• Basic Research •

IFNα2b/5-FU inhibits proliferation and cell cycle of squamous carcinoma cell line Cal27

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Abstract

● **AIM:** To investigate the pathological features of ocular surface squamous neoplasia (OSSN) and evaluate the synergistic therapeutic effects of interferon-α2b (IFNα2b) and 5-fluorouracil (5-FU) on cellular proliferation, migration, apoptosis, and cell cycle of human oral squamous carcinoma cell line Cal27.

● **METHODS:** Tissue specimens from OSSN were processed with hematoxylin-eosin (HE) and immunofluorescence (IF) staining to characterize pathological changes. We analyzed the expression levels of four pivotal proteins involved in 5-FU metabolism: interferon alpha receptor (IFNAR), thymidylate synthase (TS), thymidine phosphorylase (TP), and dihydropyrimidine dehydrogenase (DPD). Cal27 cell lines were treated with a spectrum of concentrations of IFNα2b and 5-FU, either in isolation or in combination. Then, cell activity was measured utilizing CCK-8 assay and dose-effect curves were calculated, while tumor cell migration was detected by cell scratch experiments. Cal27 cells were added with IFNα2b and 5-FU in a non-constant ratio drug combination design and the corresponding combination index (CI) and fraction affected (Fa) were calculated with CompuSyn software. Western blot assay was conducted to quantify the expression of TP, TS, and DPD. Cell cycle and apoptosis were measured with flow cytometry and terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling (TUNEL) assay.

● **RESULTS:** Treatment with both IFNα2b and 5-FU inhibited cell proliferation. Except for the lowest and highest doses of 5-FU, CI values for all other groups were below 1, suggesting a synergistic interaction. Low concentrations of IFNα2b and 5-FU both diminished the relative mobility of Cal27 cells, instead, a stronger inhibitory effect was observed when the two drugs were co-applied. The expression levels of TP and DPD in Cal27 cells were dose-dependently increased at a low concentration of IFNα2b. Low-dose IFNα2b combined with 5-FU significantly inhibited cell proliferation in G0/G1 phase compared to 5-FU monotherapy. Medium and high doses of IFNα2b and all concentrations of 5-FU could induce apoptosis in a concentration-dependent manner. The susceptibility to 5-FU treatment and apoptosis rates of tumor cells were elevated with low doses of IFNα2b.

● **CONCLUSION:** Both IFNα2b and 5-FU, when administered individually or in combination, effectively suppress the proliferation and migration of Cal27 tumor cells, induce cell apoptosis and arrest cell cycle. Low doses of IFNα2b increase the antitumor effects of 5-FU on Cal27 potentially through up-regulating the expression of TP, demonstrating a synergistic effect between IFNα2b and 5-FU.

● **KEYWORDS:** ocular surface squamous neoplasia; squamous carcinoma cells; interferon-α2b; 5-fluorouracil; combination index

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INTRODUCTION

Ocular surface squamous neoplasia (OSSN) is the most common non-melanocytic ocular surface cancer manifested as a unilateral, vascularized limbal tumor, and can be histologically divided into conjunctival epithelial dysplasia,

conjunctival intraepithelial neoplasia and invasive squamous cell carcinoma[1-3]. The reported incidents of OSSN range from 0.13 to 1.9 per 100 000, with extremely high morbidity in Africa, reaching 3.5 per 100 000/y[4-5]. Owing to its potential to invade intraocular and orbital region, OSSN progressively causes irreversible visual impairment and endangers life quality[6]. In the past decade, the preferred consensus regarding first-line therapy for OSSN has transformed from surgical excision with no-touch technique towards topical monotherapy with chemotherapeutic drugs[7-8]. Topical medications focusing on synergistic therapeutic effects of interferon-α2b (IFNα2b) and 5-fluorouracil (5-FU) gain reputation as effective alternatives for treating microinvasive malignancy diffusedly, facilitating tumor resolution and targeting specific lesions[9-11].

5-FU, a structural pyrimidine analog, inhibits DNA synthesis and ultimately cellular proliferation[12]. Thymidylate synthase (TS), thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD), the key enzymes of DNA synthesis and 5-FU metabolism, are associated with topical chemotherapy sensitivity, tumor progression and prognosis[13-14]. The multifaceted effects of interferon (IFN) are attributed to anti-viral, immunomodulatory, antiproliferative and anti-angiogenic properties by interacting with interferon alpha receptor (IFNAR)[15-16]. However, applying IFNα2b and 5-FU separately showed significant adverse effects and drug resistance, ultimately limits therapeutic efficacy and restricts their clinical application[17]. To maximum their efficiency and minimize side effects, the combination treatment of IFNα2b and 5-FU has been broadly explored in various fundamental and experimental researches. Our previous clinical investigation has revealed the increased tumor resolution and decreased recurrence rate of this safe and effective combination therapy of IFNα2b and 5-FU for OSSN[18]. Although their functions are hypothesized to be related to IFNα2b's effects on the key enzymes involved in 5-FU-mediated DNA damage, the specific cellular and molecular mechanisms remain elusive[19-20].

In this study, we specifically investigated the impacts of IFNα2b and 5-FU on proliferation, migration, apoptosis, and cell cycle of human oral squamous carcinoma cell lines Cal27, expecting to elucidate how chemotherapeutic drugs cooperate to modulate tumor behavior.

