Deanship of Graduate Studies
Al-Quds University

HRAS G13R transformation effect on MCF10A cells harboring different p53 variants

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Master's Thesis

Jerusalem – Palestine

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M. Sc. Thesis

Jerusalem – Palestine

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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

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Table of Content

List of abbreviations

°C	Degree Celsius
Bax	Bacl-2 associated X protein
BCL-2	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
TP53	Tumor protein 53
U	Units
TP53 WT	Wilde-type *TP53* gene
TP53 Wtres	Wild-type rescue *TP53* gene
TP53 R173H	*TP53* gene with *R173H* mutation
TP53 R273H	*TP53* gene with *R273H* mutation
TP53 KO	knocked out *TP53* gene
µL	Microliter

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

List of figures

Chapter 1

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 were 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 constituently active due to unresponsiveness to GTPase activating protein (GAPs) (Downward, 2003).

Mutated *HRAS* proteins are found in different type of cancers. However, *RAS* mutations are rare in breast cancers (Miyakis et al., 1998). Even though, they still have a lot interest due to the vital role of *HRAS, PI3K* and *MAPK* signaling pathway (Myers et al., 2016). *HRAS* gene mutations are found mainly in amino acid 12, 13, and 61 (Myers et al., 2016). Despite that these mutations are in the same domain of protein, they have distinct structural and biochemical defect. Moreover, different amino acid substitutions at any of these hotspot positions can rise into different functional consequences and oncogenic power of cancer formed (Hobbs et al., 2016). This diversity in *HRAS* mutations, pathways and power; urge the need of understanding how each hotspot mutation works (Figure 1.1).

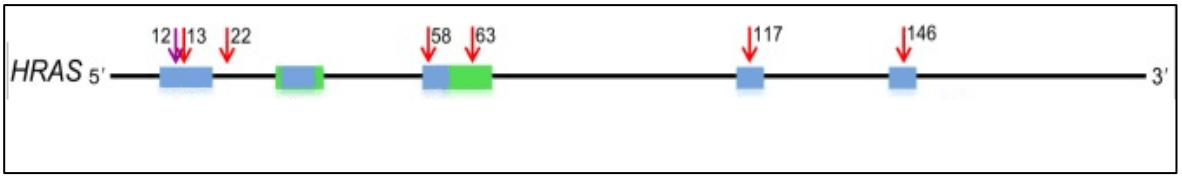


Figure 1. 1: *HRAS* most common mutations. Most common mutations in HRAS gene found in position 12,13, and 61.

HRAS G13R is a hotspot mutation that lies within the GTP binding domain of the *HRAS* protein and arise from substitution of G in position 37 with C (c.37G>C) which lead to replacement of amino acid glycine with arginine (p. *G13R*)(Hobbs et al., 2016). The *G13R* mutation forms about 85% of mutation at position 13 in *HRAS* isoform and mostly found in salivary gland cancer and less common in breast cancer (Hobbs et al., 2016). Still several studies connected between *HRAS* overexpression and aggressiveness of breast cancer. For instance; in his study, Koh M found that MCF10A cells with *HRAS* transformation showed more invasiveness and aggressiveness over MCF10A cell transformed with N-*RAS* (Koh et al., 2016).Other study showed that over expression of *HRAS* alone or with PI3K or P53 in MCF10A cells give them very high oncogenic features (Geyer et al., 2018).When focusing on mutation site, it is found that the mutation at G12 site are more stringent than at G13. Nevertheless, the *G13R* mutation has a strong effect on GTPase activity since it prevent binding of factors needed for acceleration of GTPase activity (Gremer et al., 2008).

1.4. *TP53* tumor suppressor gene

Well known as" guardian of the genome", *TP53* protein product is a tumor suppressor protein that functions in many ways to protect the cell under stress conditions (Niazi et al., 2018). This protein encoded by *TP53* gene is located on the short arm of chromosome 17 (Matlashewski et al., 1984). *TP53* coding sequence (CDS) gives a 393 amino acid protein which is composed mainly from 3 domains (1) RNA binding domain which recruits RNA polymerase and activates transcription of several genes (transactivation domain), (2) Regulatory domain which binds to specific regulatory sites present on DNA/response elements, and (3) tetramerization (TET) domain (Niazi et al., 2018).

The main role of *TP53* protein is to provide antiproliferative cellular response that may lead to either, cell-cycle arrest, senescence, modulation of autophagy, or apoptosis in response to cellular stress such as DNA damage, oncogene activation, or hypoxia (Yee and Vousden, 2005). Still, this response depends on many factors such as type of cell, microenvironment, and tumor evolution events. So *TP53* targets can be altered depending on the above mentioned factors. Simply, p53 function starts with stabilization process; in which under stress p53 is freed from its negative regulator MDM2 through phosphorylation of p53 amino acid terminus (Appella and Anderson, 2001). Then, p53 binds to specific sequences on DNA. This step will lead to the third step in which p53 will activate or repress its target genes (el-Deiry et al., 1992, Iyer et al., 2004). But this understanding is very humble and thing are much more complicated. For example; it's found that p53 bind through its carboxy-terminal domain even in unstressed conditions, which means it can control gene expression in stressed and unstressed conditions,(Appella and Anderson, 2001). Also, p53 does not only affect transcription of its target genes through traditional transcriptional factors, but also interacts with transcriptional activators and repressors to modulate transcription. One clear example on this mode of gene expression modulation is p300-p53 interaction that facilitates both histone and p53 acetylation, leading to a more open chromatin conformation near p53 targets and a more active p53 protein, respectively (Chan and La Thangue, 2001).

TP53 expression is regulated through protein-protein interaction between MDM2 and *TP53*. This interaction is formed as loop in which p53 bind to MDM2 promoter and starts its transcription so increase MDM2 levels. In its turn, MDM2 binds to *p53b* and prevents it from interacting with transcriptional co-activators; keep it out of nucleus, and targets *TP53* ubiquitination and degradation. In other words p53 produce its inhibitor. This loop is functional and maintains cellular balance by maintaining low p53 level during unstressed conditions (Fig1) (Haupt et al., 1997, Honda et al., 1997, Kubbutat et al., 1997, Nag et al., 2013).

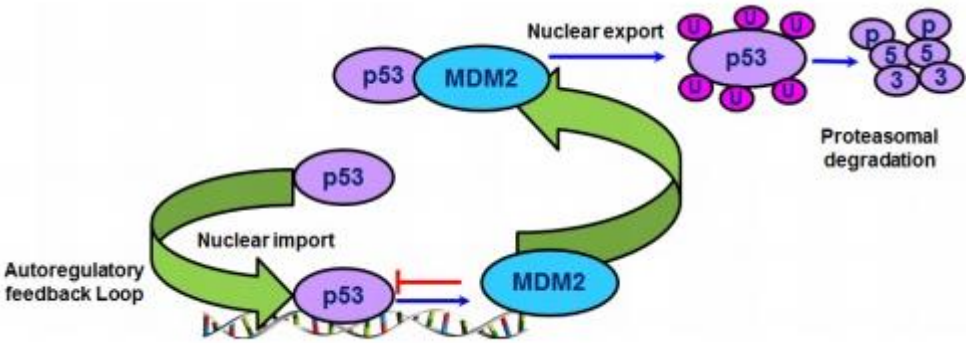


Figure 1. 2*: MDM2-TP53* regulatory pathway. The feedback regulation involving the *TP53* and MDM2 (Nag et al., 2013)(adapted).

Since *TP53* discovery, researchers have studied *TP53* target genes thoroughly. To date, with recent genome-wide analysis from hundreds to thousands of potential p53 target genes have been identified (Wei et al., 2006, Li et al., 2012). The p53 regulation of its targets can be either by activating, repressing, or both. For instance, one study shows that from 346 p53 targets 246 were reported to be activated by p53 and 91 repressed while 9 found to be both activated and repressed by *TP53*(Haupt et al., 1997). Among studied p53 targets there are high-confidence p53 targets. These targets function in many cell processes such as cell cycle arrest, DNA repair, apoptosis, metabolism, autophagy, translation control, and feedback mechanism (Fig1.2) (Haupt et al., 1997, Fischer, 2017).

