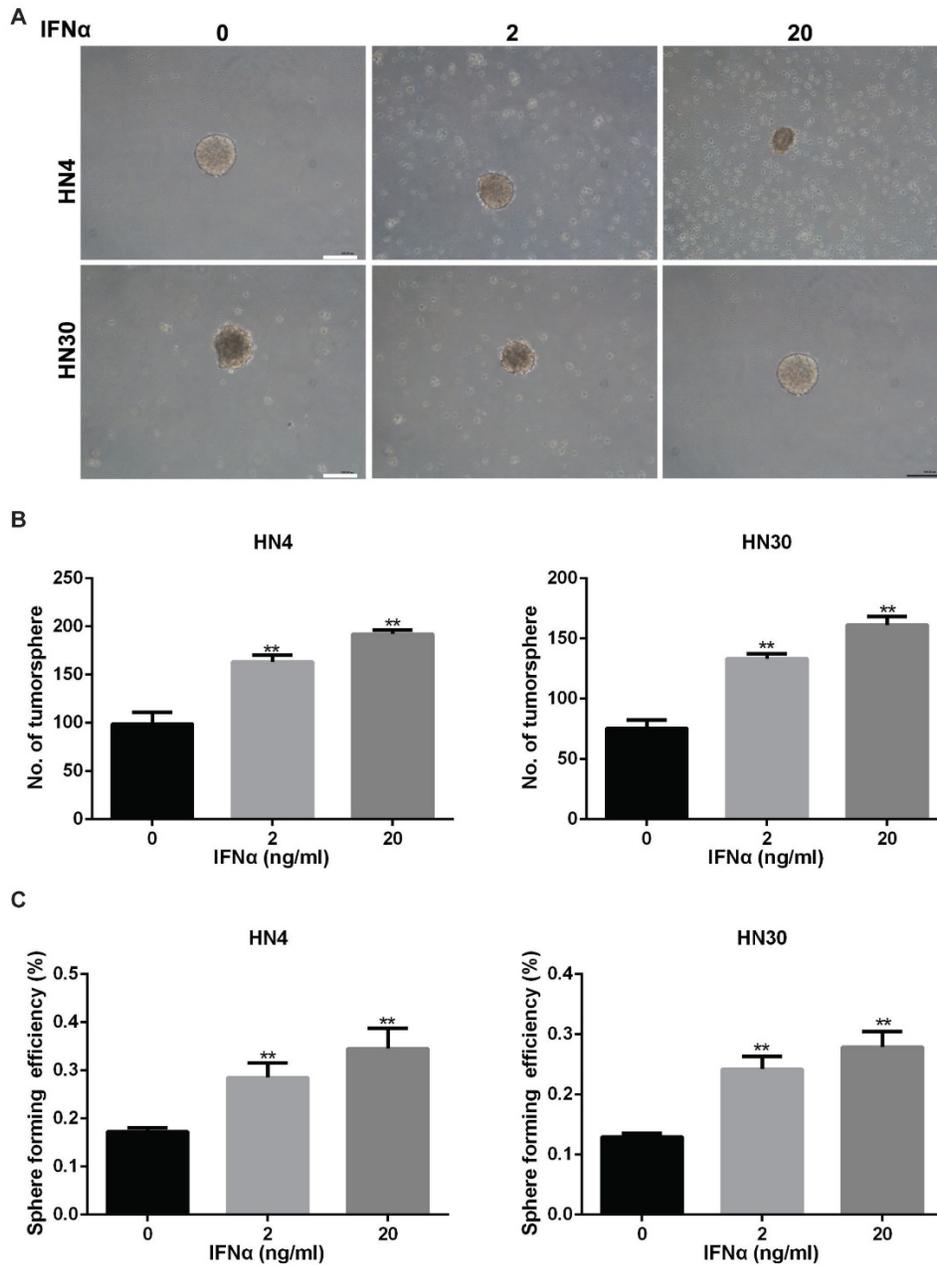
Figure 4. IFNα enhances tumorsphere formation ability in HN4 and HN30 cells. (A) Representative images of tumorsphere formation in conditioned medium with indicated concentration of IFNα (2ng/ml; 20ng/ml) for 2 weeks. Scar bar: 1mm. (B) Quantitation of the number of tumorsphere formation in HN4 and HN30 cells in the presence and absence of IFNα incubation. (C) Sphere forming efficiency of HN4 and HN30 cells in the presence and absence of IFNα incubation.
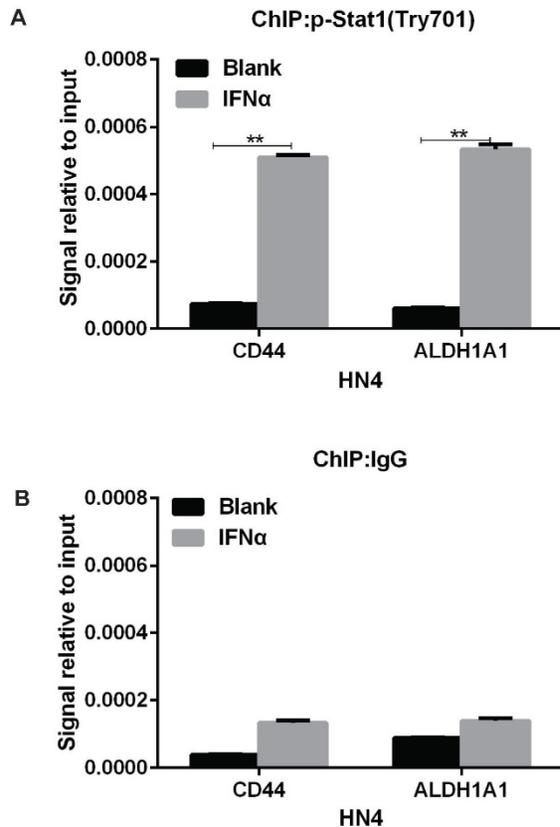
A