PARTICIPANTS AND METHODS

Ethical Approval This retrospective study was approved (UHCT22048) by the Ethics Committee of Wuhan Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, and the study was conducted in accordance with the tenets of the Declaration of Helsinki. The institutional review board waived the requirement for informed consent owing to the retrospective nature of the study.

Human Tumor Tissue Preparation Fresh tumor tissue samples (n=6) diagnosed as OSSN from June 2021 to June 2022 at Union Hospital were collected and healthy conjunctival tissues from donors (n=3) during the same period were taken as the control group. All tissues were preserved in 4% paraformaldehyde or quickly frozen in liquid nitrogen after comprehensive irrigating with sterile saline solution several times. Comprehensive ophthalmic examinations were performed on OSSN cases and anterior segment photographs were captured before surgical intervention.

Histology and Immunofluorescence Staining Tissue samples were fixed in 4% paraformaldehyde for 24h and dehydrated with a series of sucrose before being embedded in optimum cutting temperature (OCT) compound (Sakura Finetek, Japan) for sectioning at a thickness of 6 μm. Then hematoxylin and eosin (HE) staining was employed to assess the morphology of the sections.

The sections were blocked in 10% donkey serum (AntGene, China) for 30min at room temperature, and incubated with rabbit polyclonal antibodies against IFNAR1 (ABclonal, China), IFNAR2 (ABclonal), TP (Proteintech, USA), TS (Proteintech), and DPD (Affinity Biosciences, USA) at 4℃ overnight. After washing with tris-buffered saline and Tween 20 (TBST), samples were stained with donkey anti-rabbit IgG (Thermo Fisher Scientific, USA) for detection. Then 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Beijing Solarbio Biotech Co., China) was utilized for nucleus staining.

Cell Culture and Cell Viability Assay Human oral squamous cell carcinoma cell line Cal27, purchased from FuHeng BioLogy (Shanghai, China),was cultivated in DMEM high glucose medium containing fetal bovine serum (HYCEZMBIO, China) at 37℃ with 5% CO_2/95% air. Cal27 cells were passaged upon reaching 80% confluence.

Cell viability was assessed *via* the cell counting kit-8 assay (CCK8; Abbkine, Wuhan, China). Cal27 cells were seeded in 96-well plates, with negative blank wells containing medium and CCK8 only, and positive control wells containing untreated cells, medium and CCK8. After cultivated for 24h, cells were simultaneously treated with IFNα2b, 5-FU or a combination of both at different concentrations for 48h. Concentration gradients of IFNα2b were 2.56×10, 1.28×10^2, 6.4×10^2, 3.2×10^3, 1.6×10^4, 8×10^4, 4×10^5, 2×10^6 IU/mL, and the set gradients of 5-FU were 0.064, 0.32, 1.6, 8, 40, 200, 1000, 5000 μg/mL. Subsequently, 20 μL of CCK8 solution per well was added and incubated at 37℃ for another 2h. The optical density (OD) values were measured at 450 nm using a microplate reader (PerkinElmer, USA). The dose-effect curves were drawn, and fraction affected (Fa) and combination index (CI) value of the combination drugs were calculated by CompuSyn software.

Western Blotting Different concentrations of IFNα2b were added to the Cal27 cells at 1×10^5, 1×10^7, 1×10^9 IU/L for 48h and the expressions of key enzymes TP, TS and DPD were analyzed. Proteins were extracted using RIPA lysis buffer supplemented with PMSF and their concentrations were measured. Proteins were separated by electrophoresis on 12% SDS-PAGE gels and transferred to PVDF membranes. The samples were blocked by 5% fat-free milk for 1h, followed by incubation with primary antibody [(TP, 1:2000, Proteintech; TS, 1:5000, Proteintech; DPD, 1:1000, Affinity; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 1:50000, 60004-1-IG)] and HRP-conjugated goat anti-rabbit IgG antibody (1:5000, Proteintech). The products were visualized with the gel imaging system (Bio-rad, USA).

Flow Cytometry For the analysis of cell apoptosis and cycle, Cal27 cells were treated with various concentrations of drugs, 1×10^5 IU/L IFNα2b, 1×10^7 IU/L IFNα2b, 1×10^9 IU/L IFNα2b, 0.2 μg/mL 5-FU, 2 μg/mL 5-FU, 20 μg/mL 5-FU and 1×10^5 IU/L IFNα2b+0.2 μg/mL 5-FU, respectively for 48h. Untreated Cal27 cells served as a control.

After two washes with phosphate buffered saline (PBS), adding 100 μL of 1×binding buffer to resuspend cells. Totally 5 μL Annexin FITC and 2 μL PI were added and mixed gently. Then, 400 μL of 1×binding buffer was added, mixed and samples were placed on ice. Flow cytometry was performed within 1h.

Cell suspension at a density of 5×10^5 to 1×10^6 was slowly added into precooled 70% ethanol, fully mixed, and incubated overnight at 4℃. Monitor the fluorescence intensity with a flow cytometer to detect cell cycle.