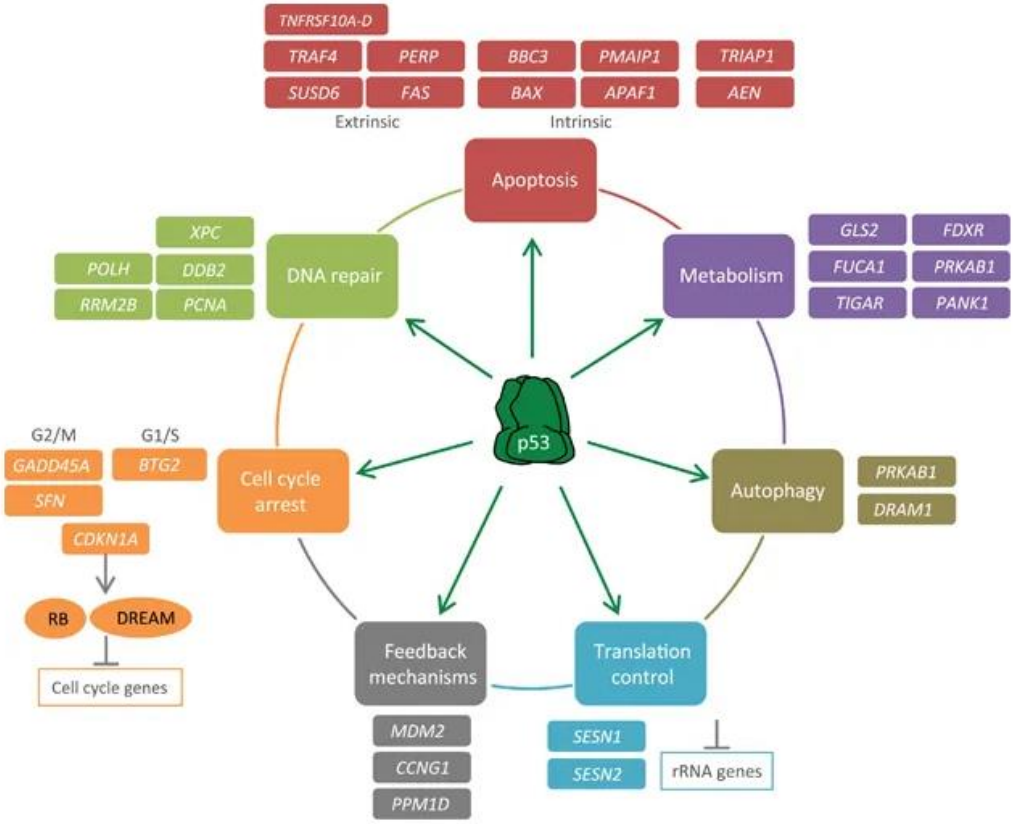


Figure 1. 3: Various *TP53* target genes and their functions. *TP53* target genes produce proteins that regulate many cell activities like cell cycle arrest, DNA repair, apoptosis, metabolism, autophagy, translation control and feedback mechanisms(Fischer, 2017)(adapted)

Mutations in *TP53* can lead to dramatic changes in cellular activity. It's the most mutated gene in cancer. It's found to be mutated in about 50% of cancers (Fischer, 2017). Interestingly, only about 15% are defined as "disruptive mutations" while 85% of *TP53* mutations are missense mutations and most of them are in DNA-binding domain. In addition, some of them are considered "gain of function" mutations in which *TP53* not only lose its function as tumor suppressor gene but also gain a new oncogenic function. That's why when *TP53* was first discovered it was considered as an oncogene (Fischer, 2017, Perri et al., 2016). Back to breast cancer, mutations in *TP53* are found in 28% of most aggressive breast tumors (Silwal-Pandit et al., 2014). Moreover status of *TP53* gene is considered a strong marker of prognosis (Borresen-Dale, 2003, Olivier et al., 2006). The distribution of mutations in *TP53* gene in early breast cancer is listed in Fig (1.3) with majority of mutations fall in the DNA-binding domain (Fountzilas et al., 2016).

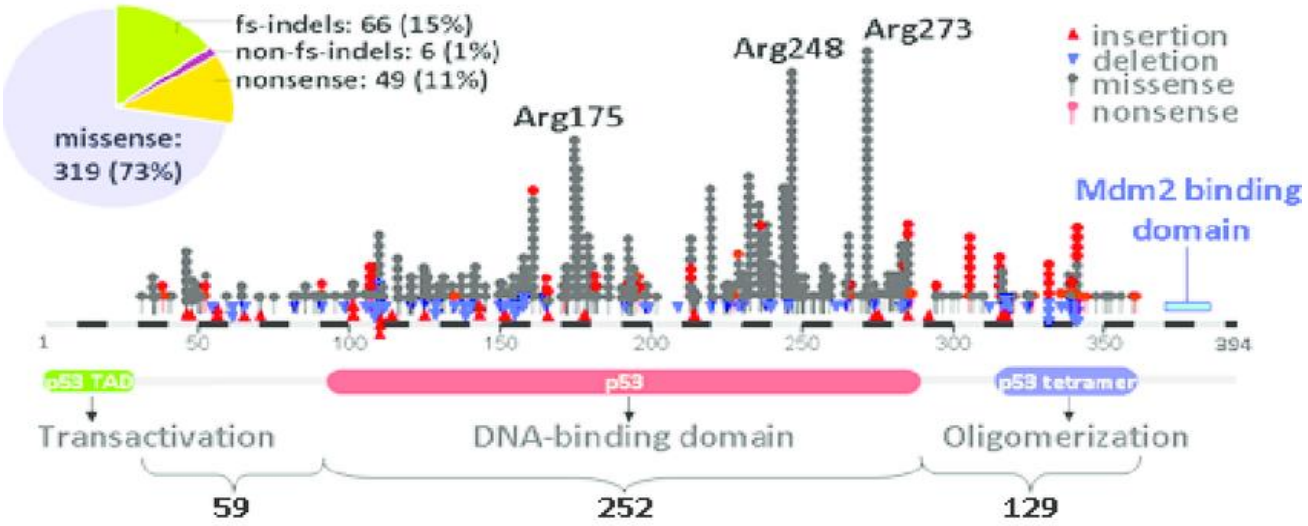


Figure 1. 4: *TP53* mutation that occur in early breast cacner. Mainly TP53 mutations were aggregated in the area coding for the DNA binding domain of the protein.(Fountzilas et al., 2016)(adapted)

In addition, *TP53* mutation status can also determine therapeutic strategies for breast cancer treatment since *TP53* mutation status can predict response to treatment. For example, breast cancer patients with wild type *TP53* show less survival than patient with mutant *TP53* when treated with chemotherapy, while the addition of hormonal therapy to chemotherapy increases wild-type *TP53* patient survival compared to mutant *TP53* patients (Ungerleider et al., 2018).

Among all *TP53* mutations there are four hotspot mutations at positions 175, 245,248,273 (Hainaut and Hollstein, 2000). While R273H mutation is a contact mutation R175H is a structural mutation (Sigal and Rotter, 2000). Both R175H and R273H mutations are gain of function mutations that were studied extensively due to their high oncogenic activity and their worse clinical effect. For example, patients with R175H mutation show worse prognosis than those with *TP53* null mutation (Alsner et al., 2001, Poeta et al., 2007) .It's well documented that Gain of function mutations can promote cancer formation and progression by increasing cell proliferation, metastasis and migration, resistance to apoptosis, and induction of genome instability (reviewed) (Zhou et al., 2019).

1.5. *TP53* variants and *HRAS* combination role in cancer transformation

TP53 has a wide range of targets and mutant *TP53* interacts with oncogenes efficiently when transfected into normal cells. Mutant *TP53* interaction with oncogenic *HRAS* induces normal cell transformation *in vivo* (Eliyahu et al., 1984, Parada et al., 1984). In addition, *RAS* and mutant *TP53* are involved in cancer initiation and progression cooperatively, and enhance cancer hallmarks (Solomon et al., 2010). For example, mutated *K-RAS* gene overexpression proliferation is arrested in normal cell by *TP53-WT* and did not form tumor in mice, on the other hand when mutated *K-RAS* combined with inactivation of *TP53* or mutated *TP53*, tumor formation was promoted with high metastatic form in *TP53-R175H* mutated mice (Morton et al., 2010). Also , Xia and Land found in 2007 that combination between *TP53 R175H* mutation and *HRAS G12V* mutation increase cell motility and invasiveness in mouse colon cells(Xia and Land, 2007). In another study, it was found that the combination between *TP53* KO or *TP53* mutation and *HRAS G12V* results in cancer transformation (Buganim et al., 2010).

TP53 and *HRAS* interaction control many target genes because both of them have wide range of downstream targets. In one study Buganim et al using microarray expression profiling found unique gene cluster that regulate precancerous secreted molecules controlled mainly by crosstalk between *TP53* and *HRAS* (Buganim et al., 2010). Those molecules include interleukins (*IL8*, *IL6*, *IL1*b), chemokines (*CXCL1*, *CXCL2*, *CXCL3*), and extracellular matrix related genes (*MMP3*, *CLECSF2*) (Solomon et al., 2010). Another study provided evidence and molecular links between *TP53* and *RAS* pathways by revealing that mutated *TP53* cells invasiveness and motility is mediated by enhancement of integrin and epidermal growth factor receptor (EGFR) recycling and activity. Mutant *TP53*-harboring cells showed higher levels of activated EGFR, AKT, and, to a lesser extent, ERK, which are close neighbors of *RAS* in the signaling network that controls motility (Muller et al., 2009, Solomon et al., 2010).

1.6. Problem statement and motivation of study:

During the last two decades evidences viewed breast cancer as a very heterogeneous disease rather than a homogeneous entity of fast proliferating neoplastic cells (Sousa et al., 2019). Because of breast cancer heterogeneity, huge effort targeted toward revealing genetic changes and mechanisms that are associated with breast cancer epithelial cell transformation(Solomon et al., 2010). In attempt to understand how different mutations are involved in cancer prognosis and treatment regimens, it's important to study

particular mutations. In fact, many efforts toward understanding and treating breast cancer have passed through targeting *RAS* and *TP53* pathways (Suter and Marcum, 2007). In recently accepted paper from our lab, to understand how *TP53* and *HRAS* are regulated in breast cancer; using Hi-C data, we have found that *HRAS G12V* can regulate *TP53* by redistribution of chromatin binding with minor alteration in *TP53* expression level(Michal Schwartz, 2019). Since there are growing evidence that different *RAS* mutations will lead to different consequences (Hobbs et al., 2016); we believe that studying how *HRAS* G13R functions with different *TP53* variants in breast cancer could help in elucidating behavior of different tumors.

1.7. Hypothesis:

This research is part of larger project based on the assumption that different oncogenic phenotype are underlined by various gene transcription reprogramming. We hypothesized that *HRAS G13R* overexpression combined with different *TP53* variants in MCF10A cells will give different phenotypic changes based on different transcriptional reprogramming events.

