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ORIGINAL ARTICLE

Inhibition of tumor necrosis factor alpha using RNA interference in oral squamous cell carcinoma

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Summary

Purpose: *Oral squamous cell carcinoma (OSCC) is a disease with increased prevalence and unfavorable prognosis calling for development of novel therapeutic strategies. Tumor necrosis factor-a (TNF-a) is a pro-inflammatory cytokine implicated in the development and progression of cancer. The present study was designed to assess the impact of TNF-a specific inhibition using small interference RNA (siRNA) in SSC-4 cells, a representative model for OSCC.*

Methods: *The present study evaluated the effect of TNF-a inhibition using siRNA as inhibitory mechanism on SCC-4 cells. The study focused on the effect of TNF-a inhibition on apoptosis, autophagy and invasion in parallel with a panel of 20 genes involved in apoptosis and angiogenesis.*

Results: *TNF-a inhibition was related with reduction of cell viability, activation of apoptosis and autophagy in parallel with the inhibition of migration in SCC-4 cells. Evaluating the impact on gene expression levels, inhibition of FASL/FADD, NFκB, SEMA 3C, TNF-a, TGFB1, VEGFA, along with activation of PDGFB and SEMA 3D was observed. Our study confirms the important role of TNF-a and sustains that it might be a therapeutic target in OSCC.*

Conclusions: *TNF-a is a key mediator of the immune system, with important role in OSCC tumorigenesis, and might be considered as a therapeutic target using siRNA technology, particularly for those risk cases having FASL/FADD overexpressed.*

Key words: *apoptosis, autophagy, migration, SSC-4 cells, TNF-a siRNA therapy*

Introduction

OSCC is considered to be one of the common types of head and neck cancers [1-3] with nearly 500,000 new cases per year [4], localized in the tongue and floor of the mouth. It is a malignancy with a high rate of nodal metastasis [5,6]. OSCC includes the upper and lower portion of the mouth e.g. lips, cheeks, hard and soft palate, gums and salivary glands [7]. It is characterized by a high degree of local invasiveness and a high rate of metastasis to the cervical lymph nodes [3]. Tongue is the most common and deadliest site for OSCC, being related with a high metastatic rate [8,9].

Common risk factors of this disease include

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environmental toxic and biologic agents [9]. Therefore, there is an urgent demand to develop novel therapeutic strategies. One approach might be the targeting of overexpressed genes involved in carcinogenetic mechanisms. Small interfering RNAs (siRNA) represent a mechanism able to knock down the altered genes by targeting their mRNA expression [10], which underlies the uniqueness of this therapeutic approach. siRNAs are artificially made chemical structures of 19–23 nucleotides long having 2-nt-3' overhang [11] and the duplex siRNAs are unwound by helicase, being able to mimic natural processes. One of the two RNA strands is retained within the complex RNA inducing silencing complex (RISC), while the other strand undergoes degradation by exonucleases. Sensitivity of cancer cells to chemotherapeutic agents can be enhanced using combined therapy with siRNA and play a very crucial role in tumorigenesis and drug resistance [12]. Therefore siRNA therapy has shown important promise for cancer treatment as shown by preclinical and clinical data [13].

TNF-α is a pro-inflammatory cytokine [14,15] involved in the development and progression of cancer [16,17]. TNF-α expression plays a role in tumor microenvironment and promotes tumor cell migration and invasion [16,18], which characterize the metastatic behavior [14]. In many tumor types, the constitutive synthesis of TNF-α leads to the activation of cell survival signaling pathways and the synthesis of cytokines, chemokines and angiogenic factors. TNF-α, through the activation of the NFκB, is involved in drug resistance mechanisms and cell survival pathways leading to inhibition of apoptosis [16].

In this study we focused in blocking the expression of TNF-α gene, using siRNA technology, followed by investigation at cellular and molecular level of the effects caused by the gene silencing. The effect of TNF-α was evaluated in relation to apoptosis by flow cytometry, as well in autophagy which was assessed by inverted fluorescence microscopy, along with a panel of 20 key genes using RT-PCR.

Methods

Cell culture and treatment

SSC-4 cell line was the relevant in vitro model for OSCC and was used in our experiments. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with a high glucose concentration and F12 Ham 1:1, supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), glutamine 2 mM, penicillin 100 IU/mL and 1μM dexamethasone. SSC-4 cells were grown in a humidified 5% CO_2 incubator at 37 ºC.