TUNEL Assay Single cells were resuspended and seeded to 24-well plates. After incubated at 37℃ with 5% CO_2 overnight, cells were treated with multiple drugs of blank, 1×10^5 IU/L IFNα2b, 1×10^7 IU/L IFNα2b, 1×10^9 IU/L IFNα2b, 0.2 μg/mL 5-FU, 2 μg/mL 5-FU, 20 μg/mL 5-FU, 1×10^5 IU/L IFNα2b+0.2 μg/mL 5-FU, cultivated for 48h. Samples were fixed in 4% paraformaldehyde for 30min, followed by immersion in 0.3% Triton X-100 /PBS at 37℃ for 5min and two washes with PBS. Sections were stained in 50 μL of terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling (TUNEL) reaction mixture for 1h at 37℃ in the dark. After washing the specimens three times with PBS, nuclei were revealed by DAPI staining and then images were acquired.

Cell Migration For tumor cell migration assays, Cal27 cells were seeded at 3.5×10^5 cells/well in 6-well plates and cultivated at 37℃ with 5% CO_2 overnight. The cells were stimulated with different drugs of 1×10^5 IU/L IFNα2b, 1×10^7 IU/L IFNα2b, 1×10^9 IU/L IFNα2b, 0.2 μg/mL 5-FU, 2 μg/mL 5-FU, 20 μg/mL 5-FU, 1×10^5 IU/L IFNα2b+0.2 μg/mL 5-FU, respectively. Untreated Cal27 cells served as a control. The cells were then scratched with a 200 μL pipette tip, rinsed three times using PBS and added serum-free medium. Images of the cells were collected consecutively at 0, 6, 24, 48h and analyzed.

Statistical Analysis All results were expressed as mean±standard deviation (SD) from at least three independent experiments. Original experiment data were analyzed using GraphPad Prism 8 software. Statistical significance for multiple groups was assessed using one-way analysis of variance and independent sample *t*-test was used for two groups. Differences were considered statistically significant when $P<0.05$.

RESULTS

Upregulated TP Expression in OSSN Morphology of normal conjunctiva and OSSN tissue were observed with anterior segment photography and HE staining. By the pathology department of our institution, four tissues out of the collected OSSN samples were diagnosed as papilloma, one case as papilloma with dysplasia and one as squamous cell carcinoma (SCC; Figure 1). Compared to well-arranged structures of normal tissues, papillary tumors exhibited high proliferated epithelial cells, atypia and irregular arrangement. Meanwhile, SCC displayed variable epithelial cell sizes, obvious atypia, and squamous epithelial papilloma-like proliferation with infiltrative growth and numerous nutrient vessels (Figure 2). Immunofluorescence (IF) staining demonstrated that IFNAR2 was highly expressed in the membrane and intercellular substance of both normal conjunctiva and OSSN, whereas low level IFNAR1 was expressed in only two cases of OSSN. The weak expression of TP was primarily seen at the stromal margin infiltrated by tumor cells (Figure 2). In addition, the expression of TP in tumor tissues was significantly higher compared to normal tissues ($P<0.05$; Table 1). TS and DPD were predominantly localized in the cytoplasm of normal conjunctiva epithelial cells and tumor cells, with lesser nuclear localization (Figure 2). At the protein level, quantification of positive rates of IFNAR1, IFNAR2, TS and DPD revealed no statistical significance, albeit the observed rates were higher in tumor cells than normal tissues (Table 1).

These findings indicate that the high expression of IFNAR may explain the sensitivity of IFNα2b treatment on OSSN, and that up-regulation of TP is consistent with the rapid proliferation observed in tumor cells.

Synergetic Inhibition of Cal27 Cells Proliferation by IFNα2b and 5-FU Cal27 cells were treated with different gradients of IFNα2b and 5-FU for 48h. The concentrations used were as follows: for IFNα2b, 2.56×10, 1.28×10^2, 6.4×10^2, 3.2×10^3, 1.6×10^4, 8×10^4, 4×10^5, and 2×10^6 IU/mL; and for 5-FU, 0.064, 0.32, 1.6, 8, 40, 200, 1000, and 5000 μg/mL. The dose-effect curves indicated that both IFNα2b and 5-FU alone suppressed cell proliferation, and that the calculated

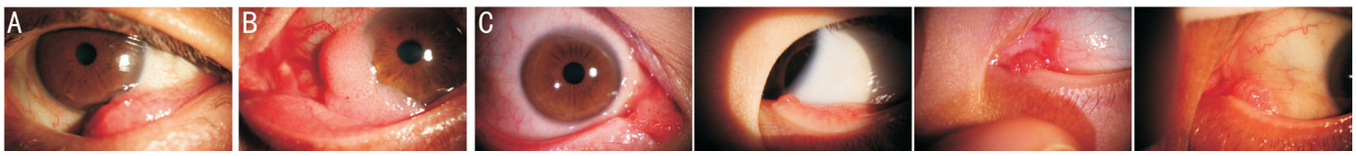

Figure 1 Anterior segment photography of appearance of OSSN A: One case of papillary tumor with dysplasia; B: One patient was diagnosed as SCC; C: Four cases were diagnosed as papillary tumors. OSSN: Ocular surface squamous neoplasia; SCC: Squamous cell carcinoma.