1.8.Aim of the study:

The main aim of this research is to create different breast cancer transformation models by infecting normal immortalized MCF10A cells harboring different *TP53* gene variants with *HRAS G13R*. The second goal is to test the differences in distinct cellular phenotypes (cancer hallmarks) caused by the combination between *HRAS G13R* and different *TP53* variants.

Chapter 2

2. Materials and Methods

2.1.Materials

Table 1 List of used materials

#	**Name**	**Company**
1	Analytical Balance	METLER TOLEDO AB104
2	Autoclave	HIRAYAMA HV-110
3	Vortexes: SA6, Genie 2, Reax top	Stuart Scientific, neoLab, Heidolph
4	Biological hood (HERA guard)	Heraeus
5	Biofuge Stratos Reconditioned	Heraeus 75005289R
6	Biofuge Fresco	Heraeus 75005521
7	Digital dry bath	Labnet
8	Elisa reader	RAL
9	Hera cell 150 CO2 Incubator	Heraeus
10	Inverted microscope	Olympus ck40-SLP
11	Labofuge 200 centrifuge	Heraeus
12	RT-PCR	(Applied Bio-systems 7500 Real Time PCR
13	GlobMax discover reader	Promega

15	Benchtop centrifuges: Labofuge 200, Biofuge fresco	Therom Fisher Scientific Heraeus
16	Cell counter CASY	Casy , Innvatis
19	Computers	hp, Fujitsu, Siemens
20	Floor centrifuge – Heraeus Multifuge 4KR	Thermo Fisher Scientific
22	Freezer -20 ℃	Libherr
23	Freezer -80 ℃ ,VIP	Sanyo
24	Freezing container	Sigma-Aldrich
25	Fridge 4℃	Liebgerr
26	Horizontal roller shaker ,RM5	neoLab
27	Ice machine	Hoshizaki
28	Image Xpress microscope	Molecular Device
30	Liquid nitrogen storage system	Cryotherm
32	MilliQ Biocel Water Purification System	Millipore
33	Multichannel pipette	Eppendorf
34	Multistep pipette Biohit (5-100 µl)	Biohit
35	Multistep pipette Ripette (200 µl – 5 ml)	Ritter Medical
36	Thermomixer comfort	Eppendorf
38	Pipettes (0.5 – 1000 µl)	Eppendorf , Gilson
40	Scanner	Epson
41	Suction device for cell culture	neoLab
42	SPIN-micropipette site	Nano Spinreact
43	Ultracentrifuge	BECKMAN COULTER optima LE80H

44	Water Bath Orbital Shaking	Grant OLS200
45	Water Baths	Grant LTD6G, Julabo TW20
46	PCR machine 96 well	Applied Biosystem #9902
47	Neubauer count chamber (0.1 mm depth)	BLAUBRAND

Table 2: Reagents and Chemicals

#	**Name**	**Company**
1	DMEM-F12 medium	Biological industries , Gibco
2	DMEM medium	Invitrogen
4	RPMI (1640) medium	Gibco
5	Horse serum	Biological industry
6	Fetal bovine serum (FBS)	Gibco
7	Hydrocortisone	Sigma-Aldrich
8	Insulin	Sigma -Aldrich
9	Epidermal growth factor (EGF)	Sigma -Aldrich
10	Cholera toxin	Sigma -Aldrich
11	L-Glutamine	Biological industries
12	Penicillin/streptomycin	Biological industries
13	Dimethyl sulfoxide	Sigma -Aldrich
14	Sterile phosphate buffer saline PBS	Biological industries
15	Puromycin ,	Sigma -Aldrich
16	XTT kite	Biological industries
17	Trypane blue	Biological industries

18	Isopropanol biological gradient	Sigma -Aldrich
19	Ethanol biological gradient	Sigma-Aldrich
20	Chloroform biological gradient	Sigma -Aldrich
21	qScript™cDNA synthesis kit	Quanta Biosciences
22	SYBR® Green	Applied Biosystems
23	TRIZOL	Sigma -Aldrich
24	Diff-Quick System	Sigma -Aldrich
25	0.5% and 0.05% Trypsin-EDTA	Invitrogen
26	Agrose	Sigma-Aldrich
27	BCA protein assay kit	Thermo Fisher Scientific
28	Deoxynucleoside triphosphates (dNTPS)	New England Biolab
29	DNA Loading dye (6x)	Thermo Fisher Scientific
30	GeneRuler 1 kb DNA Ladder	Thermo Fisher Scientific
31	GeneRuler 100 bp DNA Ladder	Thermo Fisher Scientific
32	Geneticin G148 (Neomycin)	Gibco
33	Methanol	Sigma-Aldrich
34	Nuclease Free Water	Ambion
35	Phosphate buffered saline (PBS)	Gibco
36	OptiMEM	Invitrogen
37	Lipofectamine	Invitrogen
38	Matrigel matrix	Corning
39	Anti-p53	Santa Cruz Biotechnology

40	Anti-B-Actin	Santa Cruz Biotechnology
41	Hygromycine 50mg/dl	Sigma
42	Mini-prerp kit	invitrogen
43	Trypton	sigma
44	Yeast extract	sigma
45	AGAR	sigma
46	poly-lysine-coated 100 mm dish	
47	poly-lysine-coated 75 cm^2 flasks	
48	DMSO	Sigma
49	Mirus	ivitrogen
50	Polyhema	sigma
51	HiPure plasmid Maxiprep kit	invetrogen

Table 3: Cell lines

#	**Name**	**Origin**
1	MCF10A *TP53*KO	Sigma-Aldrich
2	MCF10A Wtres	DKFZ,Genomic and Proteomics core facility
4	MCF10A Mutated *TP53 (R175H)*	
5	MCF10A Mutated *TP53 (R273H)*	
6	2^{nd} generation of HEK293T(phoenix)	Invitrogen
7	MCF10A parental	Sigma-Aldrich

Table 4: Plasmids &Bacteria

#	Name	Origin
1	rwpLX305_hRAS_*G13R*_Hygro	DKFZ,Genomic and Proteomics core facility
2	PCL-ECO	
3	VSVG	
4	DH5-Alpha	DKFZ,Genomic and Proteomics core facility

2.2.Methods

2.2.1. Cell culture

Stable MCF10A cell lines expressing different *TP53* mutations were established by the Genomics and Proteomics Core Facility at the DKFZ- Germany. These lines were generated by overexpressing different *TP53* constructs in MCF10A *TP53KO* cells from Sigma-Aldrich (catalogue number CLLS1049). Overexpression was achieved by using the lentiviral vector pLx305 that expresses either FLAG-tagged *TP53 R175H* or *TP53 R273H* or wild-type *TP53*.

All MCF10A cell lines were cultured in DMEM-F12 supplemented with 5% horse serum, 20 ng/mL EGF, 0.5µg/mL hydrocortisone, 1% L-glutamine, 100ng/mL cholera toxin, 1% penicillin/streptomycin (50 U/µL penicillin and 50µg/mL streptomycin as final concentration) and 10µg/mL insulin. RPMI medium supplemented with 1% L-glutamine, and 1% penicillin/streptomycin were used for culture of phoenix cells

Cells were passed every 3-4 days. For this purpose, the medium was eliminated and cells were washed with 1 mL PBS X1 one time. Afterwards, 0.5 mL trypsin (0.05% concentration) was used followed by incubation for 5 to 10 minutes in 37 ℃, 5% CO_2 incubator. Then, 5 mL full growth medium was added

to detach the cells. Cells were counted by using Neubauer counting chamber (0.1 mm depth) and $0.5x10^6$ cells were seeded in $75cm^2$ flasks.

2.2.2. Cells cryopreservation

To prepare the freezing medium, full growth DMEM-F12 medium supplemented with 20% horse serum and 10% DMSO was used. Trypsinized cells were collected and then counted with Neubauer counting chamber and spun down. The preferred quantity of the cells (1 – 2 x 106 cells/mL) was suspended in the freezing medium and was distributed into cryo-vials. To obtain a gradually cooling rate of the cells (1℃ /min), cryo-vials were placed inside freezing container with isopropanol and left into a -80℃ deep freezer. Finally, cells were moved to liquid nitrogen tank for long term storage.

To restore and thaw the cells from freezing, a cryo-vial holding cells were suspended in 1 mL full growth medium, centrifuged at 1600rpm, then supernatant were removed and new 5ml of growth medium were added. Finally cells were transferred into 75 cm2 flask having 6 mL full growth medium.

2.2.3. Generation of stable cell lines pools

Plasmids preparation (Maxiperp)

To prepare plasmid and increase their concentration plasmids were transformed into DH-5 alpha bacteria by mixing 20ng of plasmid with 50ul of bacteria, then bacteria was incubated for 30 min on ice, then subjected to heat shock at 42℃ for 1 min. Afterward, bacteria were centrifuged and sediment was cultured on LB agar containing ampicillin for 24 hours in order to select bacteria than get the plasmid. Then two colonies of bacteria were cultured on LB broth at 37 with shaking for 17 hours. Finally, plasmids were extracted from bacteria using Invitrogen maxiprep kit according to manufacturer instructions.