TNF-α siRNA was transfected in SSC-4 cells, based on the reverse transfection method. $0.5x10^6$ cells were seeded in a 6-well plate and treated with TNF-α siRNA (Ambion, Austin, TX, USA). We used 5 μL siPORT NeoFX transfection agents for each well, dissolved in 95 mL Opti-MEM (Gibco-Invitrogen, Paisley, UK). For each well, 2.5 μL TNF-α siRNA (40μM) were diluted in 97.5 μL OptiMEM after 10 min of incubation at room temperature, and mixed with the transfection agent in order to achieve 50 nM in the cell culture medium. At room temperature we incubated the mixture for 10 min and then immediate distributed it on the plate. Before analysis, cells were cultured in a total volume of 2 mL OptiMEM/well for 24 hrs for apoptosis evaluation by flow cytometry and gene expression evaluation, while for the rest of the test a volume of 200 ml/well was used and cells were seeded in 96-well plates.

Apoptosis evaluation by fluorescence microscopy and flow cytometry

Annexin V-FITC/PI assay kit (AbD SeroTec, UK) and Multi-Parameter Apoptosis Assay Kit (Cayman cat no. 600330, Estonia) were used using tetramethyl rhodamine ethyl ester (TMRE) and Hoechst staining for microscopical evaluation according to the manufacturer's instructions for both kits.

For flow cytometry a staining protocol with Annexin V-FITC/PI assay kit (AbD seroTec, UK) was used. Each experiment was performed in triplicate and after 24 hrs of treatment with TNF-α siRNA apoptosis was evaluated. The cells were resuspended in 300 mL Binding Buffer and 1 μL Annexin V-FITC and afterward they were removed from the culture plates and incubated for 15 min at room temperature in the dark. One μL of propidium iodide (PI) was added just before flow cytometry analysis. Using FACS Canto II flow cytometer the rate of apoptosis was evaluated and the data obtained were analysed with BD FACS Diva software.

Autophagy evaluation

For the autophagy evaluation a protocol was used based on monodansylcadaverine staining. The staining procedure was done according the manufacturer's instructions (Autophagy/Cytotoxicity Dual Staining Kit; Cayman cat no. 600140).

Cell migration assays

Cell migration was assessed using Transwell plates (Corning, USA). The 24-well plate (8 μm size pore polycarbonate membranes) in the upper compartment was coated with 1% matrigel, then was covered with OptiMem (GIBCO™, USA) and was incubated for 24 hrs at 37 ºC. As chemoattractant RPMI was used,

supplemented with 20% FCS and fibronectin (10μg/ml). Cells from the upper compartment were removed using a cotton swab. Migrated cells that attached to the underside of the Transwell plates were stained with FDA (Fluoresceindiacetate) and evaluated by fluorescence microscopy (x200).

Gene expression evaluation

Gene expression was evaluated at 24 hrs post-transfection. RNA extraction was done using TriReagent and RNA quantity and quality control was done using Nanodrop-1000 spectrophotometer (ThermoScientific), and Agilent Bioanalyzer 2100. cDNA synthesis was done starting from 500 ng of total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland), based on the manufacturer's instructions, then qRT-PCR amplification was done using LightCycler®TaqMan® Master kit (LightCycler 480, Roche). As housekeeping gene we used β–Actin. Specific primers were used for each gene of interest (Table 1).

Statistics

Analysis for the quantification of the relative gene expression for assessed genes was performed using ΔΔCt method [1]. The altered gene expression levels were analyzed using Ingenuity System Pathway Analysis (www.ingenuity.com).

Gene expression data are shown as means±standard deviation (SD). Statistical analyses were carried out using Student's t-test and p values<0.05 were considered significant using Graph Pad Prism free trial.

Results

TNF-a siRNA specifically activates apoptosis

The results obtained from flow cytometry indicate that blocking the expression of TNF-α in the SSC-4 cells leads to specific activation of apoptosis. At 24 hrs posttransfection it was observed an increased percent of late apoptotic cells, a fact confirmed by microscopic evaluation of the apoptosis using Annexin V/PI staining, as presented in Figure 1. After 24-h treatment of the SSC-4 cells with TNF-α siRNA, 29.9% late apoptotic cells and 61.6% viable cells were confirmed, compared to the control group, where the viable cells were 95.7%.