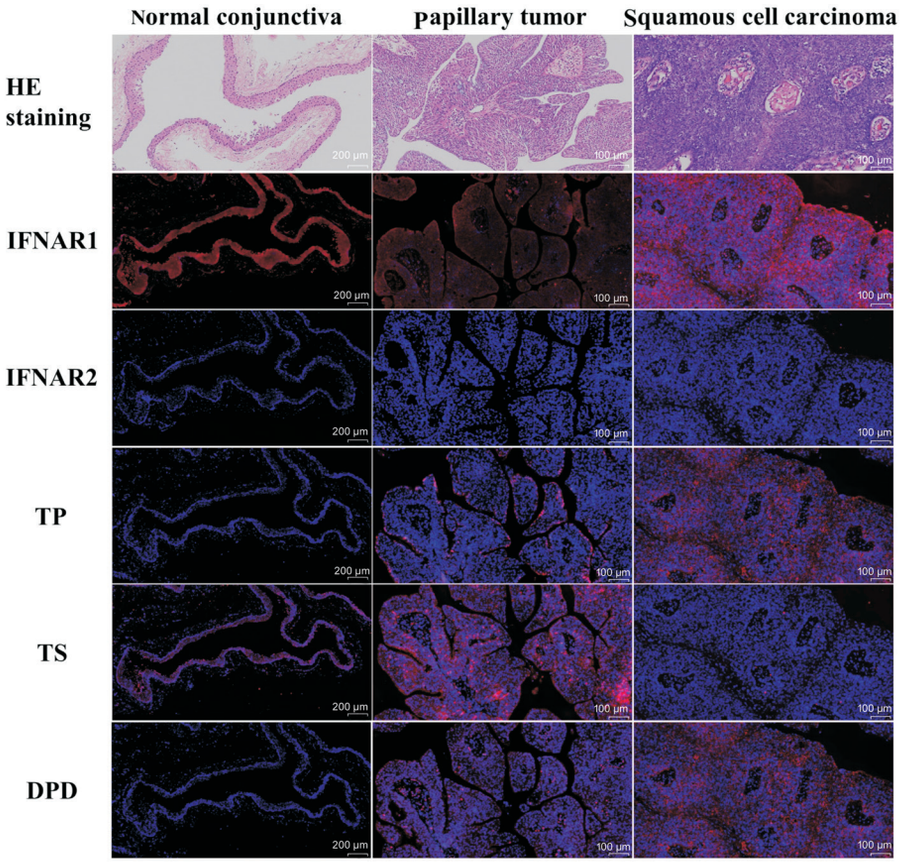

Figure 2 HE and IF staining of normal conjunctiva and OSSN tissues HE staining for normal conjunctiva, papillary tumor and SCC. Representative merge images of IF staining against IFNAR1, IFNAR2, TP, TS, and DPD. Normal scale bar =200 μm. Tumors scale bar =100 μm. HE: Hematoxylin-eosin; IF: Immunofluorescence; SCC: Squamous cell carcinoma; IFNAR: Interferon alpha receptor; TS: Thymidylate synthase; TP: Thymidine phosphorylase; DPD: Dihydropyrimidine dehydrogenase.

Table 1 The positive rates of IFNAR and 5-FU metabolic enzymes in donor conjunctiva and OSSN tissues

Parameters	*n*	IFNAR1		IFNAR2		TP		TS		DPD	
		Positive rate	*P*	Positive rate	*P*	Positive rate	*P*	Positive rate	*P*	Positive rate	*P*
Donors	3	0	0.5	100%	>0.99	0	0.048	33.3%	>0.99	33.3%	0.226
OSSN	6	33.3%		100%		83.3%		50%		83.3%	

IFNAR: Interferon alpha receptor; TS: Thymidylate synthase; TP: Thymidine phosphorylase; DPD: Dihydropyrimidine dehydrogenase; OSSN: Ocular surface squamous neoplasia.

IC50 of IFNα2b and 5-FU were respectively 8×10^4 IU/mL and 7 μg/mL (Figure 3A, 3B). To confirm IFNα2b potential to enhance inhibitory ability of 5-FU on tumor cells, Cal27 cells were exposed to a non-constant combination ratio treatment, consisting of 100 IU/mL IFNα2b in combination with different concentrations of 5-FU for 48h. Then the CI value of both drugs was calculated based on Chou-Talalay method by CompuSyn software[21]. Except for the lowest dose of 5-FU at 0.032 μg/mL and the highest dose at 2500 μg/mL, CI values of all other groups were below 1, indicative of a synergistic effect (CI<1). The combination of 5-FU at 2500 μg/mL with IFNα2b at 100 IU/mL exhibited an approximately additive effect (CI≈1, Figure 3C, 3D). At the equivalent fraction affected (Fa=0.57), the required 5-FU dosage could be significantly reduced when used in combination with IFNα2b, amounting to only 1/13. Totally 1601 of that required for 5-FU monotherapy (Table 2).