Viral particles production and Transduction

Phoenix cells ($2 X 10^5$) were seeded on poly-lysine-coated 100 mm dish with full growth medium. Next day (when cells growth reach 30% of plate), 1.2ug of VSVG plasmid (envelope) and 3.3ug of pwzl-

hygro-*HRASG13R* (plasmid contain target gene) were mixed together at RT. Meanwhile, 670ul of SFM and 13.4ul of mirus were mixed together and incubated for 5min at RT. Then plasmids mixture and mirus-SFM were mixed together and incubated at RT for 15 min. Finally, this mixture added to 5ml full growth medium and replaced old medium on cells. Then cells were incubated for 24 hour in 5%CO2, 37C incubator. Next day, media changed on plate by aspirating old media and adding new 5ml of full growth medium, then cells incubated for 24 hours. Then (1^{st} day of collection), media on plate (containing generated virus) aspirated and collected into sterile conical tube and new 5ml of media added then cell were incubated for 24 hours. Next day (2^{nd} day of collection), media collected in same sterile conical tube from last day and plate discarded. Finally, collected virus was centrifuged for 10min at 3500 rpm then supernatant was filtered using 0.45um filter, and virus was stored at -80.

Infection of MCF10A cells

For infection, $5X10^5$ cells were seeded in poly-lysine-coated 100 mm dish in full growth medium. Next day 2ml of medium containing virus were added to cells for two days at 37 ℃, 5% CO_2 incubation.

Selection of infected cells

To select for clone pools, the transduction medium was changed with fresh full growth medium containing the appropriate selection antibiotics, Hygromycin (stock = 50 mg/mL, diluted 1:500, final concentration 100μg/ml) and Geneticin (Neomycin, stock = 50 mg/mL, diluted 1:100, final concentration 500 μg/ml). The selection antibiotic was kept on cells until control non infected cells totally died.

2.2.4. Gelatin coating

To increase cell adhesion in specific assays (Cell count, XTT, Wound healing, and Colony formation) used cell culture plates were coated with 0.1%gelatin prepared by dissolving 0.2 g of gelatin in 200ml of DW. Then gelatin was placed on plate for 45 min and washed twice with DW. Finally coated plates were sterilized by UV.

2.2.5. Cell count

30000 cells from each cell line were cultured with full growth medium in 12 wells of 6 well plates. Then each day 3 wells were trypsinized and cells in each well were centrifuged at 1600rpm for 10 min then sediment were suspended with new 1ml media and counted under microscope using Neubauer chamber. So each cell line were counted for four continuous days named as day 0 , day 1, day 2, and day 3(3 wells/day for 4 days= 12 well). Then the mean and standard deviation of cells count of each day wells were calculated.

2.2.6. XTT test

Triplicate of $2x10^3$ cells/well were seeded in 96 well plate and incubated at 37℃ , 5% CO_2 for 24, 48, 72 hours and cell proliferation was assessed using XTT stain according to manufacturer instructions by aspirating old media then adding 50 ul of prepared XTT reagent (reagent prepared by adding 1 part of activation reagent to 50 part of XTT reagent), then cells were re-incubated for 2 hours at 37℃. Finally, the reagent transferred to ELISA plate, and absorbance of samples was red against control blank at 450 nanometer using ELISA reader.

2.2.7. Wound healing assay

Triplicates of $2x10^5$ cells/well were seeded in 12 well plates in full growth medium and incubated at 37 ℃, 5% CO_2 until having 100% growth confluence. To generate wound, cell monolayer was scratched using 10µl pipet tip. Afterwards, floating cells were washed out using PBSX1. Finally, cells were supplied with starvation medium that lacks serum and EGF, then wound was photographed over 24 hrs.

2.2.8. Survival assay (colony formation assay)

Triplicate of 200 and 400 cells were seeded in 6 well plates in full growth medium. Every 3-4 days the medium was changed until visible colonies were seen. Later, the growth medium was removed and the plates were washed using PBS x1. Next, cells were fixed for 15 minutes by using absolute methanol and then left to air dry. After fixation, colonies were stained using Coomassie blue, washed and counted using naked eye.

2.2.9. Adhesion assay

Triplicate of 100000 cells were seeded in 6 well cell culture plates and incubated for 5 hours at 37 ℃, 5% CO_2. Then supernatant were discarded and remaining cells were collected and counted using Neubauer chamber.

2.2.10. Anoikis (Anchorage-Dependent Cell Death) assay

Triplicate of 100000 cells were seeded in 6 well coated with polyhema cell culture plates and incubated for 48 hours at 37C , 5% CO2. Then supernatant and adhered cells were collected, centrifuged at 1600rpm for 10min. Then old media discarded and new 1ml media was added and cells were stained using typan blue . Then dead and alive cell were counted and ratio between them were calculated

2.2.11. Soft agar assay

The assay medium is composed of a lower and upper agar layers. The lower layer was prepared by using RPMI 1640 medium containing 1% Agar. 2 mL of this mixture was poured in each well of 6 well plates and kept at 4 °C for at least one hour. Then, $2X10^4$ cells were mixed with 1.2 mL of the upper layer that contained 0.7% agar. This cell mixture was then added on the top of the lower layer and put back in CO2 incubator. Cells then were checked daily, and media was added every 2 days until colonies were visible to naked eye.

2.2.12. RNA Extraction

Cells were cultured in 10 cm cell culture plates in full growth medium and incubated at 37 °C and 5% CO_2 for 24 hours. Next day, the medium was aspirated and 1 mL Trizol was added to the plates and incubated on ice for 5 minutes. The cells collected in RNase free tubes and incubated on ice and mixed with 200 µL chloroform using vortex and incubated for 15 minutes on ice. Then, tubes were centrifuged for 15 minutes at 12000 RPM at 4 °C, then the supernatant was transferred to new RNase free tubes and 500 µL pre-cooled isopropanol was added, mixed and incubated for 15 minutes on ice. Then tubes were centrifuged at 12000 RPM for 15 min at 4 °C. Finally, supernatant was discarded and 500 µL 70% Ethyl alcohol was added. Thereafter, the tubes were centrifuged at 12000 RPM for 10 min at 4 °C and the pellet

left to dry for 1 minute and the pellet was suspended with DEPC treated water and incubated at 60 °C for 5 minutes in dry bath.

2.2.13. cDNA synthesis

RNA was diluted to get concentration of 0.2ug/ul then in PCR tubes, 5µl (1ug) of extracted RNA was mixed with 4 µL RT buffer, 1µl enzyme and ultra-pure water added to complete the mix volume to 20 µL. Later, the tubes were put in PCR machine according to manufacturer recommendations.

2.2.14. Real time PCR

In 96 q-PCR well plate, 3 µL of diluted cDNA (1:10), 1 µL of primer (10 µM), 10 µL SYBR® Green and 6 µL ultra-pure water were added per well. Then, the q-PCR plates were sealed and centrifuged for 5 minutes. Lastly, the plate was put in q-PCR machine with pre-conditioning at 50 °C for 2 minutes, activation at 95 °C for 10 minutes, denaturation at 95 °C for 15 seconds and annealing and extension at 60 °C for 1 minute. Primer 3 software (http://primer3.ut.ee/) was used to design primers for each target gene.

2.2.15. Statistical analysis

All statistical analysis was done using Microsoft EXCEL 2010. Student t.test used to measure p value to detect statistical significance. Regarding real time PCR analysis, delta-delta-ct value was used to measure fold of gene expression for each target gene.

Chapter 3

3. Results and Discussion

3.1.Generation of *HRAS G13R* MCf10A clones harboring different *TP53* variants.

In order to investigate the effect of *HRAS G13R* overexpression in MCF10A cells harboring different *TP53* variants, we infected the MCF10A cells with *HRAS G13R* retroviral vector (pwzl-hygro-*HRAS-G13R*). Our control cells were generated by infecting all our cell lines with empty retroviral vector (pwzl-hygro-*EV*), Here the used cell lines were *TP53* knockout MCF10A cells that are overexpressing either Wild-type *TP53* (MCF10A *TP53 Wtres*), *TP53 R273H* mutation (MCF10A *TP53 R273H*), *TP53 R175H* mutation (MCF10A *TP53 R175H*), or lack of *TP53* gene (MCF10A *TP53 KO*). Afterward created clones were placed under selection of hygromycin to eliminate uninfected cells. As shown in figure 3.1 while infected cells was resistant to hygromycin treatment control non-infected cells died after selection. These results indicate that we were able to generate *HRAS G13R* overexpressing and control clones. To ensure the generation of *HRAS G13R* clones we did real-time PCR using primers that target *HRAS*. Here our results show that cells infected with *HRAS G13R* overexpress *HRAS* more than those with EV by 5 folds at least; so only cells infected with *HRAS G13R* are overexpressing *HRAS* compared to *EV* control cells (Figure 3.2).

