

“Effect Of Fucoxanthin On Head And Neck Squamous Cell Carcinoma Cell Line: In Vitro Study“

Thesis

Submitted to the Oral Pathology Department, Faculty of Dentistry, Ain Shams University for partial fulfillment of requirements of the Doctor Degree of Philosophy (PhD) in Oral and Maxillofacial Pathology

By

Mai Hafez Mohamed Hafez

B.D.S (Faculty of Dentistry, Ain Shams University, 2009)

M.Sc. in Oral Pathology

(Faculty of Dentistry, Ain Shams University, 2017)

Dr.maihafez.op@gmail.com (01147992390)

Supervisors

Dr/ Ihab Saeed Abd Elhamid

Professor of Oral Pathology

Faculty of Dentistry, Ain Shams University

[Dean of Faculty of Dentistry, Badr University]

Dr/ Shaimaa Eliwa Ghazy Eliwa

Lecturer of Oral Pathology

Faculty of Dentistry, Ain Shams University

Dr/ Marwa Matboly Sayed

Associate Professor of Biochemistry and Molecular Biology

Faculty of Medicine, Ain Shams University

Faculty of Dentistry
Ain Shams University
2020

Acknowledgments

First, and foremost, my deepest gratitude and thanks should be offered to "**ALLAH**", the most kind and most merciful, for giving me the strength to complete this work.

I would like to express my sincere gratitude to **Dr/ Ihab Saeed Abd Elhamid**, Professor of Oral Pathology, Faculty of Dentistry, Ain Shams University and Dean of Faculty of Dentistry, Badr University, for his continuous support and guidance for me to present this work. I'm really honored to work under his generous supervision.

I acknowledge with much gratitude to **Dr/ Shaimaa Eliwa Ghazy Eliwa**, Lecturer of Oral Pathology, Faculty of Dentistry, Ain Shams University, for the efforts and time she has devoted to accomplish this work.

I would like also to thank **Dr/ Marwa Matboly Sayed**, Associate Professor of Biochemistry and Molecular Biology, Faculty of Medicine, Ain Shams University, for her great supervision and unlimited help to provide all facilities to accomplish this work.

Dedication

I would like to dedicate this work to all members of my family, specially my parents and my sisters for pushing me forward in every step of my life.

Also to my friends for their support and encouragement

List of Contents

<i>Subject</i>	<i>Page No.</i>
List of Abbreviations	i
List of Tables.....	iii
List of Figures	v
Introduction	1
Review of Literature	7
Aim of the Study	44
Material and Methods	45
Results	79
Discussion.....	100
Conclusions	114
Summary	115
Recommendations.....	118
References	119
Arabic Summary.....	—

List of Abbreviations

Abbrev.	Full term
APC	: Adenomatous polyposis coli
BAX	: Bcl2 associated X
BMF	: Bcl2 modifying factor
BCR	: B cell receptor
CDK	: Cyclin dependent kinase
Cox2	: Cyclooxygenase 2
CXCR4	: Chemokine receptor 4
CYP1A1	: Cytochrome family 1 sub family A
EBV	: Epstein-Barr virus
EGFR	: Epidermal growth factor receptor
Fx	: Fucoxanthin
Fxol	: Fucoxanthinol
GADD	: Growth arrest and DNA damage inducible protein
GJIC	: Gap junction intracellular communication
GRB2	: Growth factor receptor-bound protein 2
HER2	: Human epidermal growth factor receptor 2
HNSCC	: Head and neck squamous cell carcinoma
HPV	: Human papilloma virus
HRK	: Harakiri gene
HUVE	: Human umbilical vein endothelial cells
ICAD	: Inhibitor of caspase activated DNA
IO	: Immuno-oncology
ITAM	: Immune receptor tyrosine based activation motif
KLF4	: Kruppel like factor 4