Upregulation Expression of Metabolism-Related Enzymes TP and DPD by IFNα2b Based on the dose-effect curve,

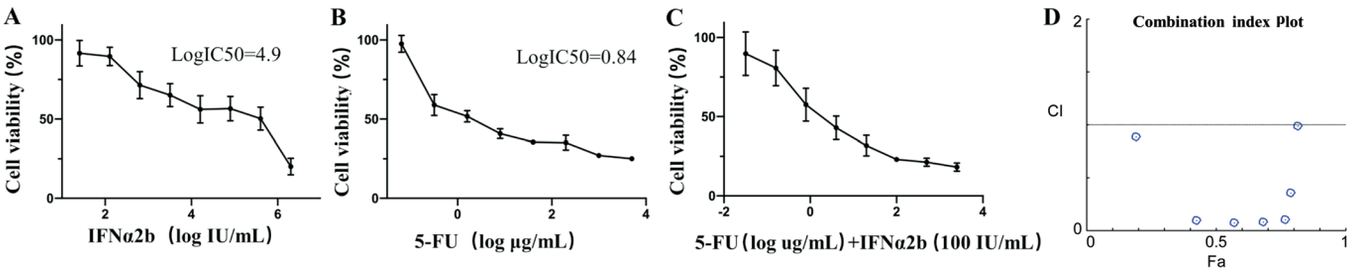

Figure 3 Combination influence of INFα2b and 5-FU on cell proliferation A, B: Dose-effect curves after 48h treatment with IFNα2b alone (A) and 5-FU alone (B); C: Dose-effect curves after 48h treatment with 100 IU/mL IFNα2b in combination with 5-FU of different concentrations (0.032, 0.16, 0.8, 4, 20, 100, 500, 2500 μg/mL); D: Fa-CI curve of each group of IFNα2b and 5-FU in non-constant combination ratios. The data were shown as the mean±SD. IC50: Half maximal inhibitory concentration; IFNα2b: Interferon-α2b; 5-FU: 5-fluorouracil; Fa: Fraction affected; CI: Combination index; SD: Standard deviation.

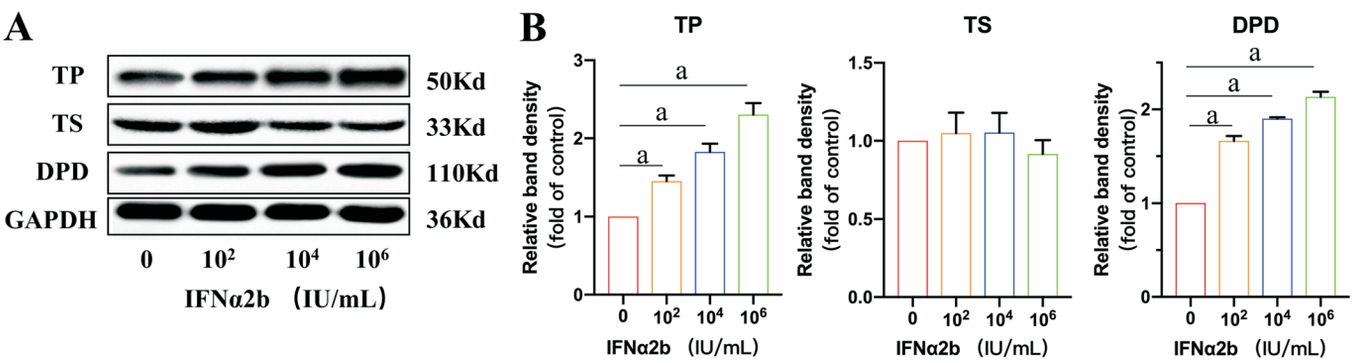

Figure 4 Effect of INFα2b on the key enzymes of 5-FU synthesis and metabolism A: The expressions of TP, TS, and DPD using GAPDH as the loading control at the protein level by Western blot; B: Relative band density for quantitating the protein expression. The data were shown as the mean±SD. n=6. ^{a}P<0.05. TS: Thymidylate synthase; TP: Thymidine phosphorylase; DPD: Dihydropyrimidine dehydrogenase; IFNα2b: Interferon-α2b; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Table 2 Single drug dosage and the rate of reduction dose for the combined drug corresponding to the same fraction affected Fa=0.57

Fa	Dose IFNα2b	Dose 5-FU	DRI IFNα2b	DRI 5-FU
0.102	39.1576	0.02183	0.39158	0.68220
0.193	502.525	0.23121	5.02525	1.44504
0.425	24032.5	8.26515	240.325	10.3314
0.57	177961	52.6436	1779.61	13.1609
0.683	940568	245.471	9405.68	12.2736
0.769	4178701	974.801	41787.0	9.74801
0.788	6097335	1382.50	60973.4	2.76501
0.818	1.169E7	2524.27	116919	1.00971

DRI Data for Non-Constant Combo: COM (IFNα2b+5-FU). DRI is a measure of how many folds the dose of each drug in a synergistic combination may be reduced at a given effect level when compared with the doses of each drug alone. IFNα2b: Interferon-α2b; 5-FU: 5-fluorouracil; Fa: Fraction affected; DRI: Dose reduction index.

Western blot assay was conducted to detect the expression of the 5-FU target enzymes TP, TS, and DPD after incubation with IFNα2b of different doses at 10^2,10^4, and 10^6 IU/mL (Figure 4A). The expression of TP and DPD in Cal27 cells were up-regulated when cells were treated with a low concentration of IFNα2b at 100 IU/mL (P<0.05; Figure 4B). As IFNα2b concentration increased, the expression of TP and DPD enhanced in a concentration-dependent manner. In contrast, there was no statistical significance of the expression of TS in the presence of IFNα2b at any tested concentrations (P>0.05; Figure 4B).

