RESEARCH ARTICLE

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FOXD1-mTOR Signaling Pathway on Oral Squamous Cell Carcinoma and Its Inhibition by Rosemary Extract (Invitro-Study)

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Abstract

Background: FOXD1 expression in oral squamous cell carcinoma remains uncovered. The aim was to detect the anticancer effect of Rosemary Extract RE through the evaluation of FOXD1 gene expression in (OSCC) by quantitative PCR. **Methods:** OSCC cell line was served as a control group. Moreover, the OSCC cell line (SCC-15) was treated with RE (OSCC/ RE group) at 24, 48, and 72 hs time intervals. We assessed the antioxidant activity of RE by evaluation of lipid peroxidation (MDA) and superoxide dismutase (SOD) levels. The cytotoxic effects of RE were examined by MTT assay. mTOR and LC3 I/II autophagy protein markers were assessed by western blot. Apoptosis activity was assessed. **Results:** The study results were statistically assessed. Intergroup comparisons were analyzed, whereas intragroup comparisons were conducted utilizing one-way repeated measures ANOVA, followed by multiple pairwise paired t-tests with Bonferroni correction revealed a significant increase of FOXD1 gene expression in the control OSCC group in comparison to the OSCC/RE group (p-value <0.001). A significant decrease of mTOR/LC3I/II proteins expression in the OSCC/RE group compared to the control OSCC group (p-value <0.001). **Conclusion:** FOXD1 can be considred a diagnostic biomarker for OSCC. RE inhibits autophagy of oral human cancer cells via mTOR/LC3I/II-dependent pathways and decrease caspase -3 apoptotic level.

Keywords: Oral squamous cell carcinoma- FOXD1- Rosemary extract- mTOR/LC3I/II- apoptosis- Caspase 3

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Introduction

Oral squamous cell carcinoma (OSCC) is the most common oral malignancy, representing up to 80-90% of all malignant neoplasms of the oral cavity (Szliszka et al., 2011). Major advances in the early detection of cancer and its therapeutic interventions demonstrate that there is still a lack of effective OSCC biomarkers to be potentially used for diagnosis and prognosis prediction. There is also some strong evidence that most malignancies are diagnosed and treated at late stages, which substantiates the clinicians' urgent need to improve the patient's prognosis to discover new biomarkers and therapeutic targets for OSCC (Quan et al., 2012).

Previously, it has been thoroughly explored the biological functions and other associated molecular mechanisms of FOXD1 concerning tumor development. Additionally, it has been demonstrated that FOXD1 plays a pivotal role in cancer progression, tumorigenesis, and metastasis across different types of human cancers, especially that its abnormal overexpression is directly related to cell proliferation, epithelial-to-mesenchymal transition (EMT), tumor invasion and metastasis, and poor prognosis (Quintero-Ronderos et al., 2018). Furthermore, the study revealed that FOXD1 is related to some solid cancer including colorectal, breast cancer, and non-small lung cancer (Pan et al., 2018). The abnormal FOXD1 overexpression may serve as an oncogene by promoting cell transformation and proliferation, facilitating the EMT process, and inhibiting apoptosis. Moreover, FOXD1 further induces cancer stem cell-related properties including therapeutic resistance, tumor initiation, and self-renewal capacities on mesenchymal glioma cells (Koga et al., 2019). The research gap, thus, lies in the impact of FOXD1 as a potential new oncogene in several types of human cancer. A deep investigation of FOXD1 should demonstrate its role and highlight its clinicopathological significance in OSCC.

Searching for new natural products with antineoplastic characteristics represents a fascinating field of research

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in oncology (Lazarević et al., 2019). Plants and herbs are the origins of compounds with a prospective anticancer activity that can arrest, reverse, or retard carcinogenesis at various stages. Several studies documented that numerous natural compound present anticancer effects and reduce tumor cell viability efficiently. For example, rosemary (Rosmarinus officinalis L.) is a woody plant of the Lamiaceae family, which has well-established chemoprotective effects against cancer. In this respect, recent research trends have focused on the pharmaceutical capacities of rosemary, such as its anti-inflammatory, anti-infective or anticancer action (Gonzalez-Vallinas et al., 2013). More particularly, its anticancer effect has been directly related to diverse positive mechanisms such as the increase of antioxidant effects, antiangiogenic properties, and epigenetic actions, in addition to the better regulation of the immune and anti-inflammatory responses, the modification of specific metabolic pathways, and the increase in the expression of onco-suppressor genes (Mielo et al., 2020).

The chemical composition of Rosemary Extract (RE) is unique with its several phytocompounds, presenting different pharmacological activities. The antioxidative activity of RE is mainly extracted from two key antioxidant components belonging to the classes of phenolic acids, flavonoids, and diterpenoids, namely carnosol (CAS No 5957-80-2, molecular formula C20H28O4) and carnosic acid (CAS No 3650-09-7, formula C20H28O4) (Sousa-Borges et al., 2019). The other phytocompounds mostly reported included caffeic acid, chlorogenic acid, monomeric acid, oleanolic acid, rosmarinic acid, ursolic acid, alpha-pinene, camphor, eucalyptol, rosmadial, rosmanol, rosmaquinones types A and B, secohinokio, and derivatives of eugenol and luteolin. The carnosic acid and carnosol which are (rosemary polyphenol) carry out chemo-preventive, antioxidant, and anticancer activities against different cancers in the prostate, skin, breast, leukemia, lung, urinary bladder, and colon, which occurs through the genes that encode the antioxidant phase II enzymes (Minero et al., 2020). Furthermore, Rosmarinic acid, a depside of caffeic acid, and hydroxyhydrocaffeic acid are all considered as other RE compounds differentiated from solvent-based procedures that have shown complementary antioxidant activity.