ChIP:p-Stat1(Try701)

B

ChIP:IgG

Figure 5. IFNα transcriptionally activates *CD44 and ALDH1A1* expression through inducing the binding of phosphorylated STAT1 to *CD44 and ALDH1A1* promoters. (A) ChIP assay of p-STAT1 (pTry701) in promoter regions of *CD44 and ALDH1A1* were under IFNα treatment for 48 h. (B) ChIP assay of control IgG in promoter regions of *CD44 and ALDH1A1* were under IFNα treatment.

Discussion

In this study, we demonstrated that IFNα can upregulated CSC markers of oral cancer cells and primed-IFNα sensitized the therapeutic effect of CDDP, Erlotinib and Nimotuzumab. CSCs have stem features such as self-renewal, high migration capacity, drug resistance, high ability of tumorigenicity. In OSCC, CSCs have been increasingly shown to have an integral role in tumor initiation, disease progression, metastasis and treatment resistance [22]. So targeting for the eradication of CSCs was critical. In haematopoietic stem cells, three factors-granulocyte colony-stimulating factor, IFNα and arsenic trioxide - have been shown to efficiently activate dormant stem cells and thereby could break their resistance to anti-proliferative chemotherapy [14]. IFNα up-regulated the expression of the CSC markers CD24, CD44 and CD133 in *in vitro* and *in vivo* models of pancreatic ductal adenocarcinoma [23], which was consistent with our results. Our results suggested that IFNα promoted the expression of CSC markers through transcription activation in HN4 cells (Figure 5). However, we failed to detect IFNα-induced

binding of STAT1 to the promoter of CSC markers in HN30 cells (data not shown). The difference may come from cellular heterogeneity, such as wild-type p53 expression in HN30 [24]. IFNα may promote the expression of ALDH1A1A in HN30 through other STAT members, which perhaps can bind the promoter region of ALDH1A1. Furthermore, IFNα may promote it through post-transcriptional regulation, posttranslational regulation and the modulation by interacting proteins. A number of studies have demonstrated that IFNα had a direct cytotoxic effect on various tumors and acted as a chemo-sensitizing agent [25, 26]. So IFNα can enrich CSCs in tumors while directly kill the bulk of the tumor cells. This distinctive feature of IFNα can provide a new method for CSC-targeted therapy in OSCC.