LAB	: Linker for activation of B cells
LAT2	: Linker for activation of T cells
LCK	: Lymphocyte specific protein tyrosine kinase
LFA1	: Lymphocyte function associated antigen 1
MMP	: Matrix metalloproteinases
MiRNA	: Micro ribonucleic acid
NK	: Natural killer
NFAT	: Nuclear factor of activated T cells
NOS	: Nitric oxide synthase
NTAL	: Non T cell activation linker
OD	: Optical density
PARP	: Poly ADP ribose polymerase
PIAS1	: Protein inhibitor of activated STAT1
PIM	: Proviral integration site for Moloney murine leukemia virus-1
PUMA	: P53 upregulated modulator of apoptosis
ROS	: Reactive oxygen species
SLP76	: SH2 domain containing leukocyte protein of 76 KDA
SOCS1	: Suppressor of cytokine signaling 1
STAT	: Signal transducer and activator of transcription
TAA s	: Tumor associated antigens
TCR	: T cell receptor
TNF	: Tumor necrosis factor
TRAIL	: Tumor necrosis factor related apoptosis inducer ligand
TRAP s	: Transmembrane adaptor proteins
VEGF	: Vascular endothelial growth factor
WHO	: World health organization
XIAP	: X linked inhibitor of apoptosis protein
ZAP70	: Zeta chain of T cell receptor associated protein kinase

List of Tables

Table No.	Title	Page No.
Table (1):	Effect of STAT1 on tumorigenesis.....	30
Table (2):	Absorbance and viability mean of fucoxanthin used to detect IC50 at 24 hours	56
Table (3):	Absorbance and viability mean of fucoxanthin used to detect IC50 at 48 hours	56
Table (4):	Fucoxanthin IC50 results at 24&48 hours	57
Table (5):	Different groups of the present study	
Table (6):	Reverse Transcription reaction preparation.	68
Table (7):	Reaction mix for SYBR green based miRNA reaction.....	71
Table (8):	Cycling conditions for real-time qPCR.....	71
Table (9):	Reaction Mix for QuantiTect SYBR Green PCR.....	75
Table (10):	MTT cell viability assay in different groups.....	80
Table (11):	The median, range values and results of Mann-Whitney U test for comparisons between the two time periods and Kruskal-Wallis test for the comparison between viability % of the different groups.....	83
Table (12):	The median, range values and results of Mann-Whitney U test for comparisons between the two time periods and Kruskal-Wallis test for the comparison between MIR-155-5P fold change of the different groups.....	86
Table (13):	The median, range values and results of Mann-Whitney U test for comparisons between the two time periods and Kruskal-Wallis test for the comparison between STAT fold changes of the different groups.....	89

Table (14): The median, range values and results of Mann-Whitney U test for comparisons between the two time periods and Kruskal-Wallis test for the comparison between LAT fold changes of the different groups.....92

Table (15): Results of Spearman’s correlation coefficient for the correlation between viability % and MIR-155-5P.....94

Table (16): Results of Spearman’s correlation coefficient for the correlation between viability % and STAT195

Table (17): Results of Spearman’s correlation coefficient for the correlation between viability % and LAT296

Table (18): Results of Spearman’s correlation coefficient for the correlation between MIR-155-5P and STAT197

Table (19): Results of Spearman’s correlation coefficient for the correlation between MIR-155-5P and LAT2.....98

Table (20): Results of Spearman’s correlation coefficient for the correlation between STAT1 and LAT2.....99

List of Figures

Figure No.	Title	Page No.
Figure (1):	Structure of fucoxanthin	11
Figure (2):	Fucoxanthin on cell cycle arrest.....	16
Figure (3):	Fucoxanthin and the apoptosis signaling pathway	19
Figure (4):	The JAK-signal transducer and activator of transcription (JAK-STAT) signaling pathway	23
Figure (5):	IFN/Stat1 pathway.....	26
Figure (6):	LAT2 and TCR signaling payhway	34
Figure (7):	Diagram showing the transcription of the miRNA gene by RNA pol II to form a pri-miRNA transcript, which is processed by the RNase III Drosha and DGCR8, yielding the premiRNA.	37
Figure (8):	A diagram demonstrating an overview of miRNAs documented in the literature regulating the transformation of normal squamous epithelial cells into carcinoma cells, ultimately resulting in metastasis	40
Figure (9):	Screenshot for the search of STAT1 in head and neck neoplasia retrieved from gene atlas database	46
Figure (10):	Screenshot for the search of LAT2 expression retrieved from gene atlas data base	46
Figure (11):	MIR-155-P targeting STAT1 retrieved from MiRTarBase microRNA Targets database	47
Figure (12):	miR-155-P targeting LAT2 retrieved from MiRTarBase microRNA Targets database	47
Figure (13):	Pathway enrichment analysis of miR-155-5p retrieved from daiana database	48
Figure (14):	Photograph showing tissue culture flask containing SCC15 cell line.	50
Figure (15):	Photograph showing anti-hsa-miR-155-5p.....	51

