

**Deanship of Graduate Studies
Al-Quds University**



***HRAS G13R* transformation effect on MCF10A cells
harboring different p53 variants**

Ahmad Mousa Ismael Jaffal

Master's Thesis

Jerusalem – Palestine

2020 AD/1441 Hijri

***HRAS G13R* transformation effect on MCF10A cells
harboring different p53 variants**

Prepared by:
Ahmad Mousa Ismael Jaffal

B.Sc. Medical Technology, Al-Quds University-Jerusalem

Supervisor: Dr. Zaidoun Salah

A thesis Submitted in Partial fulfillment of requirement for
the degree of Master of Biochemistry and Molecular
Biology, Faculty of Medicine -Al-Quds University.

2020 AD/1440 Hijri

**Al-Quds University
Deanship of Graduate Studies
Biochemistry and Molecular biology**



Thesis Approval

**HRAS G13R transformation effect on MCF10A cells
harboring different p53 variants**

Prepared by: Ahmad Mousa Jaffal


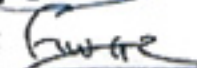
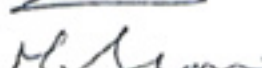
Student ID: 21511561

Supervisor: Dr. Zaidoun Salah

Master Thesis submission and acceptance date:

The names and signatures of examining committee members:

- 1. Head of committee: Dr. Zaidoun Salah**
- 2. Internal Examiner: Dr. Fawwaz Awwad**
- 3. External Examiner: Dr. Mahmoud Srour**

Signature: 
Signature: 
Signature: 

**M. Sc. Thesis
Jerusalem – Palestine
1441Hijri/ 2020AD**

Dedication

To my mother and father...

To my wife

To my brothers and sisters...

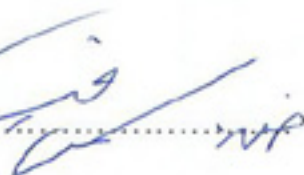
To my friends...

Ahmad Mousa Ismael Jaffal

Declaration

I certify that this thesis submitted for the degree of Master, is the result of my own research, except where otherwise acknowledged, and that this thesis has not been submitted for higher degree to any other university or institution.

Signed:



Derar Sameeh Abdel-Aziz Khader

Date: 21/12/2019

Acknowledgements

Praise be to God for His grace, praise until the end of praise, Thanks God for helping me to do this work.

I would like acknowledge and thank my wife Salam for encouraging me to complete my postgraduate study and for her support during last 4 years with her love and patience. Also I would like to thank my mother and father and family for being always beside me and for their forgiveness despite my dereliction.

I am very thankful to Dr. Zaidoun Salah for accepting me in his team allowing and teaching me how to use all equipment's. Also I would like to thank him for teaching me new methods and techniques and make me familiar with many research methods in addition to his help during my project design and work.

Special thanks my colleague Mr. Mahmoud Zahayka for his great help during all steps of this master project, he was always there when I need him. Also thanks to Dr. Zaidoun lab team during last four years (Yusef Turman, Noor El-deen Tarade, Abrar Jamous, Sharihan Ereqat, and Sondos).

Also I would like to thank both external and internal examiners Dr. Mahmoud Srour and Dr. Fawwaz Awwad

Finally I would like to thank Al-Quds University “Biochemistry and Molecular Biology Program” supervisors Dr. Suhair Arekat, and program teachers, Dr Imad Matouq, Dr Rola Abdulghany, and Dr Kefaya Azmi.

Table of Content

Dedication	IV
Declaration	I
Acknowledgements.....	II
Table of Content	III
List of abbreviations.....	V
Abstract.....	VIII
List of tables	IX
List of figures	IX
1. Introduction	1
1.1. Cancer transformation	1
1.2. Breast Cancer	1
1.3. The Oncogene-RAS	2
1.4. <i>TP53</i> tumor suppressor gene	4
1.5. <i>TP53</i> variants and <i>HRAS</i> combination role in cancer transformation	8
1.6. Problem statement and motivation of study:	8
1.7. Hypothesis:	9
1.8. Aim of the study:	9
2. Materials and Methods.....	10
2.1. Materials	10
2.2. Methods	15
2.2.1. Cell culture	15
2.2.2. Cells cryopreservation	16
2.2.3. Generation of stable cell lines pools.....	16
Selection of infected cells	17
2.2.4. Gelatin coating.....	17
2.2.5. Cell count	18
2.2.6. XTT test	18
2.2.7. Wound healing assay	18
2.2.8. Survival assay (colony formation assay)	18
2.2.9. Adhesion assay	19
2.2.10. Anoikis (Anchorage-Dependent Cell Death) assay	19