Apoptosis evaluation by Hoechst/TMRE staining confirms the activation of the apoptotic process induced by TNF-a siRNA

Hoechst staining showed that most control cells had round and intact nuclei, whereas TNF-α siRNA-treated cells mostly had condensed and fragmented nuclei. TMRE staining for mitochondrial membrane revealed that most control cells had undisrupted mitochondrial membrane, and an increasing TMRE fluorescence intensity, whereas TNF siRNA-treated cells mostly had diminished membrane potential, a characteristic preceding mitochondrial injury and, finally, apoptosis (Fig-

Table 1. Primers list used for qRT-PCR evaluation

Gene	*Reverse/forward primer*
β–Actin	AGG AAT GGA AGC TTC CGG TA/AAT TTT CAT GGT GGA TGG TGC
AKT3	GGGAGGCCAAGGTAGATGA/ TCA CAC CTA TAA TCC CAC ATG C
BCL	TTG ACA GAG GAT CAT GCT GTA CTT/ ATC TTT ATT TCA TGA GGC ACG TT
CASP8	TAG GGG ACT CGG AGA CTG C/ TTT CTG CTG AAG TCC ATC TTT TT
FADD	CCG AGC TCA AGT TCC TAT GC/ AGG TCT AGG CCG CTC TGC
FASLG	GGC CAA GTT GCT GAA TCA AT/ GAG ACG AGC TCA CGA AAA GC
NFkB	TCC AGG TCA TAG AGT GGC TCA/ GGC TGG CAG CTC TTC TCA
PDGFBB	TGA TCT CCA ACG CCT GCT/ TCA TGT TCA GGT CCA ACT CG
TP53	AGG CCT TGG AAC TCA AGG AT/CCC TTT TTG GAC TTC AGG TG
PIK3C3	GGG GAA GCA GAG AAG TTT CA/ TCT TCC CTT CCA AGC TTC CT
SEMA3C	ATA TTC TCG CCC TGG AAC TT/ TCC TTG GTG GTT CGC ATA TT
SEMA3D	AAG GCC AGA CTG ATT TGC TC/ TGT GGG GAG TAA ATA AAT ATC CTT GAA
SEMA3G	CCT GAG GAA GTG GTT CTG GA/ CAT TTC GGT GAT AGG TGT TGG
SMAD7	ACC CGA TGG ATT TTC TCA AA/ AGG GGC CAG ATA ATT CGT TC
SPHK	CCA TCA CAG CCT GTA AGA AGG/ TTC TGC AAC CAG TAG TCT GTT GA
TGFB1	CAG CCG GTT GCT GAG GTA/ AGC AGC ACG TGG AGC
TNF-α	CAG CCT CTT CTC CTT CCT GAT/ TGG GGA ACT CTT CCC TCT G
VEGFA	CTA CCT CCA CCA TGC CAA GT/ CCA CTT CGT CGA GAT TCT GT
WNT11	AGC TCG CCC CCA ACT ATT/ ATA CAC GAA GGC CGA CTC C
XIAP	GCA AGA GCT CAA GGA GAC CA/ AAG GGT ATT AGG ATG GGA GTT CA

Table 2. The alteration of gene expression pattern as a response of siRNA treatment in SSC-4 cells at 24 hours post-treatment. *denotes the statistically significant downregulated genes and **the overexpressed ones, based on a fold change (FC) of >0.25 and p<0.05

TNF siRNA	*FC*	*p value*
AKT3	0.96818	0.275766
Bcl-2	1.11626	0.525949
CASP8	1.28683	0.221779
FADD*	0.712023	0.0098987
FAS*	0.45514	0.002935
NFKB*	0.711746	0.041418
p53	0.891065	0.109886
PDGFBB**	2.357387	0.008361
PIK3C3	0.941926	0.201118
SEMA 3C*	0.570372	0.034098
SEMA 3D*	1.363609	0.000641
SeMA 3G	0.69978	0.703431
SMAD7	1.043509	0.696544
SPHK	0.995538	0.374935
TGFB1	0.622808	0.034874
TNF-α*	0.141139	0.005864
VEGFA*	0.229852	0.012019
WNT11	0.71178	0.067619
XIAP	1.132525	0.327674

ure 2). TMRE, a fluorescence dye, is highly sensitive and translocates into the mitochondria but it is easily released when the mitochondria are disturbed and the membrane potential diminished.