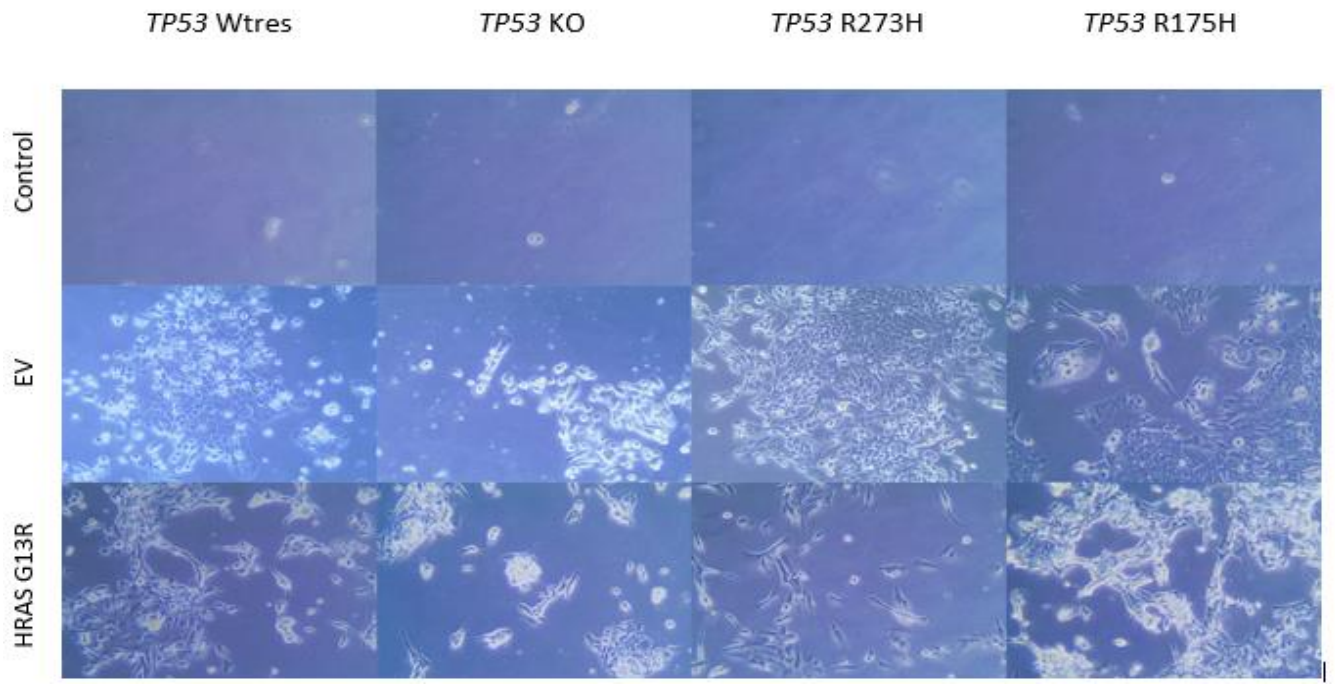


Figure 3. 1:MCF10A cells with different *TP53* variant infection and selection. Representative Image shows MCF10A cells harboring different *TP53* variant after infection with either pwzl-hygro-*HRASG13R* or pwzl-hygro-*EV*, and selection with hygromycin compared to non-infected control

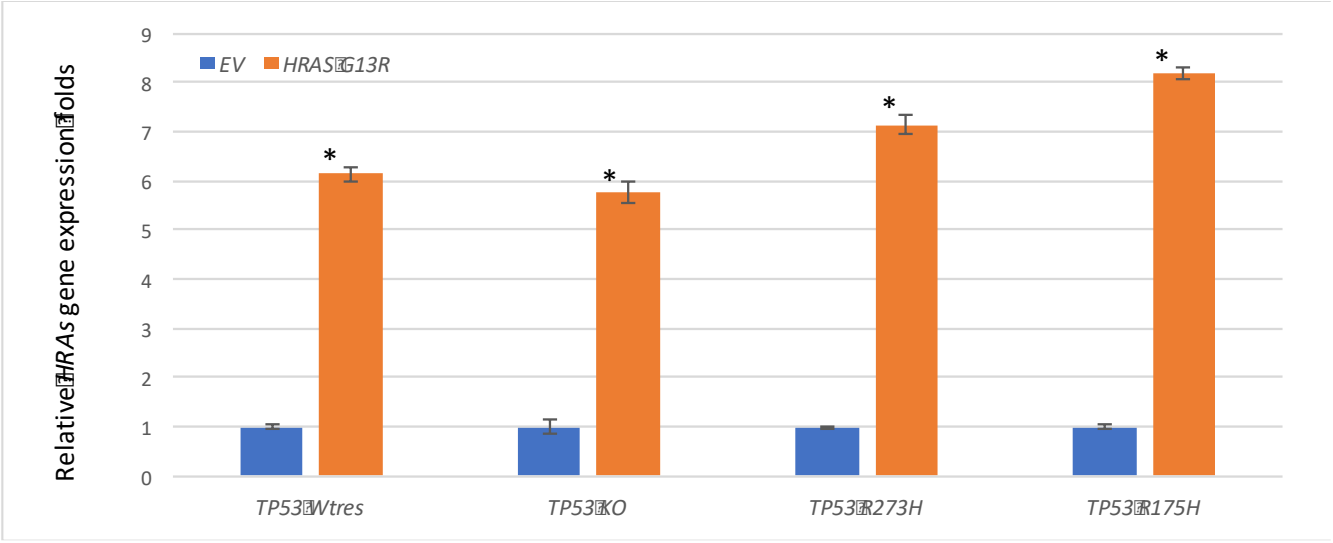


Figure 3. 2: *HRAS* expression in MCF10A transduced cells. Real time PCR results showing the relative expression level of *HRAS* gene correlated to the house keeping gene *UBC*. All gene expression folds were calculated relative to the expression of level in normal MCF10A cell (parental). Then Final 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)

Table 3.1 shows the list of each created clone and its control. Here we have included in comparison MCF10 *TP53 Wtres* in order to consider any effect come from *TP53* null background since mutant *TP53* variant should be tested in *TP53*-null to avoid effect of *WT TP53*(Freed-Pastor and Prives, 2012). Overall our results show that we were able to generate 10 stable Clones; 4 overexpressing *HRAS G13R* with different *TP53* variants (*TP53 Wtres, TP53 KO, TP53 R273H,* and *TP53 R175H*); and 4 with retroviral empty vector with different *TP53* variants used as control.

Table 5: List of created clones names and their control

Created MCF10A clones	Control
MCF10A *TP53 Wtres + HRAS G13R*	MCF10A *TP53 Wtres + EV*
MCF10A *TP53 KO + HRAS G13R*	MCF10A *TP53 KO + EV*
MCF10A *TP53 R273H + HRAS G13R*	MCF10A *TP53 R273H + EV*
MCF10A *TP53 R175H + HRAS G13R*	MCF10A *TP53 R175H + EV*

3.2.Characterization of phenotypic changes in *HRAS G13R* transduced MCF10A cells.

Previous studies showed that activation of *RAS* signaling pathway usually results in transformed phenotype in MCF10A cells (Basolo et al., 1991, Giunciuglio et al., 1995) . To examine the effect of *HRAS G13R* overexpression on MCF10A cells with different *TP53* variants, we have done several functional assays to test different cancer hallmarks, those assays were: total cell count, XTT, wound healing (migration), survival, anchorage-dependent cell death (Anoikis), and soft agar assays.

3.2.1. *HRAS G13R* overexpression in combination with different *TP53* variants effect on MCF10A cells proliferation.

One of most important cancer hallmarks is uncontrolled growth and proliferation(Hanahan and Weinberg, 2011).While *HRAS* was previously found to increase cell growth and proliferation(Karnoub and Weinberg, 2008), *TP53* limits cell proliferation(Levine and Oren, 2009). We hypothesized that

different combination between *HRAS G13R* and different *TP53* variants will affect cell growth and proliferation in different manners. To examine this hypothesis, total cell count and XTT assays performed. At the beginning, we were not able to get reliable results compared to eye observation because cells overexpressing *RAS* formed clumps (data not shown). To overcome this problem, we coated cell culture plates with 0.1% gelatin. Our total cell count results show that MCF10A *TP53 Wtre*s cells with *HRAS G13R* overexpression have higher proliferation rate than cells infected with *EV*, while MCF10A *T KO* and mutated *TP53* cells show almost same proliferation rate as control (Figure 3.3). To further investigate proliferation rate we performed XTT assay. In this assay we also obtained the same results as in total cell count assay (Figure 3.4). Our results go in line with previous studies which showed that *HRAS* mutations including *G13R* lead to cell transformation into aggressive metastatic phenotypes,(Hobbs et al., 2016). In addition Sonal Datta, et.al found that MCF10A-*HRAS* cell produced enlarging tumors when injected into mammary fat bad (Datta et al., 2007). Regarding MCF10A *TP53 KO*, MCF10A *TP53 R273H*, and MCF10A *R175H* cells phenotype, it seems that *TP53* manipulation induces the highest cell proliferation capacity that cannot be increased by *HRAS G13R* overexpression.

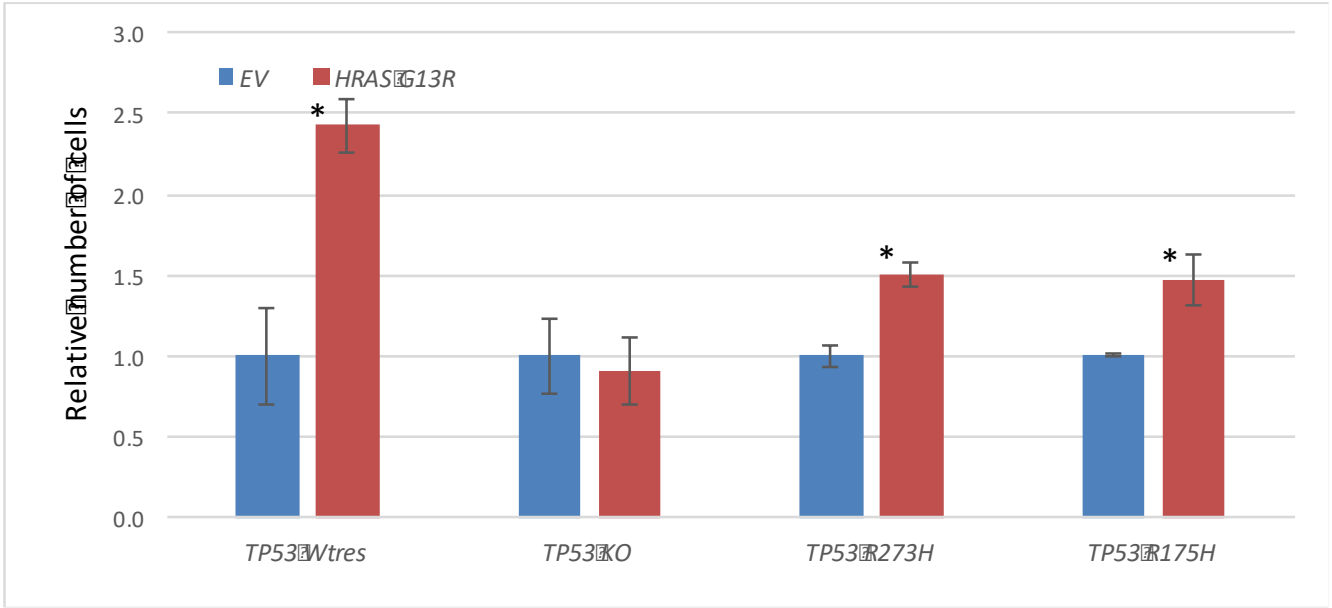


Figure 3. 3: Relative proliferation rate for the transduced MCF10A clones using total cell count assay: The bars show the effect of *HRAS G13R* over expression on cell proliferation of MCF10A cells with different *TP53* variant. Cells were counted in triplicate; the relative proliferation rate index 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).