In addition to the antioxidants, RE also contains several reference volatiles that is responsible for its flavor and odor. The components of reference for volatiles include 1 and 8-cineol (eucalyptol), camphor, borneol, verbenone, and bornyl acetate, which also help in antioxidative function. In addition to the previously listed components, the RE also contains residual organic material from the rosemary plant such as proteins, lipids, resins and waxes, carbohydrates, and inorganic constituents (Andrade et al., 2018).

The present study aimed to clarify the anticancer effects of RE through the assessment of FOXD1 gene expression in OSCC. To do so, we examined the antioxidant activity, cytotoxicity, autophagy, and apoptotic caspase-3 marker expressions upon exposure of OSCC to RE.

Materials and Methods

Cell Culture

The oral squamous cell carcinoma cell line (OSCC) used in the present study was (Human tongue SCC cell line (SCC-15), which was obtained from American Type Culture Collection (ATCC®CRL-1628, USA). This was selected to study the effects of RE on cells that represent human squamous cell carcinoma of oral cavity. The samples were cultured in DMEM containing 1% of PS (Penicillin-streptomycin) and 10% of bovine calf serum (Gibco BRL) at 37°C with 5% of CO_2 in the atmosphere. For the subsequent subcultures, the cells were rinsed with 0.25% (w/v) of Trypsin and 0.53 mm of EDTA solution to remove all the serum traces consisting of the Trypsin inhibitor. Trypsin EDTA solution was added to the flask (2-3 ml). Then, the cells had been observed under an inverted microscope until the cell layer was dispersed (usually within 5 to 15 minutes). The cell suspension was centrifuged at approximately 125×g for 5 to 10 minutes to discard the supernatant (full speed of the equipment: 2,166 x g). A fresh growth medium was used to re-suspend the cell pellet. Additionally, appropriate aliquots of the cell suspension were added to new culture flasks. The cells were seeded in 6-well plates for further assays. Cells were treated with 20 µg/mL of RE (Naturalin Code: NAT-185 Naturalin Bio-Resources Co., Ltd.) for 24 hours, 48 hours, and 72 hours in order (Pérez-Sánchez et al., 2018). The groups of our study that controlled the OSCC group were cultured for 24 hours, 48 hours, and 72 hours, likewise, the OSCC/RE group was cultured for 24 hours, 48 hours, and 72 hours. This study was approved by the Research Ethics committee at Faculty of Dentistry, October 6 University, Giza, Egypt (RECOU/6-2020).

Colony Formation Unit Assay (CFU Assay)

The OSCC (5×10^3 cells/well) samples were seeded into 6-well plates. After being attached to the plate, the cells were treated at 20 µg/mL of RE for 24 hours, 48 hours, and 72 hours, then cultured with fresh medium for 7 days. The medium was removed, and cells were washed twice with PBS. The colonies were fixed with 95% ethanol for 10 min, dried, and stained with 0.1% crystal violet solution for 10 min. Colonies on the plate were counted under an inverted microscope (Leica, Germany). Each colony consisted of more than 50 cells.

FOXD1 and Caspase-3 analysis by real-time PCR

The total RNA was extracted from the cell pellets of the studied groups. RNA extraction was performed on in vitro cells. Centrifugation of SSC-15 cell lines cells was performed for 3 minutes at full speed. The supernatant was removed and transferred to a new microcentrifuge tube. One volume of 70% ethanol (300 µl) was added to the clear lysate. About 700 µl of the sample was transferred to the RNeasy spin column that was placed in a collection tube and centrifuged for 15 seconds at ≥ 8,000 rpm. RNase-free water was added directly to the spin column membrane and centrifuged for 1 minute at ≥ 800 rpm to isolate the RNA. The isolated sample was

transferred to a new Eppendorf tube and stored at -80 c. Quantification of RNA was performed in duplicate using spectrophotometry at 260 nm (dual-wavelength Beckman, spectrophotometer, USA).

Super-Script IV One-Step RT-PCR kit (Cat# 12594100, Thermo Fisher Scientific, Waltham, MA USA) was utilized for reverse transcription of extracted RNA. Step One instrument (Applied Biosystem, USA) was used in a thermal profile as follows: 10 minutes at 45ºC for reverse transcription, 2 minutes at 98ºC for RT inactivation and initial denaturation by 40 cycles of 10 seconds at 98ºC, 10 seconds at 55ºC and 30 seconds at 72ºC for the amplification step. After the RT-PCR run, the data were expressed in Cycle threshold (Ct) for the target genes and housekeeping gene. Normalization for variation was conducted in the expression of the target gene; FOXD1 was performed referring to the mean critical threshold (CT) expression value of the GAPDH housekeeping gene by the ΔΔCt method. The relative quantitation (RQ) of the target gene was quantified according to the calculation of the $2^{-\Delta\Delta Ct}$ method. The specific primer sequences in the experiment were as follows: FOXD1: 5′-TGAGCACTGAGATGTCCGATG-3′ (forward primer) and 5′-CACCACGTCGATGTCTGT TTC-3′ (reverse primer), Caspase-3: 5′-TGACAGCCAGTGAGACTTGG-3′ (forward primer) and 5′-GACTCTAGACGGCATCCAGC-3′, GAPDH: 5′-CCATGGAGAA GGCTGGGG-3′ (forward primer) and 5′- CAAAGTT GTCATGGATGACC -3′ (reverse primer).