TNF-a siRNA activates autophagy

Microscopic evaluation of monodansylardaverine staining for vacuoles was done at 24 and 48 hrs. Monodansylsadaverine staining revealed increased fluorescence intensity in the case of *TNF-a siRNA* treated cells (Figure 3), demonstrating the increased autophagy rate for *TNF-a siRNA* treated cells.

TNF-a siRNA inhibits cell invasion

An *in vitro* matrigel migration test was carried out in order to determine the influence on cell migration potential in the presence of TNF-α siRNA treatment (Figure 4). Compared to control, the number of TNF-α siRNA-treated cells significantly decreased, as we observed by microscopic evaluation of the cells that passed through the insert membrane and stained with FDA in the matrigel layer.

Gene expression alteration at 24 hrs post treatment with TNF-a siRNA

RT-PCR assay was used to examine the transcript levels of 20 genes involved in apoptosis.

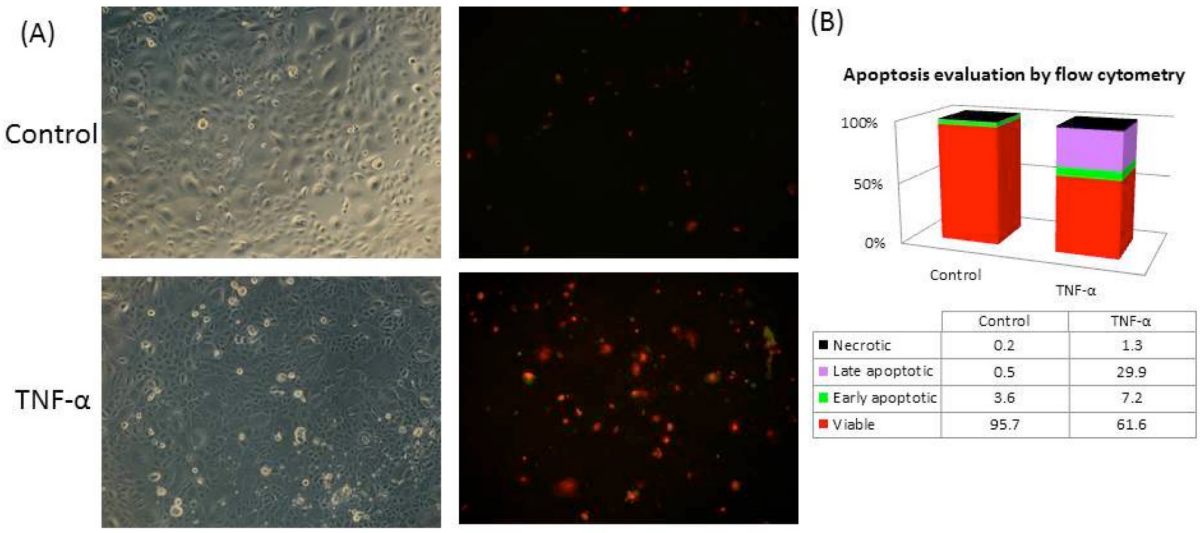

Figure 1. Induction of apoptosis by TNF-α siRNA transfection evaluated using Annexin V FITC/PI staining by **(A)** inverted fluorescence microscopy (100x) and **(B)** flow cytometry. Representative microscopic images of TNF-α siRNA induced apoptosis in SSC-4 cells after 24-h incubation with TNF-α siRNA **(A)**. Microscopic determination of late apoptotic cells, doubled-stained with Annexin V FITC and PI, confirmed by flow cytometry **(B).**