IFNα2b and 5-FU Effects on Apoptosis and Sensitivity of Tumor Cells Cal27 cells were treated with low, medium and high concentrations of IFNα2b (10^2, 10^4, 10^6 IU/mL), 5-FU (0.2, 2, 20 μg/mL), and with a combination of 100 IU/mL IFNα2b and 0.2 μg/mL 5-FU. Cell apoptosis was evaluated with flow cytometry by Annexin V-fluorescein isothiocyanate and propidium iodide staining. It has been revealed that medium and high doses of IFNα2b, along with all concentrations of 5-FU facilitated apoptosis, with the rates rose as drug concentration increased (Figure 5A, 5B). Low concentration of IFNα2b at 100 IU/mL had no significant impact on cell apoptosis. Consequently, we chose low concentration of IFNα2b combined with 0.2 μg/mL 5-FU to verify IFNα2b function on 5-FU treatment, and the relevant apoptosis rate was (18.91±0.61)%, markedly higher than that of 5-FU alone at (11.62±0.78)% (P<0.05; Figure 5). TUNEL assay labeled the apoptotic morphology and quantified the proportion of apoptotic cells (Figure 5C, 5D). Low dose of IFNα2b did not

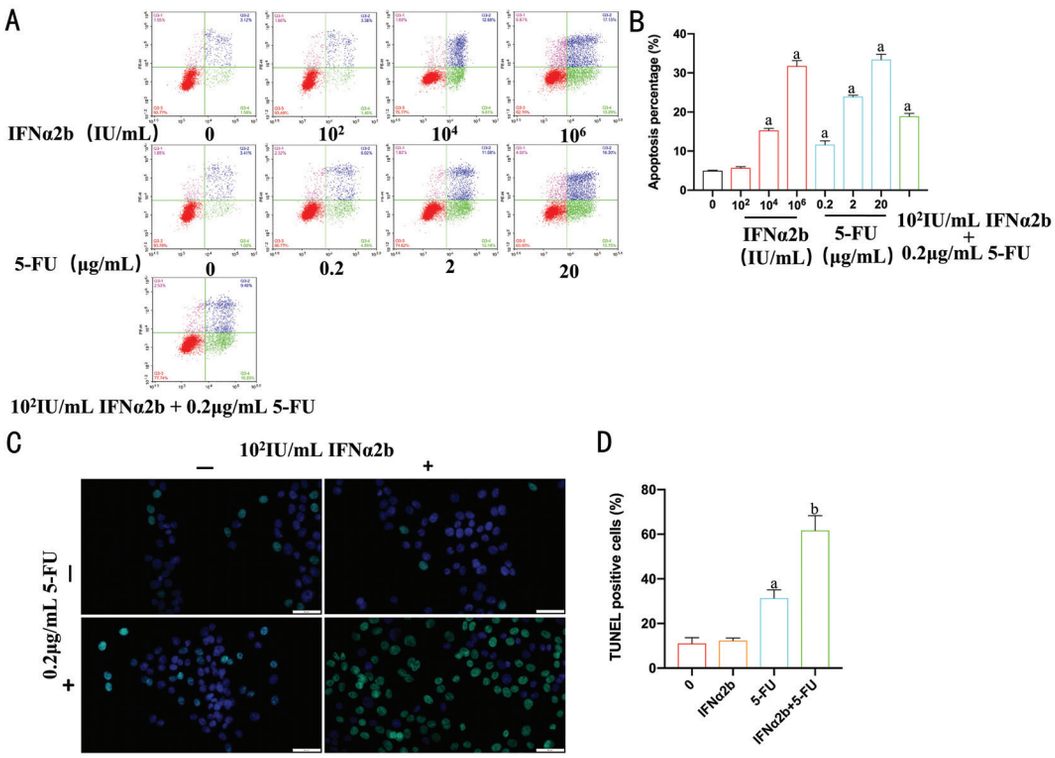

Figure 5 Effect of INFα2b and 5-FU on cell apoptosis A, B: The apoptosis of Cal27 cells treated with different concentrations of IFNα2b and 5-FU alone or in combination for 48h was determined by flow cytometry; C, D: The apoptosis of Cal27 cells (green) was detected by TUNEL assay. The data were shown as the mean±SD. n=6. ^{a}P<0.05 *vs* negative control, ^{b}P<0.05 *vs* 0.2 μg/mL 5-FU. Scale bar=50 μm. IFNα2b: Interferon-α2b; 5-FU: 5-fluorouracil.

motivate cell apoptosis, instead it enhanced the sensitivity of tumor cells to 5-FU and increased the apoptosis rate of Cal27 cells from (31.10±3.09)% to (61.60±5.44)% (P<0.05).