Western Blot

We assessed mTOR and LC3I/II autophagy markers. The Ready PrepTM protein extraction kit (total protein) provided by Bio-Rad Inc (Catalog #163-2086) was employed according to manufacturer instructions and was added to each sample of the cell pellets of the two assessed groups. Bradford Protein Assay Kit (SK3041) for quantitative protein analysis was provided by Bio basic Inc (Markham Ontario L3R 8T4 Canada). 20 µg protein concentration of each sample was then loaded with an equal volume of 2x Laemmli sample buffer containing 4% SDS, 10% 2-mercaptoehtanol, 20% of glycerol, 0.004% of bromophenol blue, and 0.125 M Tris HCl. Polyacrylamide gels were performed using TGX Stain-Free™ FastCast™ Acrylamide Kit (SDS-PAGE), which was provided by Bio-Rad Laboratories Inc Cat # 161-0181. The gel was assembled in a transfer sandwich in the following order from below to the top (filter paper, PVDF membrane, gel, and filter paper). The blot was run for 7 minutes at 25 V to allow protein bands to transfer from gel to membrane using BioRad Trans-Blot Turbo. The membrane was blocked in tris-buffered saline with Tween 20 (TBST) buffer and 3% of bovine serum albumin (BSA) at room temperature for 1 hour. The components of the blocking buffer were as follows: 20 mM Tris pH 7.5, 150 mM NaCl, 0.1% of Tween 20, and 3% of bovine serum albumin (BSA). Primary antibodies of mTOR and LC3I/II (Santa Cruze) were purchased. Incubation was done overnight in each primary antibody solution against the blotted target protein, at 4°C. Incubation was done in the HRP-conjugated secondary antibody (Goat anti-rabbit IgG- HRP-1mg Goat mab -Novus Biologicals) solution against the blotted target protein for 1 hour at room temperature. The chemiluminescent substrate (Clarity TM Western ECL substrate Bio-Rad cat#170-5060) was applied to the blot according to the manufacturer's recommendation. Briefly, equal volumes were added from solution A (Clarity western luminal/ enhancer solution) and solution B (peroxidase solution). The chemiluminescent signals were captured using a CCD camera-based imager. Image analysis software was used to read the band intensity of the target proteins against the control sample beta-actin (housekeeping protein) by protein normalization on the ChemiDoc MP imager.

MTT Assay Methodology

Plate cells (cells density 1.2–1.8 × 10,000 cells/well) were in 96 well plates at a volume of 100µl complete growth medium ± 100 µl of the RE extract per well for 24 hours before the micro-culture tetrazolium (MTT) assay. MTT Reagent, supplied ready for use, was obtained from (Biospes, China, Cat n#BAR1005-1). A colorimetric assay was utilized for the assessment of cell metabolic activity. Using the oxidoreductase enzymes under defined conditions could reflect the number of viable cells present and those enzymes could reduce the tetrazolium dye. The absorbance was directly proportional to the number of living cells in the culture. The reduction of tetrazolium salts has been widely accepted as a reliable method for examining cell proliferation. Color absorbance was read at OD range 450 to 630 nm using an Enzyme-Linked Immuno-Sorbent Assay (ELISA) plate reader (Stat Fax 2200, Awareness Technologies, Florida, USA). In addition, duplicate readings were taken and averaged for each sample. The amount of absorbance was proportional to cell number.

ELISA

Protein content in the cell pellets of all studied groups was determined according to the method of Bradford et al. (1976) using Genei, Bangalore, protein estimation kit. Color absorbance was read at OD range 490 to 630 nm using an Enzyme-Linked Immuno-Sorbent Assay (ELISA) plate reader (Stat Fax 2200, Awareness Technologies, Florida, USA). Lipid peroxidation (MDA) was assessed using a Biovision Assay kit (#K739-100) according to manual instructions. The calculated MDA values were expressed as nmol/mg protein. Superoxide Dismutase (SOD) was estimated using the Biovision Activity Assay kit (catalog #K335-100). The calculated SOD activity (inhibition rate %) was expressed as U/min/mg protein. The apoptosis activity was assessed by measuring the level of caspase-3 in cell lysate using lifespan Bioscience Inc assay kit (Catalog No. LS-F4138) according to manual instructions. The level of caspase-3 was calculated as ng/mg protein.

Statistical Analysis

Numerical data were represented as mean and standard deviation (SD) values. Assumptions of normality, homogeneity of variance, and sphericity were confirmed

using Shapiro-Wilk's, Levene's, and Mauchly's tests, respectively. Intergroup comparisons were analyzed using an independent t-test, whereas intragroup comparisons were conducted utilizing one-way repeated measures ANOVA, followed by multiple pairwise paired t-tests with Bonferroni correction. The significance level was set at $p<0.05$ within all tests. Statistical analysis was performed with R statistical analysis software version 4.0.3 for Windows.

Results

CFU assay at different time intervals demonstrated that the mean values cell count of the control OSCC group were significantly higher than the mean values of the OSCC/RE group ($p<0.05$). Intragroup comparison at both groups showed a significant difference between various time intervals ($p<0.05$). There was no significant difference between both groups at 24 hours ($p=0.118$) time intervals. However, the other intervals OSCC group had a significantly higher value than OSCC/RE group ($p<0.05$). Within both groups, there was a significant difference between the values measured at different time intervals ($p<0.05$). Below is an illustration of the pairwise comparisons in different time intervals of CFU Table 1 and (Figures 1 and 2).

MTT assay at different time intervals showed that mean values of absorbance at 450 of OSCC group were significantly higher than mean values of OSCC/RE group ($p<0.05$). Intragroup comparison at both groups showed a significant difference between different time intervals ($p<0.05$). Below is an illustration of the pairwise comparisons in different time intervals of MTT results in (Table 2 and Figure 3-a).