Figure (16): Photograph Showing STAT1 primer assay52

Figure (17): Photograph Showing LAT2 primer assay53

Figure (18): Photograph showing the 6-well plate where SCC15 cells were treated with the phytochemical extracts.58

Figure (19): A diagram showing MiRNEASy mini procedure.64

Figure (20): Conversion of mature miRNAs into cDNA and subsequent detection.....67

Figure (21): High specificity is required when using SYBR Green since SYBR I bind all double-strand DNA (non-specific or primer dimer).....69

Figure (22): SYBR Green I Assay:.....70

Figure (23): Amplification Plot (miRNA-155 %252c SA-1).....72

Figure (24): Amplification Plot (miRNA-155%252c SA-6).72

Figure (25): Amplification Plot (miRNA-155%252c SA-21).....73

Figure (26): Amplification Plot (STAT1 %252c SA-1).73

Figure (27): Amplification Plot (LAT2 %252c SA-20).74

Figure (28): Photomicrograph of SCC 15 cells of the control group showing proliferating cancer cells at 100% confluence (origin. mag. x40).....81

Figure (29): Photomicrograph of treated SCC15 cells showing some apoptotic morphological changes: chromatin margination and nuclear fragmentation (grey) (origin. mag. x40).....81

Figure (30): Photomicrograph of SCC15 cells treated with miRNA inhibitor showing apoptotic cell death (original magnification x 40)82

Figure (31): Box plot representing median and range values for viability % of the different groups84

Figure (32): Box plot representing median and range values for MIR-155-5P fold changes of the different groups87

Figure (33): Box plot representing median and range values for STAT fold changes of the different groups90

Figure (34): Box plot representing median and range values for LAT fold changes of the different groups93

Figure (35): Scatter plot representing direct correlation between viability % and MIR-155-5P fold change.....94

Figure (36): Scatter plot representing inverse correlation between viability % and STAT fold change.....95

Figure (37): Scatter plot representing direct correlation between viability % and LAT fold change.....96

Figure (38): Scatter plot representing inverse correlation between MIR-155-5P and STAT fold changes97

Figure (39): Scatter plot representing direct correlation between MIR-155-5P and LAT fold changes.....98

Figure (40): Scatter plot representing inverse correlation between STAT and LAT fold changes.....99

Introduction

Head and neck cancer constitutes the eighth leading cause of cancer-related deaths worldwide. Its incidence varies widely among different geographical areas: in North America and the European Union, head and neck cancer accounts for 3% to 4% of all cancer diagnosis, while in developing countries its incidence is higher due to smoke and drinking habits in combination with poor socioeconomic status (*Siegel et al., 2014*).

The most common type of head and neck cancer is head and neck squamous cell carcinoma (HNSCC) which is a morbid, frequently lethal disease that affects the epithelial cell lining of the upper aerodigestive tract (UADT, i.e., the oral cavity, pharynx, and larynx) (*Leemans et al., 2011*). While HNSCC has been strongly associated with tobacco use and heavy alcohol consumption, over the past two decades, high-risk alpha human papillomaviruses (HR α -HPV) have been an important etiological factor for a subset of HNSCC arising from the oropharynx (*Gillison et al., 2000*).

Generally, HNSCC is treated by surgery and/or chemoradiation, which cause severe side-effects in more than 80 % of the advanced HNSCC patients (*Conley, 2006*). Unfortunately, other treatment options for HNSCC patients are limited to only one single targeted therapy available in the clinic and with variable benefit i.e. cetuximab, targeting epidermal growth factor receptor (EGFR) protein

(*Huang et al., 2014*). To expand personalized cancer care for HNSCC, novel targeted therapies are needed (*Razzouk, 2014*).