IFNα2b and 5-FU Effects on Cell Cycle Diverse concentrations of IFNα2b (10^2, 10^4, 10^6 IU/mL) and 5-FU (0.2, 2, 20 μg/mL), along with the combination of 100 IU/mL IFNα2b with 0.2 μg/mL 5-FU, were added to the culture medium of Cal27 cells to analyze cell distribution in various phases. There were apparent discrepancies in the proportion of G0/G1 and S phase cells among different groups (P<0.05). Compared with the control group, IFNα2b at a low dose (10^2 IU/mL) exerted no significant impact on Cal27 cell cycle (P>0.05). The proportion of G0/G1 phase cells was elevated, and the ratio of S phase cells was reduced instead in the medium and high-dose IFNα2b groups and all 5-FU treatment groups compared to the blank control group (P<0.05; Figure 6). Low dose of IFNα2b (100 IU/mL) combined with 5-FU (0.2 μg/mL) distinctively inhibited cell proliferation by inducing stagnation in G0/G1 phase compared to 5-FU (0.2 μg/mL) group alone, increased the proportion of G0/G1 phase cells from (58.50±1.67)% to (67.16±1.17)% (P<0.05; Figure 6A, 6B).

Influence of IFNα2b and 5-FU Treatment on Migration Ability To further explore the inhibitory effect of IFNα2b and 5-FU on cell migration, Cal27 cells were subjected to treatments with IFNα2b, 5-FU or a combination of IFNα2b and 5-FU for 48h, and the cell scratch assay and migration ratio were performed and measured. Low dose of IFNα2b at 100 IU/mL and 5-FU at 0.2 μg/mL individually both decreased the relative mobility of Cal27 cells. Notably, the inhibitory effect was more obvious when the two drugs were applied in combination (P<0.05; Figure 7).

DISCUSSION

With the benefits of *in vivo* confocal microscopy (IVCM) and high-resolution optical coherence tomography (HR-OCT) of anterior segment, the early diagnosis of OSSN has become more accurately and timely[22-23]. Traditional surgical excision with the double freeze-and-slow-thaw technique brings about severe complications including limbal stem cell deficiency and conjunctival scarring, however, the recurrence rates with positive margins can be diminished through adjunctive postoperative topical INFα2b or 5-FU[24-26]. Given minimal incidences of serious side effects and affordable cost, local single-agent chemotherapy emerges as a prospective adjunct and replacement, guaranteeing similar long-term advancements compared to surgical excision[27-30]. Owing to the prolonged medication duration and inferior compliance of single-agent chemotherapy, we advocate for combination chemotherapy strategy to improve outcomes and minimize toxicity, which has been proven efficient in treating colorectal cancer and hepatocellular carcinoma in published literatures[31-32].

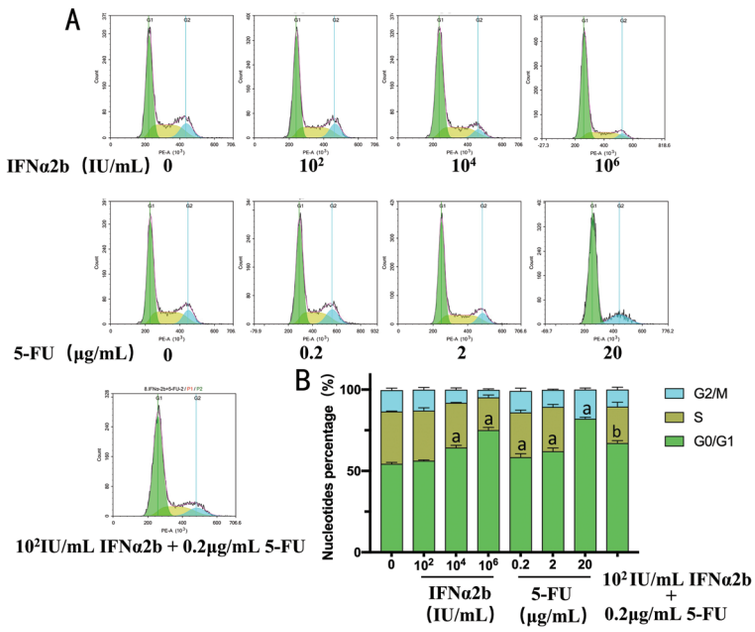

Figure 6 Effect of INFα2b and 5-FU on cell cycle A: Calculated cell numbers in different stages of cell cycle by flow cytometry; B: Percentage of nucleotides in basal conditions (0 as control group) and in the presence of different concentrations of INFα2b /5-FU. The data were shown as the mean±SD. *n*=6. [a]*P*<0.05 *vs* negative control, [b]*P*<0.05 *vs* 0.2 μg/mL 5-FU. IFNα2b: Interferon-α2b; 5-FU: 5-fluorouracil.