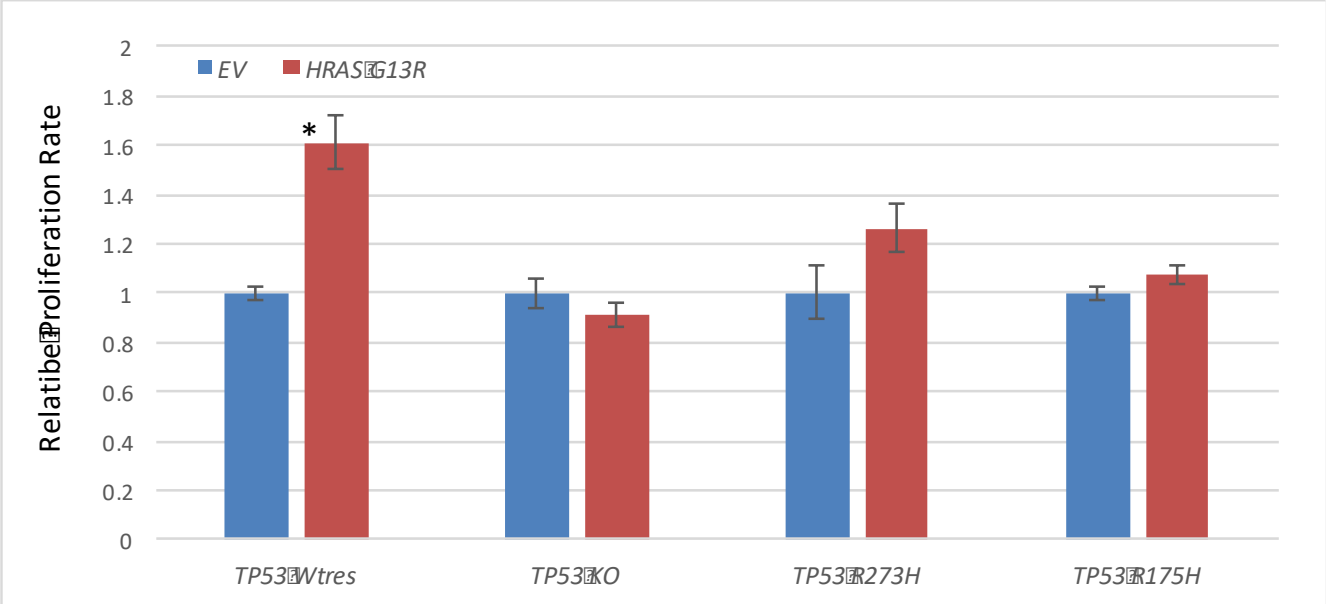


Figure 3. 4: Relative proliferation rate for the transduced MCF10A clones using XTT assay: The graph shows the effect of *HRAS G13R* overexpression on MCF10A cells with different *TP53* variants cell proliferation. The relative proliferation rate index of day3 was correlated to day 0 for each clone. 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).

3.2.2. *HRAS G13R* overexpression in combination with different *TP53* variants effect on MCF10A cells migration.

Enhanced cell mobility and cell migration phenotype is one important characteristic of cancer cells. *HRAS* overexpression is found to support this phenotype(Solomon et al., 2010).In contrast *TP53* prevent cell migration as a defense against cancer progression by different mechanisms (Gadea et al., 2002, Gadea et al., 2004, Guo et al., 2003). However, gain of function *TP53* mutation as *R175H* and *R273H* are found to promote cell migration and invasion (Dong et al., 2009, Coffill et al., 2012). Here we hypothesized that combination between *HRAS G13R* mutation and *TP53 R175H, TP53 R273H*, and *TP53 KO* will increase cell migration. In order to investigate this hypothesis, cell migration assay was performed. Our results show that combination of *HRAS G13R* with *TP53 R175H* show fastest healing pattern followed *TP53 Wtres + HRAS G13R*, while *TP53 KO*, and *TP53 R273H* overexpressing *HRAS G13R* both has almost same migration as their control and all of them have higher healing rate than *Wtres EV* (Figure 3.5).In one hand increased migration in MCF10A *TP53 Wtres* in combination with *HRAS G13R* correspond with previous studies suggest that *HRAS* over expression increase cell migration in

MCF10A cells in presence of *TP53* (Moon et al., 2000). On the other hand, the equivalent migratory level between both *HRAS G13R* and *EV* in MCF10A *TP53 KO* and MCF10A *TP53 R273H* is prove to our hypothesis that different *TP53* and *HRAS* will give different forms of transformed phenotypes. Regarding *TP53 R175H*, it was expected to have higher migration when combined with constituently active *HRAS G13R*, because previous study showed that *TP53 R175H* co-expression with *RAS* induces cell migration *in-vitro* as well as *in-vivo* (Jiang et al., 2015).This high migratory phenotype was found to be specific for *HRAS* but not *N-RAS* and determined by signaling program in hypervariable region (HVR) in *HRAS*(Yong et al., 2011). This migration phenotype is highly connected to cancer ability of metastasis, and integration within secondary organ and form colonies (Hanahan and Weinberg, 2011). Overall, *HRAS G13R* migratory phenotype was very similar to *HRAS G12V* mutation which is the usually studied *HRAS* variant in breast cancer (Koh et al., 2016).

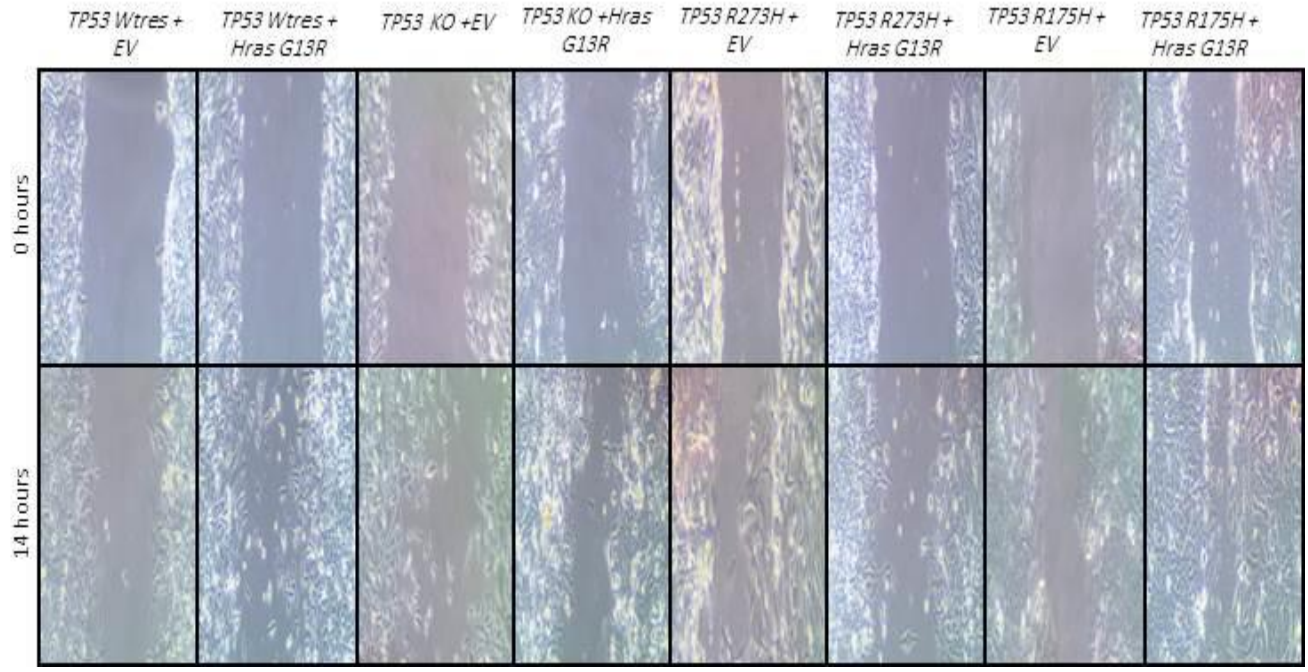


Figure 3. 5: Effect of *HRAS G13R* over expression on the migration of MCF10A cells with different *TP53* variants. Representative pictures taken for indicated cells at 0 hours and 14 hour after wounding cell monolayer.

3.2.3. *HRAS G13R* overexpression in combination with different *TP53* variants effect on MCF10A cells survival.