Regarding ELISA, mean values of SOD of OSCC/RE group at different time intervals were significantly higher than mean values of OSCC group ($p<0.05$). Intragroup comparison of OSCC/RE group showed a significant difference between different time intervals ($p<0.05$), (Figure 3-b). On the contrary, mean values of MDA and Caspase-3 of the OSCC group were significantly higher than mean values of the OSCC/RE group ($p<0.05$) at different time intervals except for the 24-hour interval ($p>0.05$). Intragroup comparisons for both groups showed

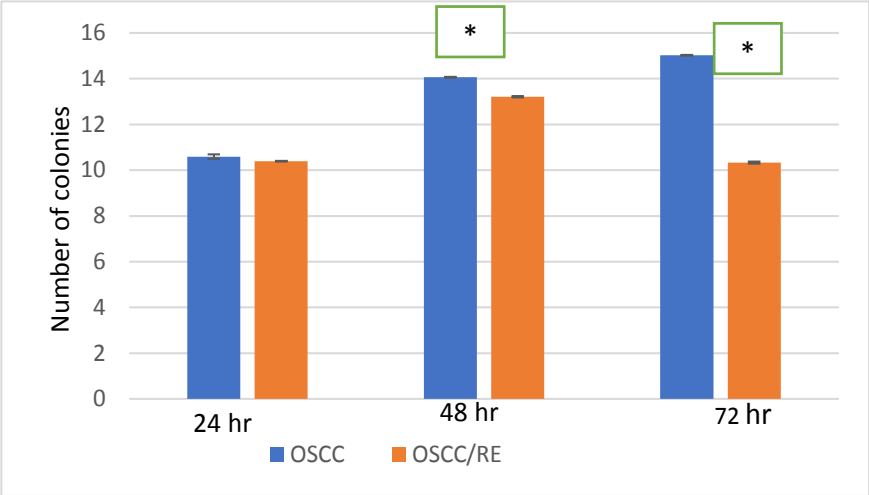

Figure 1. Bar Chart Showing CFU

Table 1. Intragroup Comparisons for Both Groups Showed a Significant Difference between Different Time Intervals of CFU

		Time	(Mean± SD) OSCC	OSCC/RE	p-value
Cell count	CFU	24 hours	10.59 ± 0.10^{C}	10.39 ± 0.01^{B}	0.118ns
		48 hours	14.06 ± 0.01^{B}	13.21 ± 0.03^{A}	0.001*
		72 hours	15.02 ± 0.02^{A}	10.33 ± 0.05^{B}	<0.001*
		p-value	<0.001*	<0.001*	

Different superscript letters indicate a statistically significant difference within the same vertical column and parameter; *significant ($p<0.05$).

Table 2. Intragroup Comparisons for Both Groups Showed a Significant Qdifference between Different Time Intervals of MTT Assay

		Time	(Mean± SD) OSCC	OSCC/RE	p-value
MTT	Absorbance at 450	24 hours	0.72 ± 0.12^{B}	0.47 ± 0.13^{A}	0.014*
		48 hours	1.46 ± 0.40^{A}	$0.25\pm0.11A^{B}$	0.002*
		72 hours	1.32 ± 0.21^{A}	0.11 ± 0.06^{A}	<0.001*
		p-value	0.009*	0.006*	

Different superscript letters indicate a statistically significant difference within the same vertical column and parameter; *significant ($p<0.05$).

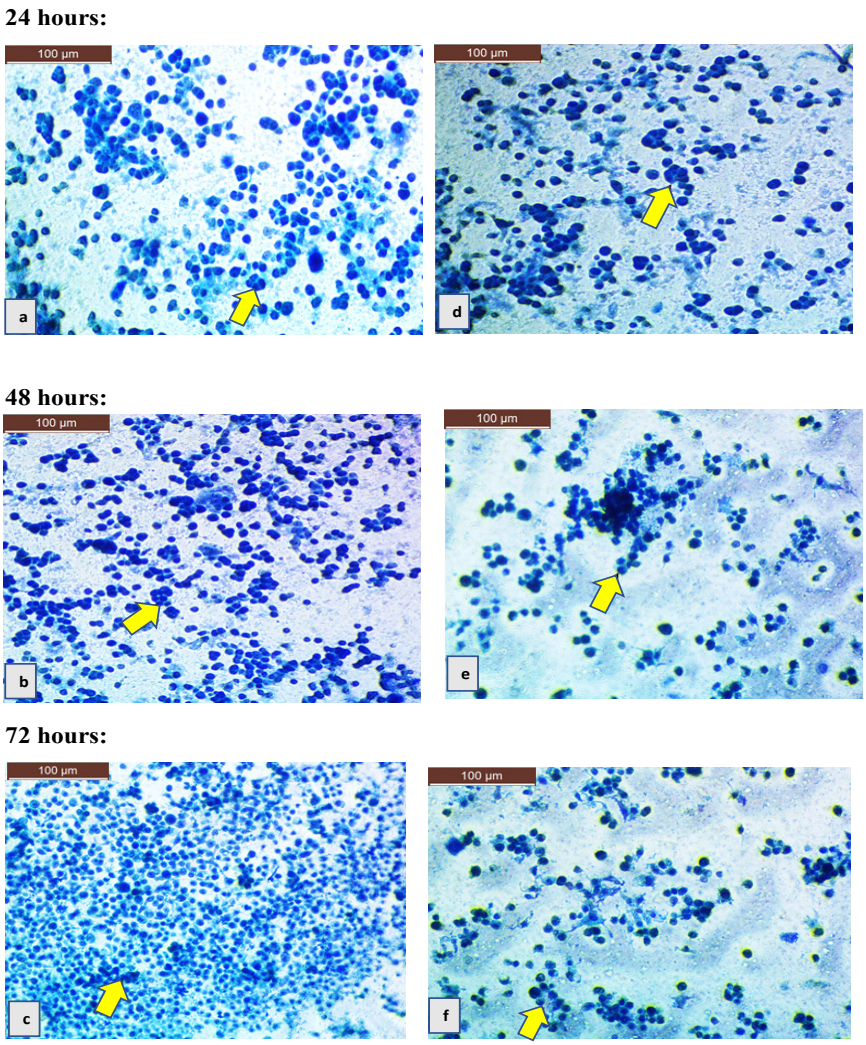

Figure 2. CFU were Sained with Crystalline Violet in All Studied Groups. A, b and c: OSCC group at 24 h, 48 h, and 72 h respectively. d, e and f: OSCC/RE group at 24 h, 48 h, and 72 h respectively. Yellow arrows showed the CFU. Each colony consisted of more than 50 cells

a significant difference between different time intervals ($p<0.05$) Table 3 and (Figures 3-c and d).