2.2.11.	Soft agar assay	19
2.2.12.	RNA Extraction.....	19
2.2.13.	cDNA synthesis.....	20
2.2.14.	Real time PCR.....	20
2.2.15.	Statistical analysis	20
3.	Results and Discussion	21
3.1.	Generation of <i>HRAS G13R</i> MCF10A clones harboring different <i>TP53</i> variants.....	21
3.2.	Characterization of phenotypic changes in <i>HRAS G13R</i> transduced MCF10A cells. 23	
3.2.1.	<i>HRAS G13R</i> overexpression in combination with different <i>TP53</i> variants effect on MCF10A cells proliferation.....	23
3.2.2.	<i>HRAS G13R</i> overexpression in combination with different <i>TP53</i> variants effect on MCF10A cells migration.	25
3.2.3.	<i>HRAS G13R</i> overexpression in combination with different <i>TP53</i> variants effect on MCF10A cells survival.....	27
3.2.4.	<i>HRAS G13R</i> overexpression in combination with different <i>TP53</i> variants effect on MCF10A cells anchorage-independent growth.	28
3.2.5.	<i>HRAS G13R</i> overexpression in combination with different <i>TP53</i> variants effect on expression of genes related to different cancer phenotypes.	31
4.	Conclusion and future outlook.	34
5.	References:	40
6.	Appendix:.....	46
6.1.	Appendix 1: plasmid construction:.....	46
6.2.	Appendix 2: Table of used primer:.....	48

List of abbreviations

°C	Degree Celsius
<i>Bax</i>	Bac1-2 associated X protein
<i>BCL-2</i>	B-cell lymphoma 2
cDNA	Complementary DNA
cm	centimeter
CGS	Cancer related gene signature
COSMIC	Catalogue of somatic mutations in cancer
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EMT	Epithelial mesenchymal transition
EMC	Extracellular matrix
ER	Estrogen receptor
ERBB2	v-erb-b2 avian erythroblastic leukemia oncogene homolog 2
g	gram
h	hour
HER2	Human epidermal receptor 2
KO	Knockout
L	liter
M	Molar
MDM2	Mouse double minute 2 homolog
mg	Milligram

min	Minute
mL	Milliliter
mRNA	Messenger RNA
ng	Nanogram
nm	Nanometer
nM	Nanomolar
nt	Nucleotide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
rpm	Round per minute
Rb	Retinoblastoma
RNA	Ribonucleic acid
SFM	Serum free media
Sec	Second
TCGA	The cancer genome atlas
<i>TP53</i>	Tumor protein 53
U	Units
<i>TP53 WT</i>	Wilde-type <i>TP53</i> gene
<i>TP53 Wtres</i>	Wild-type rescue <i>TP53</i> gene
<i>TP53 R173H</i>	<i>TP53</i> gene with <i>R173H</i> mutation
<i>TP53 R273H</i>	<i>TP53</i> gene with <i>R273H</i> mutation
<i>TP53 KO</i>	knocked out <i>TP53</i> gene
μL	Microliter

μM	Micromolar
Fig	Figure
MCF10A Wtress	MCF10A cell that wild-type <i>TP53</i> gene is knocked out from them and overexpress wild-type <i>TP53</i> gene
MCF10A R175H	MCF10A cell that wild-type <i>TP53</i> gene is knocked out from them and <i>TP53</i> gene with R175H mutation
MCF10A R273H	MCF10A cell that wild-type <i>TP53</i> gene is knocked out from them and <i>TP53</i> gene with R273H mutation
MCF10A KO	MCF10A cell that wild-type <i>TP53</i> gene is knocked out from them
MCF10A WT	Normal MCF10A cells with wild-type <i>TP53</i> gene

Abstract

Breast cancer is the most common cancer and the leading cause of death among women worldwide. Mainly imbalance between tumor suppressor genes and oncogenes lead to cancer transformation. This imbalance usually arises from mutations in one or more of either tumor suppressor genes or oncogenes. Different gene expression patterns among breast cancer subtypes lead to heterogeneity and give different phenotypes. Our preliminary data showed that different *TP53* variants resulted in different gene expression patterns. So we hypothesized here that combinations between *HRAS G13R* and different *TP53* variants will lead to different gene expression patterns and different phenotypes. To test this hypothesis, we infected MCF10A cell harboring different *TP53* variants (*TP53 KO*, *TP53 R175H*, and *TP53 R273H*) with *HRAS G13R* viral vector. Afterward we tested proliferation, migration, survival, and apoptotic resistance of manipulated cells. In addition, we tested some of phenotypic related target genes expression. Our results showed that *HRAS G13R* overexpression increases tumorigenicity of infected cells with *HRAS G13R-TP53 R175H* combination having highest tumorigenic effect. Also results of different tested assays shows that cell proliferation, migration, survival, and resistance to apoptosis was affected differentially in each of *HRAS G13R* and *TP53* variants combination. This phenotypic diversity was combined with difference in gene expression patterns between different combinations. Overall our study provides a new model that spots the light on the role of two hit system in cancer transformation and progression. In addition, this model may help in understanding *TP53* and *HRAS* crosstalk in breast cancer and help in cancer diagnosis and treatment.