While CD44 and ALDH1A1 were used as biomarkers for CSC in OSCC in our study, BMI-1 is another stem cell-related biomarker involved in the carcinogenesis in HNSCC [12]. CD133 antigen, known as prominin-1 has been also identified as a CSC marker in several cancers and particularly in the laryngeal cancer [27]. However, the combined use of ALDH1 and CD44 is more relevant for identifying CSC-like populations as it is more selective than any other marker used alone [28]. ALDH expression was seen in only 1%-7.8% of HNSCC cells, and were able to form tumors with injections of as few as 500 cells into mice. While, HNSCC tumors shown a proportion of CD44+ cells ranging from 0.1% to 41.72% of the tumor population [27]. Combination of CD44 and ALDH1A1 can identify almost all the CSC population in HNSCC, which had strong capacity of tumorigensis. So two generally accepted biomarkers - CD44 and ALDH1A1, were adopted in our investigation.

Accumulating evidences have demonstrated that IFNα possessed chemo-sensitizing characteristics under different drugs. Radical surgery followed by IFNα and 5-FU combination chemotherapy offers possibility of long-term survival despite massive hepatocellular carcinoma with multiple intrahepatic metastases [29, 30]. IFNα pre-treatment followed by a combination of IFNα plus Erlotinib significantly enhanced the sensitivity of colon cancer cell lines [31]. IFNα enhanced the sensitivity to an anti-epidermal growth factor receptor monoclonal antibody (Nimotuzumab) in a human lung cancer cell line with intermediate expression of the receptor [32]. Low dose of IFNα intensified antineoplastic effect of a histone deacetylase -inhibitor, valproic acid, and the mamm-alian target of rapamycin inhibitor everolimus in prostate cancer cells [33]. It should be noted that in these studies IFNα was administrated together with chemoradiotherapy. Patients can benefit from

unspecific activation of the immune system through IFNα to obtain better outcomes. While the future of

Figure 6. IFNα pretreatment enhances the sensitivity of OSCC cells to chemotherapy. (A) Cell viability of OSCC cells pretreated with 2 ng/ml IFNα, followed by CDDP treatment at different concentration. (B) Western blot of OSCC cells pretreated with 2 ng/ml IFNα, followed by CDDP treatment (1μg/ml). (C) Cell viability of OSCC cells pretreated with 2 ng/ml IFNα, followed by erlotinib at different concenrtartion. (D) Western blot of OSCC cells pretreated with 2 ng/ml IFNα, followed by 5 μM erlotinib incubation. (E) Cell viability of OSCC cells pretreated with 2 ng/ml IFNα, followed by nimotuzumab treatment at different concentration. (F) Western blot of OSCC cells pretreated with 2 ng/ml IFNα, followed by 200 ng/ml nimotuzumab incubation for 48 h.

enrichment of CSCs was implied with IFNα-primed in our study, only CDDP, Erlotinib and Nimotuzumab can be sensitized with pretreated with low dose IFNα in HN4 and HN30 (Figure 6). This synergistic effect should be further explored in *in vitro* study. Our results showed that p38 and ERK1/2 were activated after IFNα pretreatment, while p53 decreased in OSCC cells (Figure 6B, D, F). The activation of Akt depend the chemotherapies. p38 could be strongly activated by a wide variety of environmental and cellular stresses or by inflammatory cytokines [34]. Under certain conditions, ERK1/2 can also have pro-apoptotic functions which has lead this pathway to receive significant attention for development of cancer therapies [35]. Poly (ADP-ribose) polymerase (PARP) can be activated in cells experiencing stress and/or DNA damage. Our results showed that PARP was also elevated and activated under IFNα-primed and chemotherapy treatment. It should be noted that STAT1 that would be directly activated by IFNα was not significantly activated because of IFNα withdrawal under IFNα-primed and chemotherapy treatment. Taken together, IFNα could be given prior to chemotherapy to enrich the CSC and followed with concurrent with drugs to activate immune system. This hypothesis should be confirmed in future preclinical studies.

In summary, our studies indicate that IFNα can activate dormant CSCs in OSCC to facilitate the killing of CDDP, Erlotinib and Nimotuzumab. Our results provide experimental evidences and new treatment strategy for application of IFNα in OSCC.

Supplementary Material

Figure S1. http://www.jcancer.org/v08p2384s1.pdf

Acknowledgements

Competing Interests

The authors declare no conflicts of interest.

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