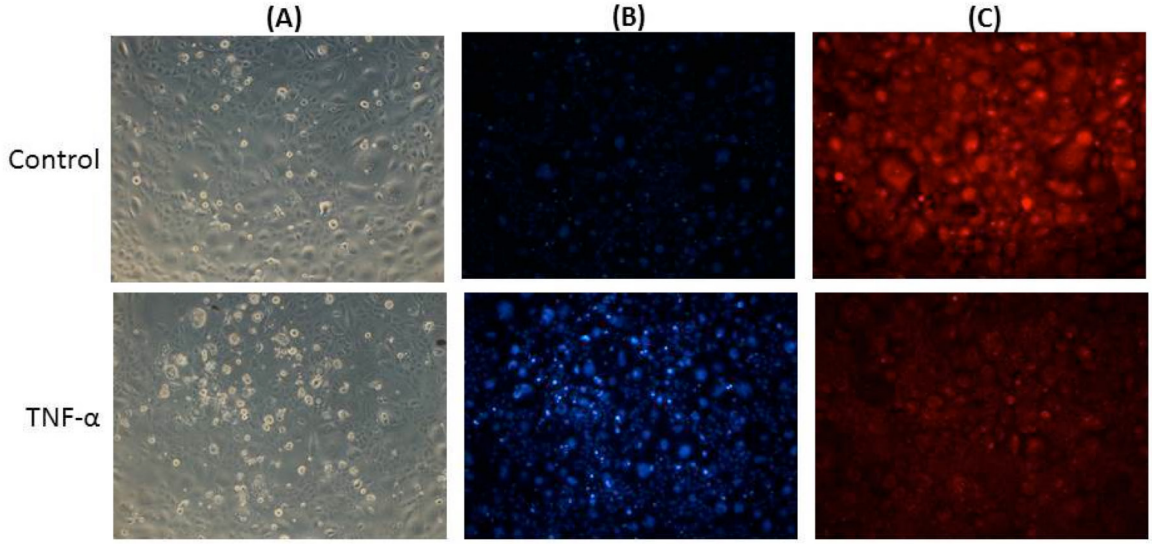

Figure 2. Induction of apoptosis by TNF-α siRNA transfection evaluated by TMRE/ Hoechst staining using inverted fluorescence microscope: **(A)** bright-field image; **(B)** Hoechst staining; **(C)** TMRE staining. Representative microscopic images: **A:** Alteration of cellular morphology after TNF-α siRNA treatment; **B:** Hoechst staining emphasizes the presence of fragmented and condensed nuclei, and apoptotic bodies after TNF-α siRNA treatment. Meanwhile, control cells remain unaffected (100x).

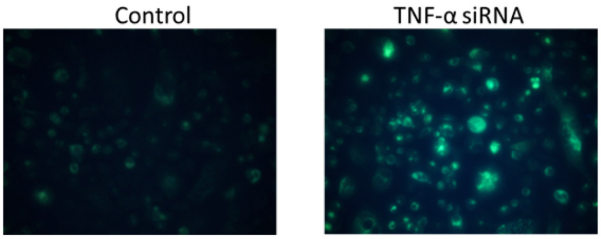

Figure 3. Activation of autophagy by TNF-α siRNA transfection evaluated using monodansylcadaverine staining for autophagy vacuoles. Although SSC-4 cells naturally express autophagy, treatment with TNF-α siRNA was correlated with increased autophagy, as displayed in the representative fluorescent photomicrographs (400x).

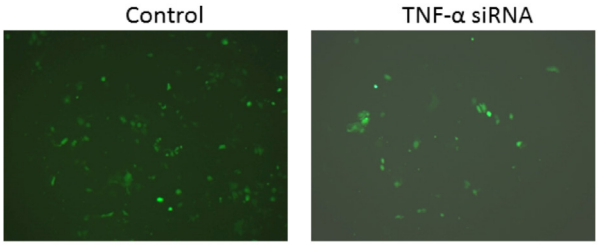

Figure 4. Inhibition of invasion by TNF-α siRNA transfection evaluated using matrigel assay. Microscopic evaluation of cells that passed through the insert membrane (FDA staining, green fluorescence). At 48 hrs posttransfection significant of reduction cell invasion was observed (100x).

After data analysis with the ΔΔCt method, we obtained a relevant p-value for 8 genes after comparing the treated with untreated groups, 7 being downregulated and one upregulated. Most of the genes from Table 2 are members of the extrinsic apoptosis pathway, mediated by its receptors and angiogenesis.

Using Ingenuity (www.ingenuity.com), the genes that had a significantly altered level were analyzed in order to display the gene interaction. TNF-α siRNA knockout revealed an interconnection among apoptosis, autophagy and angiogenesis (Figure 5).