Fucoxanthin is a marine carotenoid pigment that is produced in brown seaweeds and some microalgae (*Peng et al., 2011*). In humans, dietary fucoxanthin is mainly metabolized to fucoxanthinol, the deacetylated form of fucoxanthin (*Hashimoto et al., 2012*), and which is considered to be an active form of fucoxanthin.

Fucoxanthin possesses a unique chemical structure that includes an allene bond, 5,6-monoepoxide and acetylated group. Fucoxanthin is one of the most abundant carotenoids and fucoxanthin-containing brown algae, such as wakame (*Undaria pinnatifida*) and kombu (*Laminaria japonica*), are commonly consumed in Asia (*Satomi, 2017*).

Studies have revealed several health benefits of fucoxanthin, including anti-inflammatory, anti-obesity, anti-diabetes, hepato-protective and cardiovascular-protective activities, in addition to its anticancer activity (*Mikami and Hosokawa, 2013*).

Fucoxanthin and fucoxanthinol exert their anti-proliferative and cancer preventive influences via different molecular pathways involved in either cell cycle arrest, apoptosis, or metastasis (*Kumar et al., 2013*).

In addition, Fx has been shown to have anti-angiogenic potential using human umbilical vein endothelial (HUVEC) cells (*Ganesan et al., 2013*), thus, contributing to cancer prevention.

Immuno-oncology (I-O) is a young and growing field, the product of the many groundbreaking discoveries in immunology and cancer therapy in the last century. The novelty of this field is due to the historical controversy over whether or not the body's immune system could even respond to cancer at all. The idea was first proposed by William Coley in 1893, when he observed the remission of cancer in patients who had contracted acute bacterial infections (*Coley, 1893*); then followed by Paul Ehrlich in 1909, when he suggested that the immune system must have some role in preventing an outbreak of cancer in the body (*Ichim, 2005*). Unfortunately, their theories were opposed by those who did not see a plausible biological explanation, and who were convinced that cancer cells were indistinguishable from healthy cells to the body's lymphocytes. The breakthrough for modern I-O came in the 1960s, when it was accepted that lymphocytes are constantly eliminating precancerous cells throughout the body in a process called "immune-surveillance," and do in fact recognize them through tumor-associated antigens (TAAs) (*Baldwin, 1966*). This has gradually led to our understanding of cancer today.

The manipulation of immune checkpoints is the leading edge for the field of I-O. The activation of a lymphocyte, such as a cytotoxic T-cell, in adaptive immunity has been well-characterized and is known to involve the interaction between an antigen-presenting cell (APC) and the T-cell receptor (TCR), and associated coreceptors. Less explored are immune checkpoints, the body's natural defense against auto-immunity. This involves the binding of receptors on the lymphocyte

with associated ligands on the surface of the cancer cell that interfere with activation signals or induce apoptosis. Inhibition occurs almost as quickly as activation and balances the antigenic response of immune cells to avoid an attack on healthy cells. Tumor cells exploit this with an upregulation of inhibitory ligands, leaving them free to grow unchallenged by the immune system (*Kamta et al., 2017*).

The ability of immunotherapy to mediate cancer regression has been shown in a variety of malignancies. Head and neck squamous cell carcinoma (HNSCC) serves as a paradigm of immunosuppressive disease, as it is characterized by dysregulated cytokine profile, impaired function of immune effector cells and abnormalities in tumor-associated antigen (TAA) presentation (*Ferris et al., 2006; Allen et al., 2007*). In several countries, an increasing number of cases with oropharyngeal carcinoma (OPC) are attributed to human papillomavirus (HPV), especially type 16, rather than traditional risk factors such as tobacco and alcohol (*Weinberger et al., 2006*). However, regardless of the implicated cause, it is believed that the immune system plays a key role in tumorigenesis of HNSCC, as malignant cells evade immune surveillance using multiple mechanisms (*Srivastava et al., 2013; Bauman and Ferris, 2014*).

Signal transducer and activator of transcription (STAT) are a proteins family that mediate cellular response to cytokines, such as IL-6 and growth factors (*Turkson et al., 1998*).