List of tables

Table 1 List of used materials	10
Table 2: Reagents and Chemicals	12
Table 3: Cell lines	14
Table 4: Plasmids &Bacteria	15
Table 5: List of created clones names and their control	23

List of figures

Figure 1. 1: HRAS most common mutations. Most common mutations in HRAS gene found in position 12,13, and 61.....	3
Figure 1. 2: MDM2-TP53 regulatory pathway. The feedback regulation involving the TP53 and MDM2 (Nag et al., 2013)(adapted).	5
Figure 1. 3: Various TP53 target genes and their functions. TP53 target genes produce proteins that regulate many cell activities like cell cycle arrest, DNA repair, apoptosis, metabolism, autophagy, translation control and feedback mechanisms(Fischer, 2017)(adapted)	6
Figure 1. 4: TP53 mutation that occur in early breast cancer. Mainly TP53 mutations were aggregated in the area coding for the DNA binding domain of the protein.(Fountzilias et al., 2016)(adapted)	7

Figure 3. 1:MCF10A cells with different TP53 variant infection and selection. Representative Image shows MCF10A cells harboring different TP53 variant after infection with either pwz1-hygro-HRASG13R or pwz1-hygro-EV, and selection with hygromycin compared to non-infected control 22

Figure 3. 2: HRAS expression in MCF10A transduced cells. Real time PCR results showing the relative expression level of *HRAS* gene correlated to the house keeping gene *UBC*. All gene expression folds were calculated relative to the expression of level in normal MCF10A cell (parental). Then Final relative expression index for each *HRAS G13R* infected clone was correlated to its *EV* control. Bars indicate standard error mean (SEM) of three replicates. The statistical significance of the results was determined by measuring the p value. (* indicates p-value <0.05) 22

Figure 3. 3: Relative proliferation rate for the transduced MCF10A clones using total cell count assay: The bars show the effect of *HRAS G13R* over expression on cell proliferation of MCF10A cells with different *TP53* variant. Cells were counted in triplicate; the relative proliferation rate index of day3 was correlated to day 0 for each clone. Final relative proliferation index for each *HRAS G13R* infected clone was correlated to its *EV* control. Bars indicate standard error mean (SEM) of three replicates. The statistical significance of the results was determined by measuring the p value. (* indicates p-value <0.05)..... 24

Figure 3. 4: Relative proliferation rate for the transduced MCF10A clones using XTT assay: The graph shows the effect of *HRAS G13R* overexpression on MCF10A cells with different *TP53* variants cell proliferation. The relative proliferation rate index of day3 was correlated to day 0 for each clone. Final relative proliferation index for each *HRAS G13R* infected clone was correlated to its *EV* control. Bars indicate standard error mean (SEM) of three replicates. The statistical significance of the results was determined by measuring the p value. (* indicates p-value <0.05). 25

Figure 3. 5: Effect of HRAS G13R over expression on the migration of MCF10A cells with different TP53 variants. Representative pictures taken for indicated cells at 0 hours and 14 hour after wounding cell monolayer..... 26

Figure 3. 6 : <i>HRAS G13R</i> overexpression effect on survival of MCF10A cells with different <i>TP53</i> variants. Representative statistical analysis of the survival rate of <i>HRAS G13R</i> overexpressing cells in comparison to <i>EV</i> cells using colony forming assay. The relative colony number of each clone was correlated to MCF10 <i>TP53 Wtres + EV</i> clone. Bars indicate standard error mean (SEM) of three replicates. The statistical significance of the results was determined by measuring the p value. (* indicates p-value <0.05).	27
Figure 3. 7: <i>HRAS G13R</i> overexpression effect on anchorage-dependent cell death of MCF10A cell with different <i>TP53</i> variants. Representative statistical analysis of the anchorage-dependent cell death of <i>HRAS G13R</i> overexpressing cells in comparison to <i>EV</i> cells using anchorage-dependent cell death assay. The relative dead cell number of each clone was correlated to MCF10 <i>TP53 Wtres + EV</i> clone. Bars indicate standard error mean (SEM) of three replicates. The statistical significance of the results was determined by measuring the p value. (* indicates p-value <0.05).	29
Figure 3. 8: Soft agar assay. Representative Image at 10X show the colonies shape and size of MCF10A cells infected with <i>HRAS G13R</i> VS <i>EV</i>	30
Figure 3. 9. Real time PCR results showing the relative expression level of different genes correlated to the house keeping gene <i>UBC</i>. All gene expression folds were calculated relative to the expression of level in normal MCF10A cell (parental). Bars indicate standard error mean (SEM) of three replicates. The statistical significance of the results was determined by measuring the p value. (* indicates p-value <0.05)	39