Mean values of mTOR and LC3 I/II, which were measured using the Western blot technique, were higher in the OSCC group than the OSCC/RE group and the differences were significant at different time intervals

Table 3. Intragroup Comparisons for Both Groups Showed a Significant Difference between Different Time Intervals of ELISA

ELISA	MDA	Time	(Mean± SD) OSCC	OSCC/RE	p-value
	SOD	24 hours	1.03 ± 0.50^{A}	1.59 ± 0.17^{C}	0.041*
		48 hours	0.98 ± 0.21^{A}	2.03 ± 0.24^{B}	<0.001*
		72 hours	0.55 ± 0.17^{A}	2.95 ± 0.31^{A}	<0.001*
		p-value	0.109ns	<0.001*	
	MDA	24 hours	1.58 ± 0.37^{B}	1.43 ± 0.26^{A}	0.433ns
		48 hours	2.32 ± 0.23^{A}	1.01 ± 0.12^{A}	<0.001*
		72 hours	3.07 ± 0.65^{A}	0.43 ± 0.12^{B}	<0.001*
		p-value	0.009*	<0.001*	
	Caspase-3	24 hours	2.08 ± 0.28^{B}	1.80 ± 0.22^{A}	0.080ns
		48 hours	2.53 ± 0.19^{A}	1.35 ± 0.23^{B}	<0.001*
		72 hours	2.98 ± 0.29^{A}	0.78 ± 0.09^{C}	<0.001*
		p-value	0.003*	<0.001*	

Different superscript letters indicate a statistically significant difference within the same vertical column and parameter; *significant ($p<0.05$).

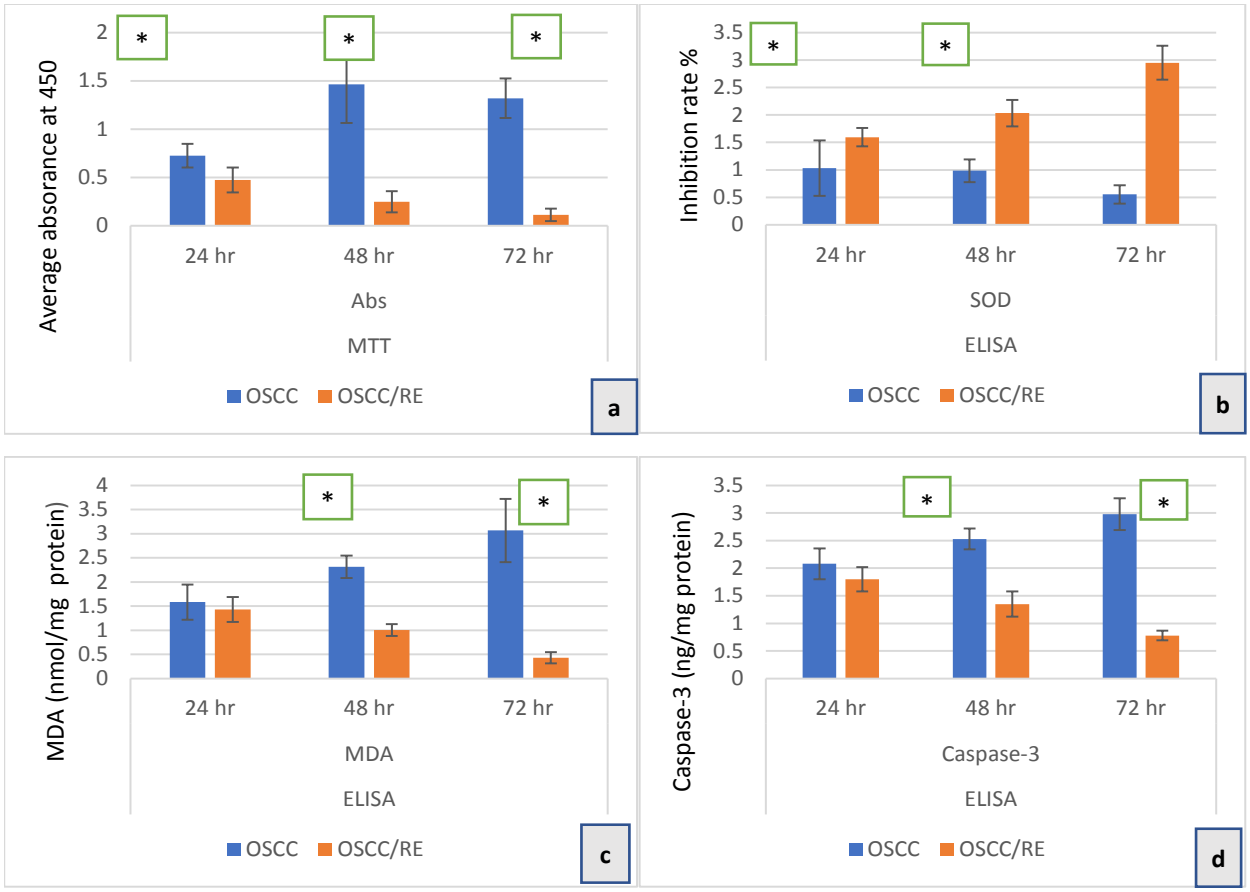

Figure 3. (a), Bar chart showing average absorbance at 450; b, Bar chart showing average SOD; c, Bar chart showing average MDA; d, Bar chart showing average Caspase-3.

($p<0.05$) except for 24 hours ($p>0.05$). Intragroup comparisons of the OSCC group showed a significant difference between different time intervals for both markers ($p<0.05$) Table 4, (Figures 4-a and b).