One of cancer hallmarks is the ability of cancer cell to grow and survive independently; without the need of exogenous growth and survival signals.(Solomon et al., 2010, Hanahan and Weinberg, 2000). To test this feature, we performed colony formation assay. In all clones with *HRAS G13R* overexpression MCF10A cell survival increased in different manners with most survival with *TP53 R175H* (Figure 3.6). Of note, all cell lines formed much more colonies compared to MCF10A *TP53 Wtres EV* cells. Cell autonomous growth is a hall mark of cancer cell growth that depends on imbalance between tumor suppressor genes like *TP53* and oncogenes like *RAS*. Previous studies have shown that *TP53* inhabits *RAS* induced cell survival (Ma et al., 2002). These findings explain the higher survival index we noticed in our *TP53* manipulated cells. Since *TP53 R175H* is gain of function mutation, it seems that this mutation in our cells has a synergistic effect on cell survival when combined with *HRAS G13R* mutation (Tan et al., 2015).

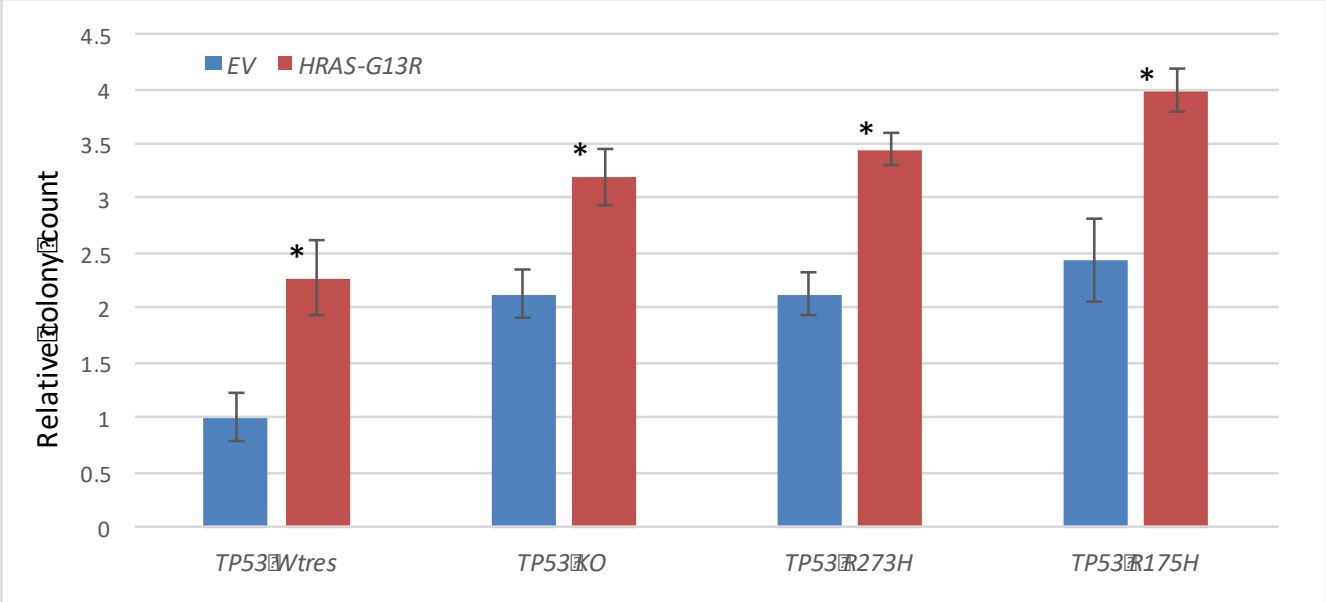


Figure 3. 6 *: HRAS G13R* overexpression effect on survival of MCF10A cells with different *TP53* variants. Representative statistical analysis of the survival rate of *HRAS G13R* overexpressing cells in comparison to *EV* cells using colony forming assay. The relative colony number of each clone was correlated to MCF10 *TP53 Wtres + EV* 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).

3.2.4. *HRAS G13R* overexpression in combination with different *TP53* variants effect on MCF10A cells anchorage-independent growth.

Anchorage-dependent cell death (Anoikis) is a form of apoptosis induced in normal cells when they detach from surrounding cell-matrix (Frisch and Screaton, 2001).Since MCF10A cells are normal epithelium breast cells, they induce anoikis and lack the ability of anchorage independent growth(Debnath et al., 2003). However, Cancer cells develop mechanisms to resist this type of apoptosis during transformation of solid tumors including breast cancer (Wise et al., 2016) and perform anchorage-independent growth in absence of extracellular matrix and solid surface(Hanahan and Weinberg, 2011). We hypothesized that with combination of *HRAS G13R* with mutant *TP53* will stimulate anchorage-independent growth and resist anoikis. To test this hypothesis, we performed anchorage-dependent cell death assay. It was clear that *HRAS G13R* overexpression increases cells resistance to anoikis in all MCF10A cells compared to *EV* (Figure 3.7). Of note, MCF10A *TP53 R175H* cell line show highest resistance to anoikis. In the same context, to more investigate the ability of live cells to form colonies with anchorage-independent growth phenotype we performed soft agar assay. Soft agar assay results show that MCF10A *TP53 Wtres + EV* failed to form colonies, while all other MCF10A cell lines were able to form visible colonies after 10 days with variable capability to do so. For example, the number of colonies formed by *HRAS G13R* infected cells is more than *EV* control cells. Also, in MCF10A *TP53 KO* and MCF10A *TP53 R273H* infected with *HRAS G13R* cells was able to form more and larger colonies than control EV (Figure 3.8). Between the *TP53* manipulated cells, *R175H* mutation resulted in the highest colony number formation compared to other cells. In fact, the ability of MCF10A clones *TP53 KO, TP53 R275H,* and *TP53 R175H* to form colonies even without *HRAS G13R* was not surprising and conforms to other previous studies which showed that knockout of *TP53* from MCf10A cell may lead to appearance of clones with ability of anchorage-independent growth (Weiss et al., 2010). So it's expected for mutated *TP53* with *HRAS G13R* clone to become more aggressive and form more colonies than in clone that express *WT TP53*. Also, other studies proved that *HRAS*-MCF10A cells gain anchorage independent growth ability even in the presence of normal *TP53* protein (Yoh et al., 2016, Moon et al., 2000). This speculation is supported by findings of one study which found that *HRAS* transformed MCF10A cells were able to form colonies in soft agar while Bmi-1-transformed cell could not. However, co-overexpression of both *HRAS* and Bmi-1 give more aggressive and invasive phenotypic changes (Datta et al., 2007), which supports our findings that demonstrated that double manipulation of *HRAS* and *TP53* gives more aggressive phenotypic changes.

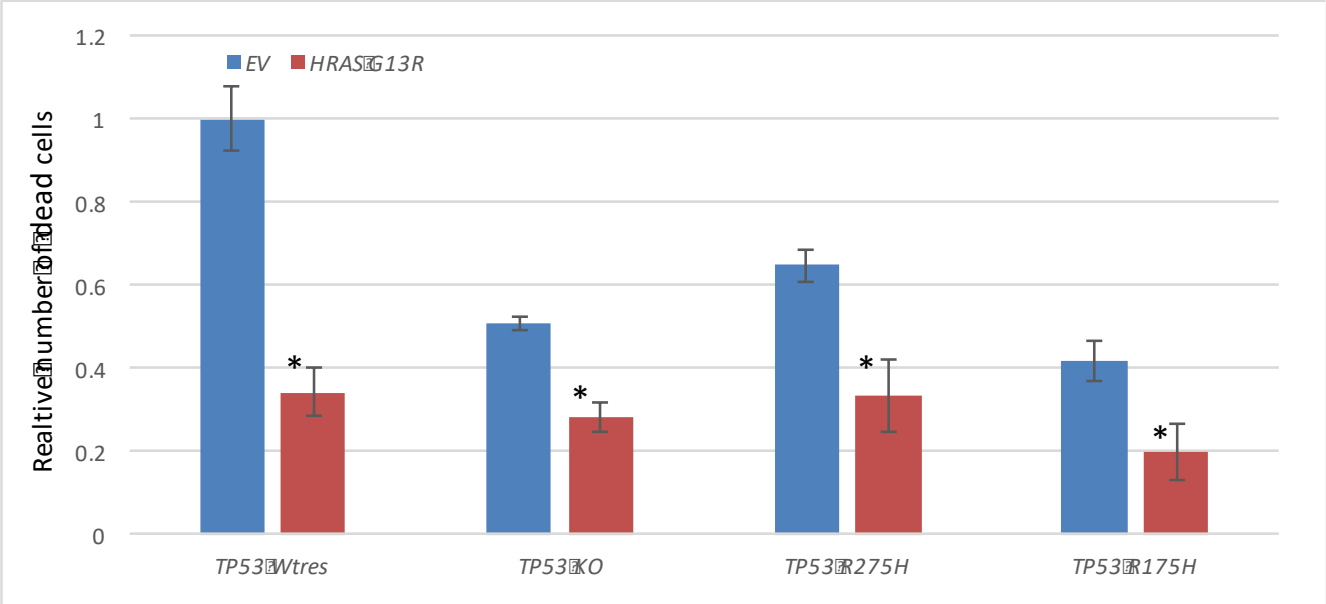


Figure 3. 7: *HRAS G13R* overexpression effect on anchorage-dependent cell death of MCF10A cell with different TP53 variants. Representative statistical analysis of the anchorage-dependent cell death of *HRAS G13R* overexpressing cells in comparison to *EV* cells using anchorage-dependent cell death assay. The relative dead cell number of each clone was correlated to MCF10 *TP53 Wtres + EV* 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).