Discussion

There are several studies that employ siRNA therapy in oral cancer but none of them are focused on TNF-α inhibition using siRNA. TNF-α, a major mediator of inflammation, plays a crucial role in the inflammation-associated cancer development and inhibits the death of precancerous or transformed cells during the development of inflammation-associated cancers [20]. The secretion of TNF-α stimulates its own formation and is involved in all steps of tumorigenesis. TNF-α induces tumor initiation and promotion by reducing susceptibility to chemotherapy, it enhances tumor cell proliferation which serves as a mutagen to promote the proliferation and survival of many tumor cell lines without inducing cell differentiation, enhances tumor angiogenesis and confers an invasive and transformed phenotype into tumor

cells [14-16]. Therefore, our study has proved that TNF-α siRNA was able to specifically target multiple mechanisms involved in tumorigenesis.

Flow cytometry confirms the reduction of cell viability as response to TNF-α siRNA treatment. The inverted fluorescence microscopy confirmed the presence of late apoptotic cells showing a high percent of cells with double staining for Annexin V/PI. It is clear that our experimental data suggest that cells undergo apoptosis after blocking the expression of TNF-α in the SSC-4 cells. As it was observed in gene expression data, inhibition of NFκB was determined. It is clear that the activation of NFκB is essential for TNF-α-induced cell survival and proliferation. Downregulation of TNF-α blocks the expression of NFκB pathway and the activation of NFκB in tumor cells is triggered by TNF-α, mechanism confirmed by previous studies [14-17].

Fas-associated death domain (FADD) represents an important adaptor for the signal transduction of activation of apoptosis via death receptors [21]. FADD was downregulated in our study, this protein being considered essential in the apical poll of the extrinsic apoptosis pathway that is connected to death receptors pathways. Clinical data present FADD overexpression to be related with unfavorable prognostic [22]. Another study showed that FAS/FASL expression was related with disease recurrence and low survival rate [23]. Our study confirmed the important role of this cytokine and sustains the idea that it could be a therapeutic target in OSCC.

Autophagy has an intrinsic function in tumor suppression and may allow tumor cells to survive under metabolic stress. Several genetic links have emerged between defects of autophagy and cancer development. It is known that autophagy activation by TGFB1 is mediated through the Smad and JNK pathways [24]. TGFB1 induced autophagy in certain tumor types, being implicated in tumor promotion in the later phase of tumori-