1. Introduction

1.1. Cancer transformation

Cancer is a generic term for a large group of diseases characterized by the growth of abnormal cells beyond their usual boundaries that can then invade adjoining parts of the body and/or spread to other organs. It accounts for 9.6 million deaths worldwide in 2018 (WHO, 2019). Many factors are responsible for cancer development and progression. All these factors lead to the main cause of cancer which is the imbalance between tumor suppressor genes and oncogenes. This imbalance can be either due to mutations in tumor suppressor genes, oncogenes or in DNA repair genes (Osborne et al., 2004). The involvement of epigenetics in this process of cancer development added more complexity to understating cancer initiation and progression mechanisms (Wu et al., 2015). So in order to make progression in cancer diagnosis and therapy, greater understanding of cancer molecular mechanisms is needed on both direct gene alterations and epigenetic alterations (Hinshelwood and Clark, 2008). During cancer transformation, a lot of phenotypic properties related to transformed cells are uncovered, these properties are called cancer hallmarks and are described as major hallmarks that in part include self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2011).

1.2. Breast Cancer

Breast cancer is the leader cause of female cancer related deaths worldwide. It's responsible for more than half million deaths among women in 2018 (15% of cancer deaths), and yearly there are about 2 million new cases of breast cancer are reported among women (WHO, Breast Cancer 2019). Several factors affect breast cancer prognosis and survival rate. Those factors include age, ethnic group, hormones, and genetic factors (Libson and Lippman, 2014). Breast cancer is a very heterogeneous type of cancer which can be classified into different categories based on breast cancer type,

appearance of the tissue, stage of cancer, and gene profile (cancer genome Atlas 2012). Huge efforts are targeted toward developing strategies to fight this cancer starting with early detection of disease and not limited to surgical procedures, radiotherapy, chemotherapy and biological and targeted therapy (Libson and Lippman, 2014). Targeted therapy is very important due to heterogeneity of the disease (Sousa et al., 2019). This heterogeneity makes the war against breast cancer more complicated, increase treatment cost in addition to decrease the chances of treatment availability to vast group of patients (especially in low-income and middle-income countries where individual therapy is not always provided) (Jamison et al., 2015). For instance, not all HER2 positive patient's respond to trastuzumab (a drug targeted toward HER2 receptor) in the same way, and survival rate are different among them. This thought to be underlined by several resistance mechanisms including heterodimerization, with other HER receptors, and bypassing HER2 signaling pathways (Baselga et al., 2012). These resistance mechanisms of cancer made researchers focus towards developing new strategies to overcome resistance mechanisms and prevent recurrence of disease (Luo et al., 2015). Moreover, it has been determined that within single breast carcinoma there are multiple cancer cell clones, harboring distinct genetic and epigenetic profiles (Sousa et al., 2019). This intra tumor heterogeneity is highly affected by tumor micro-environment (McGranahan and Swanton, 2017, Colak and Medema, 2014). Due to the mentioned reasons, standard therapies against breast cancer are not enough and do not prevent cancer recurrence (Sousa et al., 2019), and more studies focusing on different underlying molecular mechanisms of different breast cancer categories are needed.

1.3. The Oncogene-RAS

RAS genes are one of the earliest oncogenes discovered in human tumors. This family includes *K-RAS*, *HRAS*, and *N-RAS* (Downward, 2003). The protein products of *RAS* proto-oncogenes family are a group of small GTPases that play vital role in signal transduction through numerous growth factors to stimulate cell proliferation and movement. *RAS* proto-oncogene is frequently mutated in cancers and affects a variety of processes involved in cancer progression (Pylayeva-Gupta et al., 2011, Kiaris and Spandidos, 1995). Mutations in *RAS* genes were found in about 30% of all human cancers (Adjei, 2001). The mutations in *RAS* proteins make the protein product constitutively active due to unresponsiveness to GTPase activating protein (GAPs) (Downward, 2003).