The PCR analysis at 72-hour intervals only showed that the mean value of FOXD1 of the OSCC group was significantly higher than the mean values of the OSCC/RE group ($p<0.001$). Intragroup comparison of OSCC/RE group showed a significant difference between different time intervals ($p<0.05$) (Figure 5-a). Furthermore, the PCR analysis of caspase-3 at 24, 48, and 72 hours of the OSCC group showed all the values to be highly significant than the mean values of the OSCC/RE group ($p<0.001$). Intragroup comparison of OSCC/RE group showed a significant difference between different time intervals ($p<0.05$) Table 5, (Figure 5-b).

Discussion

OSCC initiation and progression have multiple consecutive histopathological stages, which are driven by oncogene activation and tumor suppressor dysfunction (Wu et al., 2018). Particularly, numerous studies have found that abnormal expression of transcription factors

Table 4. Intragroup Comparisons for Both Groups Showed a Significant Difference of Western Blot Technique

		Time	(Mean± SD) OSCC	OSCC/RE	p-value
Western blot	mTOR	24 hours	1.03 ± 0.50^{A}	1.59 ± 0.17^{C}	0.041*
		24 hours	1.73 ± 0.15^{C}	1.57 ± 0.12	0.211ns
		48 hours	2.97 ± 0.52^{B}	1.58 ± 0.28	0.026*
		72 hours	4.07 ± 0.59^{A}	1.48 ± 0.22	0.009*
		p-value	0.820ns	0.010*	
	LC3 I/II	24 hours	1.70 ± 0.50^{B}	1.03 ± 0.48^{A}	0.170ns
		48 hours	2.43 ± 0.15^{B}	0.95 ± 0.30^{A}	0.005*
		72 hours	3.70 ± 0.36^{A}	1.10 ± 0.20^{A}	0.001*
		p-value	0.810ns	0.006*	

Different superscript letters indicate a statistically significant difference within the same vertical column and parameter; *significant ($p<0.05$).

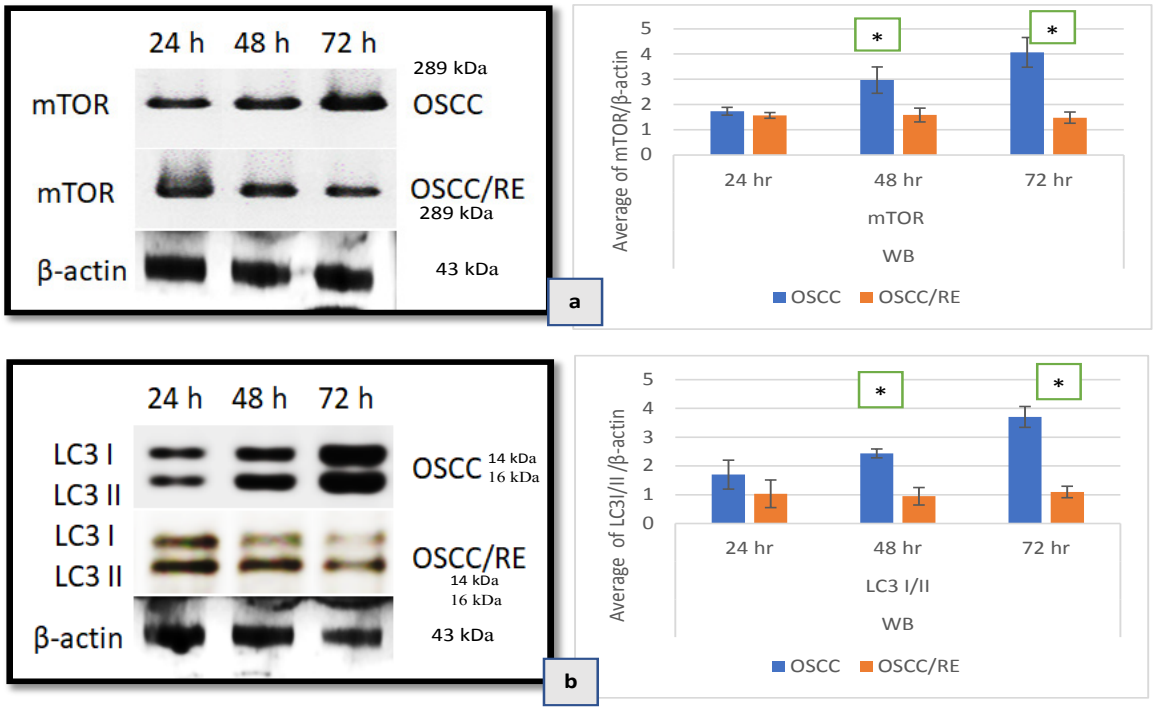

Figure 4. a, Bar chart and western blot results showing average mTOR; b. Bar chart and western blot showing average LC3 I/II.

in the FOX gene family is involved in almost all stages of human tumorigenesis. Among these tumor regulators, FOXD1 expression is demonstrated in a series of human cancers (Gao et al., 2017).

FOXD1 is a mediator of the successful progression of cell reprogramming through differentiation and

Table 5. Intragroup Comparisons for Both Groups Showed a Significant Difference in Different Time Intervals of RT- PCR

		Time	(Mean± SD) OSCC	OSCC/RE	p-value
RT-PCR	FOXD1	24 hours	1.03±0.50^{A}	1.59±0.17^{C}	0.041*
		24 hours	1.44±0.33	1.26±0.15^{A}	0.267ns
		48 hours	1.37±0.37	1.03±0.07^{A}	0.077ns
		72 hours	1.74±0.34	0.76±0.12^{B}	<0.001*
		p-value	0.129ns	<0.001*	
	Caspase-3	24 hours	2.39±0.17^{B}	1.97±0.20^{A}	0.003*
		48 hours	2.66±0.10^{B}	1.60±0.13^{A}	<0.001*
		72 hours	3.11±0.12^{A}	0.99±0.19^{B}	<0.001*
		p-value	<0.001*	<0.001*	

Different superscript letters indicate a statistically significant difference within the same vertical column and parameter; *significant (p<0.05).