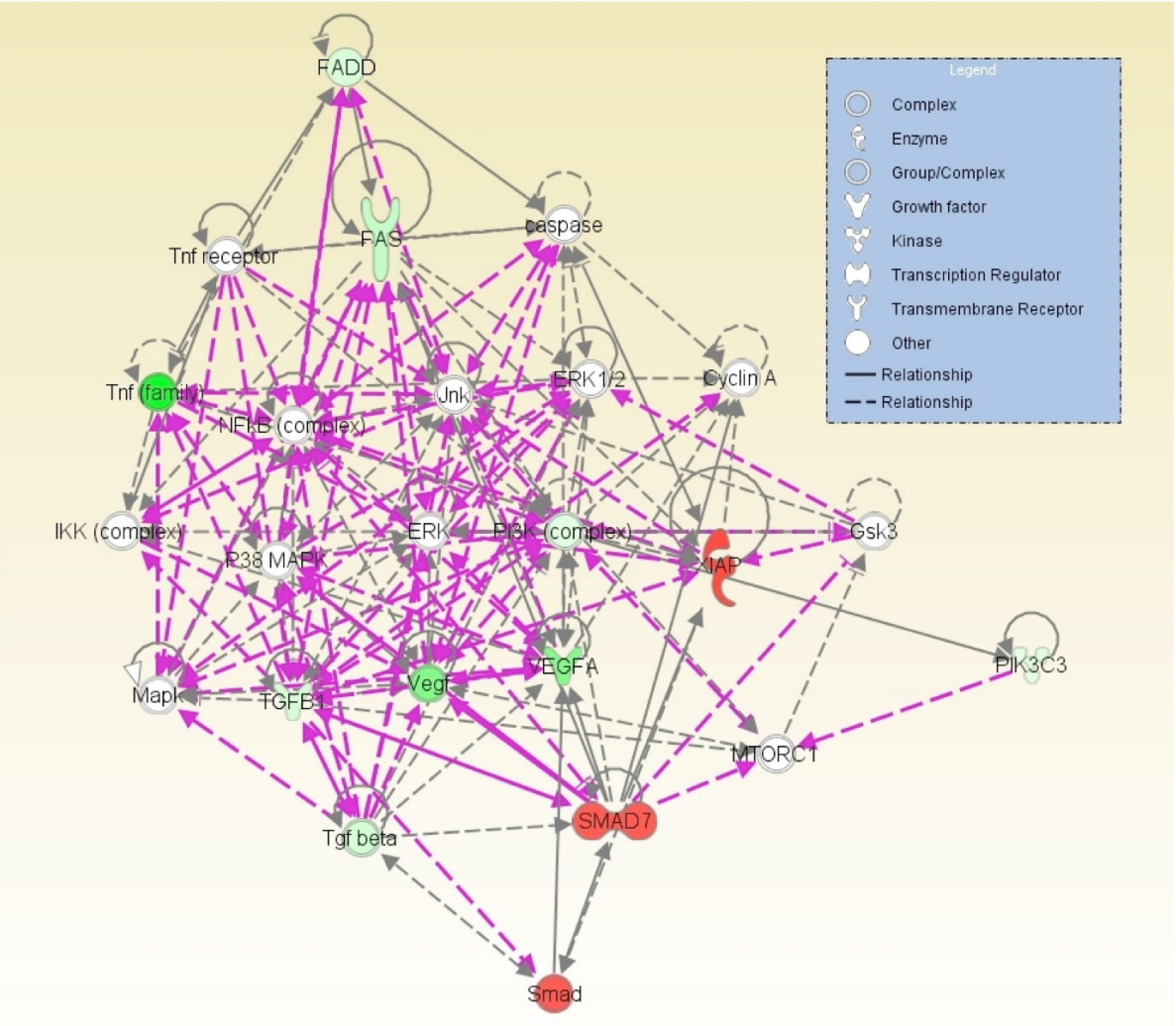

Figure 5. Analysis of gene interaction using Ingenuity software in the case of TNF-α knockout.

genesis [25]. TGFB1 downregulation might be an important event that contributes to the activation of autophagy and apoptosis.

The TGFB1-dependent immunosuppressive activity stimulates angiogenesis, increases the affinity of cancer cell to cell adhesion molecules, and creates a microenvironment favorable for tumor growth and metastasis by increasing cancer cells' invasiveness. Additionally, TGFB1 induces death of the surrounding healthy cells and thus eliminates their effect in inhibiting tumor growth. It appears that cancer cells need higher TGFB1 concentrations than normal cells to receive TGFB1-anti-mitotic stimuli [26]. TGFB1 activates autophagy before the initiation of apoptosis, and silencing of autophagy genes by siRNA attenuates the cell cycle arrest and induction of apoptosis by TGFB1. TGFB1 specifically modulates autophagy via SMAD and non-SMAD signaling pathways [27], but the present study revealed that SMAD-7 was not involved.

Vascular endothelial growth factor A (VEGFA) is a key mediator of angiogenesis and suppresses apoptosis, in addition to promoting angiogenesis [28], being inhibited in the present study. Semaphorins in tumors can promote cell apoptosis [29]. SEMA3C in ovarian cancer cells acts like an agent that increases resistance to cytotoxic drugs [30], a fact that can be extrapolated also for oral cancer based on experimental data. SEMA3D in breast cancer cells inhibits tumor development in vivo and it also inhibits anchorage-independent growth displaying antitumorigenic effects [29,30].

Conclusions

Targeting the ability of tumor cells to proliferate by blocking the intracellular cell survival pathways activated by TNF-α definitely leads tumor cells to apoptosis. A combination with other conventional or targeted therapies might be more efficient or at least stop the tumor development and metastasis.

TNF-α is a key mediator of immune system, with important role in OSCC tumorigenesis, and can be considered as an alternative for therapy, particularly for those risk case that have FASL/FADD overexpressed.

Authors' contributions

Conceived and designed the study: AII, RSC and IBN. Performed the experiments: AII, AO, VP, OS. Analyzed the data: AII, CB, OS. Wrote the paper: all the authors.

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