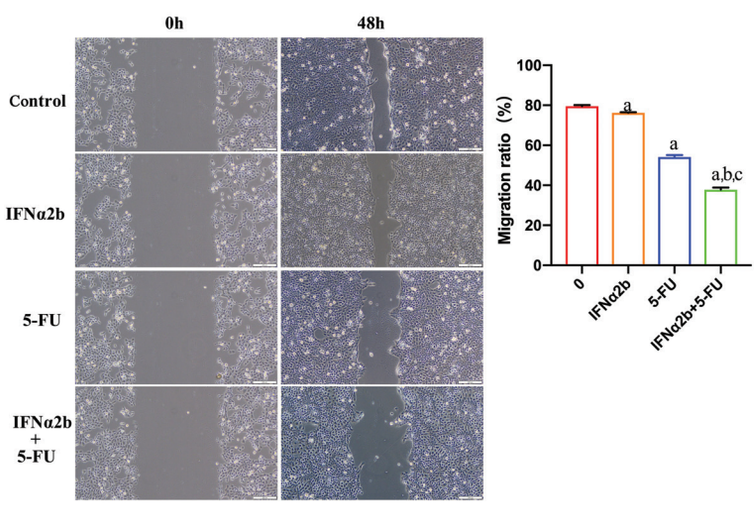

Figure 7 Effects of IFNα2b and 5-FU alone or in combination on the migration of Cal27 cells Cell scratch test result and migration ratio of Cal27 cells in different drug conditions after 48h in culture. The data were shown as the mean±SD. *n*=6. [a]*P*<0.05 *vs* negative control, [b]*P*<0.05 *vs* 0.2 μg/mL 5-FU, [c]*P*<0.05 *vs* 100 IU/mL IFNα2b. Scale bar=200 μm. IFNα2b: Interferon-α2b; 5-FU: 5-fluorouracil.

The anti-neoplastic properties of IFNα2b include direct cytotoxicity exhibition and modulation of immune responses[33-35]. IFNα2b also plays a role in inducing apoptosis by upregulating caspase and p53 in certain cell lines, targeting the most commonly mutated DNA repair gene *p53* in OSSN[36]. Considering HPV infection as a risk factor for OSSN[37], the function of IFNα2b extends beyond its antitumor function to the inhibition of virus replication in treating OSSN[38]. The activity and expression levels of IFNAR, the cell surface binding receptors imperative for the recognition and binding to target cells, directly affect the therapeutic function of IFN[39-40]. In this study, high expressions of IFNAR were detected both in the normal conjunctiva and tumor tissues, which may explain the clinical sensitivity of OSSN patients to IFNα2b. The potential anti-tumor mechanisms of 5-FU involve the inhibition of TS activity and the suppression of DNA and RNA synthesis, thereby impeding tumor cell proliferation[41]. Collectively, these approaches were consistent with our findings that low dose of 5-FU significantly inhibited the proliferation and migration, induced cell apoptosis, and disrupted cell cycle by blocking tumor cells in G0/G1 phase, ultimately leading to programmed cell death.

Quantitative studies have unraveled that INF synergistically enhances the anti-tumor activity of 5-FU through up-regulation of TP expression and inhibition of TS activity to slow down metabolism[31,42]. In our research, IFNα2b not only exhibited

direct inhibitory effects, but enhanced sensitivity of 5-FU chemotherapy at low doses, thus influencing cell migration, apoptosis and cell cycle. High expression of TP in tumor tissue will increase the sensitivity to 5-FU chemotherapy, promote angiogenesis, and increase tumor growth and invasiveness[43-44]. In addition, our result showed no significant difference in the expression levels of TP and DPD between normal conjunctiva and tumor tissue, which may be related to the low malignancy of OSSN and the limited sensitivity of reagents. On the other hand, the TP expression in tumor tissues was significantly higher than that in normal tissues, consistent with the active proliferation condition.

Combination chemotherapy is widely applied in multiple dreadful diseases such as cancer and acquired immune deficiency syndrome, with the main purposes of facilitates drug efficiency, diminishing drug toxicity and minimizing resistance[45-46]. However, a clear and formative definition of drug interactions including synergism, summation and antagonism remains inadequately established. Hence our investigation introduced the widespread and recognized CI to quantify and define drug interactions, which was put forward by multiple researches[47-50]. The theorem allows quantitative determination of drug interactions based on a fixed ratio of two drugs or the quantification of one drug, where CI<1, =1, and >1 respectively indicate synergism, additive effect and antagonism[21]. Through theoretical design and computer simulation, its simplicity and effectiveness in experimental program and data analysis mitigate the need for experimental animals and the number of patients required for clinical drug trials. This promising concept provides theoretical basis and reference for future research and proportion control of clinical combination drug. In our research, a specific low concentration of IFNα2b at 100 IU/mL was selected to exclude its intrinsic anti-tumor effect, which had no significant effect on cell proliferation, apoptosis and cell cycle (P>0.05). Our result indicated that the combination of IFNα2b at 100 IU/mL and 5-FU of the rest concentrations showed strong synergistic effect (CI<1)[51], except for the lowest concentration of 5-FU (CI=4.8), which was regarded as an experimental error considering the low drug concentration. Under the stimulation of IFNα2b at low doses, the expression of 5-FU metabolism-related enzymes such as TP increased, explaining the sensibilization of 5-FU.

The present study also acknowledges certain limitations. Due to the shortage of stable proliferating OSSN cell lines, lack of pathological tissue samples and challenges in primary cell culture, we employed human oral squamous cancer cell lines Cal27 which were easily accessible for this research. Although oral mucosal epithelial cells resemble keratoconjunctival epithelium in physiological structures and biomarkers, they cannot fully represent the proliferation characteristics of OSSN tumor cells[52-53].

In conclusion, this fundamental study testified the underlying reasons behind our clinical investigation, and the proved synergy between IFNα2b and 5-FU substantiated that topical combination chemotherapy would be a promising new therapeutic option for OSSN. Further research is needed to further elucidate the specific mechanism of the integration treatment of IFNα2b and 5-FU, aiming to achieve long-lasting and sustainable effects in the precise treatments targeting OSSN.

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