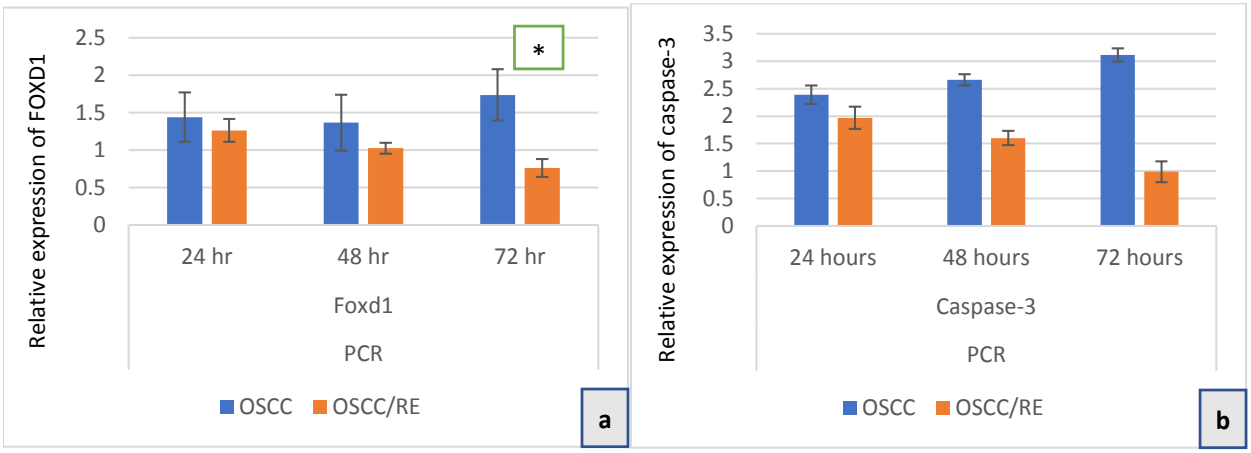

Figure 5. a, Bar chart showing average FOXD1; b, Bar chart showing average Caspase-3.

self-renewal. It has been firstly identified in the forebrain neuroepithelium (Fetting et al., 2014; Koga et al., 2014). Moreover, FOXD1 is associated with carcinogenesis, tumor progression, and metastasis in numerous cancers (Van der Heul-Nieuwenhuijsen et al., 2009, Nakayama et al., 2015 and Qiu et al., 2021).

The management of cancer can successfully be achieved through early diagnosis. Concealment of the anatomical site and lack of specific and reliable indicators cause more than half of the HNSCC patients to reach advanced stages at the time of diagnosis (Kreppel et al., 2016). Therefore, the identification of biomarkers for the early diagnosis of HNSCC is of great importance. In the current study, RT-PCR was calculated to evaluate FOXD1 expression as a potential diagnostic marker of HNSCC.

Our FOXD1 results were consistent with Perri et al., (2017) and Lin et al., (2020) results showing upregulation of FOXD1 expression in oral squamous cell carcinoma cell lines. Furthermore, the study heavily suggested that FOXD1 would most probably play a key role during the OSCC tumorigenesis, delineating it as a novel diagnostic and prognostic biomarker for OSCC that is closely related to tumor invasion and metastasis. Moreover, our findings in respect of FOXD1 worked in unison with the results of Qiu et al., (2021). These results confirmed that FOXD1 mRNA was significantly overexpressed in HNSCC tissues, which was due to the specific function of FOXD1 as an oncogene in HNSCC.

Autophagy is an intracellular degradation process that allows cancer cells to survive under stress conditions. Autophagy plays an important role in tumor progression and helps to maintain the malignant state against anti-cancer therapies (Hippert., 2006). Although several autophagy markers have been associated with oral squamous cell carcinoma, it remains unclear whether up or down-regulation of autophagy favors its progression. Autophagy levels during oral squamous cell carcinoma are both timing- and cell-specific (Peña-Oyarzu´ n et al., 2020). In the present study, we examined the autophagosome marker proteins, LC3-I/II, and mTOR in both groups. Our results of LC3-I/II confirmed those of Sambandam et al., (2016) who postulated that autophagosome formation occurs in OSCC tumor cells.

mTOR, which is an autophagy molecule, seems to be a highly promising marker to handle patients with solid malignancies, based on applying targeted therapeutic strategies (Mastronikolis et al., 2017). Our mTOR results agreed with Yin et al., (2019). They stated that the overexpression of mTOR protein in OSCC was clarified as a coincidence of upstream oncogene overactivation combined with suppressor gene downregulation, whereas its pure mutations are very rare. Furthermore, Harsha et al., (2020) revealed that mTOR represses autophagy under normal nutritional conditions and the increase in mTOR was due to an impairment of autophagic flux. Terabe et al., (2018) also reported the expression of autophagy-related markers (LC3-I/-) at the surgical margin of OSCC and concluded that it might be an indicator of local recurrence and a poor prognosis in human OSCC.

In the present study, MDA and SOD were examined using the ELISA test. In confirmation of our results, Huo et al., (2014) showed elevation of MDA expression and the decrease of SOD expression in the OSCC group. This can be explained that the detection of antioxidant enzyme levels might be a valuable marker in cancer prognosis, which could be a great resource for improving therapeutic strategies in oral cancer.

Many recent studies have addressed the pharmaceutical capacities of rosemary due to its anti-inflammatory, anti-infective or anticancer action (Allegra et al., 2020). It can act on free radicals and could defend against the oxidative damage of DNA, proteins, and lipids. Although, as subsequently observed, the derivatives of rosemary are, in some conditions, capable of inducing a cytotoxic effect precisely through the release of reactive oxygen species (ROS). Several studies have demonstrated that RE and its polyphenols carnosic acid and rosmarinic acid would have potent anti-cancer effects (Petiwala et al., 2015).

Our results of RE in treated OSCC cell lines with all examined techniques showed an excellent effect of RE on treatment acting as anticancer material. Colony formation assay demonstrated the greatest effect on OSCC/RE in the number of cancerous cells. In agreement with our results were (Tumur et al., 2015) results, which illustrated that RE pretreatment significantly reduces EGFR activation in cancer cells, providing a rationale for RE antiproliferative effects and reduce tumor metastasis. Moreover, the antitumor effect of rosemary was found to be related to the reduction of serum miR-15b, a possible marker for controlling the effect of rosemary. Likewise, in agreement with our results, (Moore, et al., 2016) stated that rosemary can inhibit the activation of the Akt/mTOR/p70S6K signaling pathway, which in turn would lead to reduced cell proliferation and affect the anticancer effect.

Apoptosis, also known as programmed cell death, is a process with typical morphological characteristics including a reduction in cell size, condensation of the cytoplasm, blebbing of the plasma membrane, and fragmentation of chromatin and DNA into oligonucleosomes (Julian and Olson, 2015). Caspase cascades play a crucial role in apoptosis as they are highly associated with cancer development and prognosis. However, the role of expression and activation of various caspases in tumorigenesis remains multi-faceted (Liu et al., 2017). Several studies indicated that the caspase-3 expression is associated with many pathological outcomes. In the present study, we examined caspase 3, which is considered a major effector in apoptotic pathways that is useful in scoring the apoptotic index.

On the one hand, an elevated expression of caspases was observed in tumor tissues of several cancer types, including breast carcinomas, pancreatic ductal carcinoma, non-small cell lung carcinoma, and oral carcinoma (O'Donova et al., 2003; Satoh et al., 2000; Tormanen-Napankangas et al., 2001; Coutinho-Camillo et al., 2011). On the other, the level of caspase-3 was found to have low expression in tumors compared to normal in hepatocellular carcinoma (Fujikawa et al., 2000), pancreatic cancer (Jakubowska et al., 2016), and prostate carcinoma (Winter et al., 2001). These findings demonstrate that the roles of caspases in tumorigenesis might be varied. They are likely depend on the type of cancer and complex tumor

microenvironment.

Following previous studies results, the results of our study showed that the levels of expression of caspase-3 were higher in the OSCC group than in OSSC/RE group, suggesting that apoptosis-related caspases were elevated to promote tumor growth. Moreover, Fu et al., (2016) confirmed our results and added that the high expression of caspase-3 was associated especially with advanced stage in OSCC and was dependent on the site of cancer. Also, Hague et al., (2004) and Leite et al., (2016) result revealed that OSCC had a higher apoptotic area index compared to other types of lesions. Hence, apoptosis plays a dual role in cancer development and malignancy. Moreover, the role of apoptosis-related caspases particularly in oral tongue squamous cell carcinoma remains controversial, (Liu et al., 2017).

In contrast, some studies suggested that the number of apoptotic cells decreased in oral lesions because of increased molecular abnormalities in epithelial cells and cancer.Tanda et al., (2000) and Poomsawat et al., (2014) explained explained this difference and identified contrasting results, given the multiple differences in apoptosis detection methods, analyses modes, and study models used in the investigations.

To the best of our knowledge, most studies underscored a great effect of RE on apoptosis in different types of cancers such as Moore et al., (2016) who studied its effect on lung cancer cells and got it effect on apoptosis through the Akt phosphorylation enzyme. In addition, Barni et al., (2012) examined the effect of RE on colorectal cancer cells, through its carnosic acid component of RE. In sum, carnosic acid inhibited the proliferation and migration capacity of human colorectal cancer cells by down-regulating the expression of COX-2. Furthermore, in agreement with our results were Levine e al., (2017) results, which illustrated the remarkable effect of RE on the canine neoplastic cancer cell line.

Regarding the results of caspase-3 on the OSCC/RE group, our results came following the results of Tsai et al., (2011), which showed a decrease in its expression in human neuroblastoma. The apoptotic effect of RE was mainly due to carnosic acid exhibiting antitumor properties in both in vivo and in vitro conditions. Moreover, the study noticed that it neutralizes reactive oxygen species, including hydroxyl and lipid peroxide radicals, and thus, protects biologic membranes against lipid peroxidation. In addition, Choi et al., (2019) also proved the great apoptotic effects of RE through its carnosol component on human melanoma cells.

More results of RE apoptotic effects were documented in Abd El Wahab et al., (2020) study, evaluating the apoptotic effect of rosmarinic acid, which is also a component of RE to treated hamsters induced OSCC. This was clarified that rosmarinic acid may act through numerous mechanisms such as exerting anti-inflammatory and antioxidant effects, in addition to inhibiting cell proliferation, migration, and inducing cancer cells apoptosis.

Limitations

Other biological entities than cell lines and other types of cell lines may be used to evaluate the anticancerous effect of Rosemary extract.

In conclusion, the present study results revealed that FOXD1 could potentially be used as a valuable diagnostic biomarker for OSCC. Moreover, Rosemary extract can be utilized as an anticancer treatment by inhibiting the proliferation of OSCC cells. Furthermore, RE suppresses OSCC progression by motivating autophagy as well as inhibiting cell proliferation and suppressing caspase-3 apoptotic marker.

Author Contribution Statement

Y. A: Manuscript topic creation and a major contribution to whole manuscript writing.

D. S: Practical part of the study and methodology writing.

S.H: Revision of the whole manuscript.

A.M: Revision of the practical part.

Availably of data and materials: Available.

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Ethics approval

It was approved by Research Ethics Committee at Faculty of Dentistry, October 6 University, Giza, Egypt (RECOU/6-2020).

Conflict of Interest

We declare no conflict of interest.

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