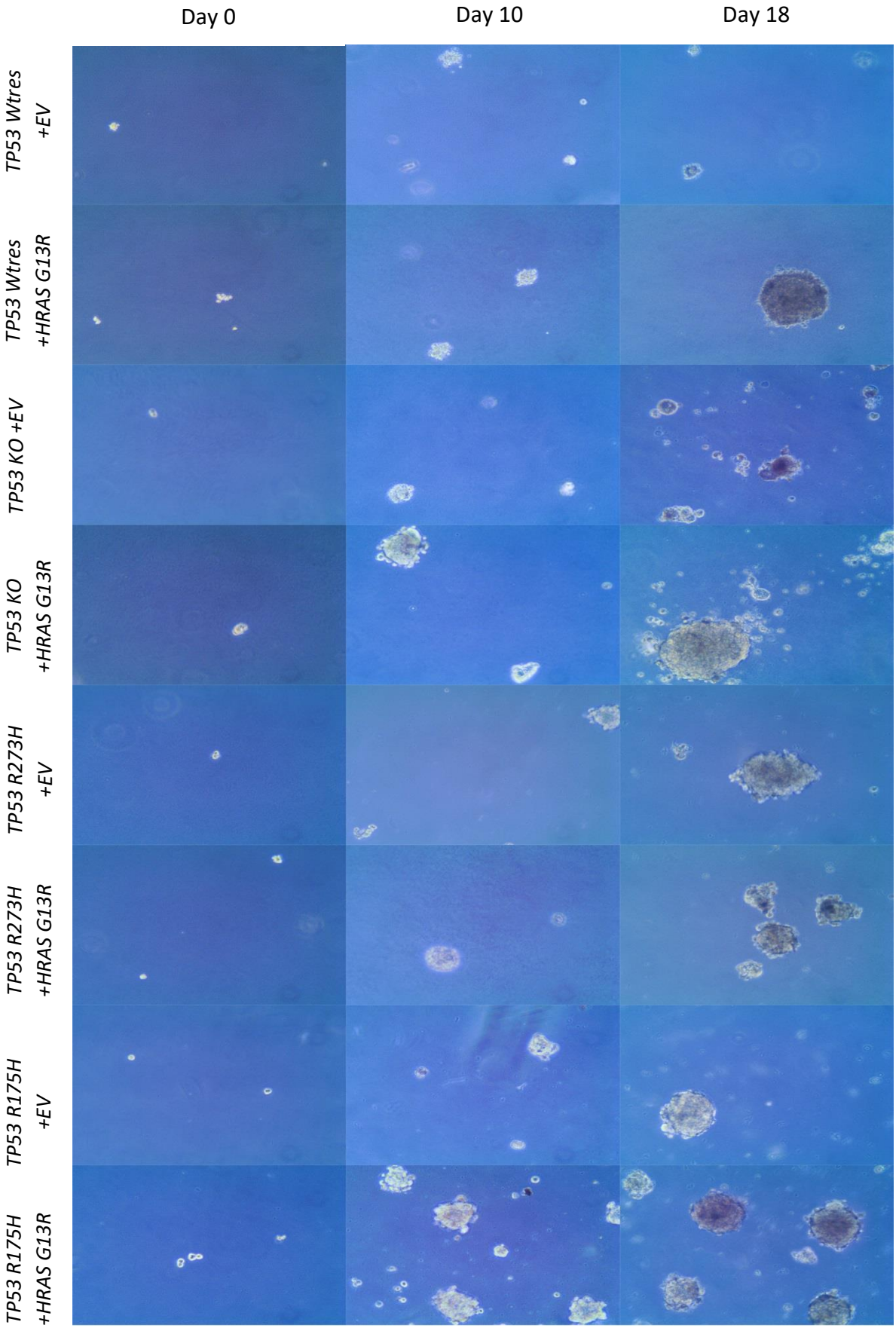


Figure 3. 8: Soft agar assay. Representative Image at 10X show the colonies shape and size of MCF10A cells infected with *HRAS G13R VS EV* .

3.2.5. *HRAS G13R* overexpression in combination with different *TP53* variants effect on expression of genes related to different cancer phenotypes.

In order to investigate the gene expression pattern responsible for different phenotypes in MCF10A cells with different *TP53* variants infected with *HRAS G13R*, we did real time PCR for specific genes related to different cell phenotypes that we examined. The tested target genes were divided into groups according to their functions: cell proliferation related genes, epithelial to mesenchymal transition (EMT) related genes, and cell death and survival related genes. First of all, when taking a look at all gene expression; it was clear that different MCF10A cells express some genes in different manners which comply with our hypothesis that different combination may lead to diversity in gene expression as well as appeared phenotype. On the other hand, other genes were expressed in the same manner in all cells. And here, this group of genes supports our hypothesis behind our bigger group which speculates that the presence of common changes and phenotypes between different transformation models could be explained, in part, by common gene expression pattern that stems from transcriptional reprogramming. Such common reprogramming can be used as molecular markers for cancer diagnosis, prognosis and targeted therapy (Figure 3.9).

After looking at the global pattern of gene expression, we looked at the expression pattern related to specific cell phenotypes. Firstly, we looked at proliferation related genes. *P21* decreased in *KO* and mutant *TP53* MCF10A cells regardless of presence of *HRAS G13R*. This was expected, because *P21* is a known *TP53* target. Thus when *TP53* is deleted or mutated p21 expression is downregulated (Macleod et al., 1995).Of note, we noticed that the *P21* expression is increased by 1.5 fold in cells harboring *Wtres TP53* after over expression of *HRAS G13R compared to EV cells*. This observation is in concordance with a previous study which sowed that *RAS* induction of *P21* depend on the presence of *WT TP53*(Swarbrick et al., 2008). The downstream *RAS* signaling pathway *AKT* gene was found overexpressed significantly (about 1 fold increase compared to EV cells) in all kind of MCF10A cell infected with *HRAS G13R* except MCF10A *TP53 R175H* in which the expression was the same as control, this could be explained since the *TP53 R175H* mutation found to induce *HRAS* expression which will lead to *AKT* expression even in *EV* cells (Solomon et al., 2012). Other important result is that Cyclooxygenase 2(*COX-2*) gene expression increased in all *HRAS G13R* infected MCF10A cells with at least 1.5 folds compared to *EV*. This gene encodes an enzyme known as prostaglandin endoperoxide synthase that catalyzes the rate limiting step in prostaglandin biosynthesis by converting arachidonic acid to prostaglandin endoperoxide (prostaglandin H2). Prostaglandin was found to be higher in malignant

breast tumors than benign tumor or normal breast tissue. One study relates the increased prostaglandin in breast tumors to increased cox expression and the use of aspirin is known to beneficial in reduction of *COX* related breast cancer risk (Hwang et al., 1998). Thus our results could demonstrate that *HRAS* can increase prostaglandins in breast tumors via *COX-2* dependent mechanism (Figure 3.9).

CYCLINB1 expression is correlated in several studies with poor breast cancer prognosis (Aaltonen et al., 2009).Its downregulated by *WT TP53* protein while both *R175H* and *R273H* mutation increase *CYClINB1* expression (Innocente et al., 1999)and previous studies suggest that *HRAS* enhance cell cycle through *CYCLINB1* dependent pathway (Santana et al., 2002). In this context, we examined expression of *CYCLINB1*. Generated models show different changes in expression pattern of *CYCLINB1*. While with MCF10A *TP53 Wtres* the *HRAS G13R* overexpression increases *CYCLINB1* by 0.5 fold compared to *EV*, it has little effect on MCF10A *TP53 KO*, MCF10A *TP53 R273H*, and MCF10A *TP53 R175H*. This expression can be correlated to insignificant difference that we observed in cell proliferation rate in those three models. Also we have investigated expression of *C-MYC*, which also promote cell cycle and proliferation. In addition, it's upregulated in high-grade breast cancer and connected to cancer resistance to anti-cancer therapy (Fallah et al., 2017). Also, it's known that *TP53* represses *C-MYC* expression through miR-145(Sachdeva et al., 2009). On contrast, *HRAS* enhances *C-MYC* activity (Sears et al., 2000). Here, the effect of *HRAS* overexpression was almost the same as EV in all generated models. This can be explained by previous study result, which found that *HRAS G12V* can enhance *C-MYC* by stabilizing protein product not by increase it on transcriptional level. However to elucidate how *HRAS G13R* regulate *C-MYC* further investigation is needed (Kapeli and Hurlin, 2011) (Figure 3.9).

Previous reports found that *TP53* control several genes related to EMT, migration and invasion. Those genes include *SLUG and SNAIL*(Muller et al., 2011). Other reports demonstrated that *RAS* oncogene induce migration and invasion by altering specific genes like, *SNAIL* and *SLUG* (He et al., 2015, Horiguchi et al., 2009, Lamouille et al., 2014). In our effort to reveal the molecular changes that produce the different migration and invasion phenotypes we tested expression of genes which are important in breast cancer progression like *SLUG, SNAIL, and WNT5A (Fernandez-Cobo et al., 2007, Huang et al., 2017, Vuoriluoto et al., 2011, Wang et al., 2016).* In addition, we tested *TP63, and TIMP2*. Our results show differential expression of different EMT markers. While *SNAIL* was almost not affected, *SLUG* shows higher expression in *TP53 R175H* cells (8 folds), which is in concordance with the migration phenotype we observed with this cell line (Figure 3.5). *WNT5A* was overexpressed in all MCF10A with different *TP53* variants after *HRAS G13R* overexpression. The expression folds of WNT5A was variable

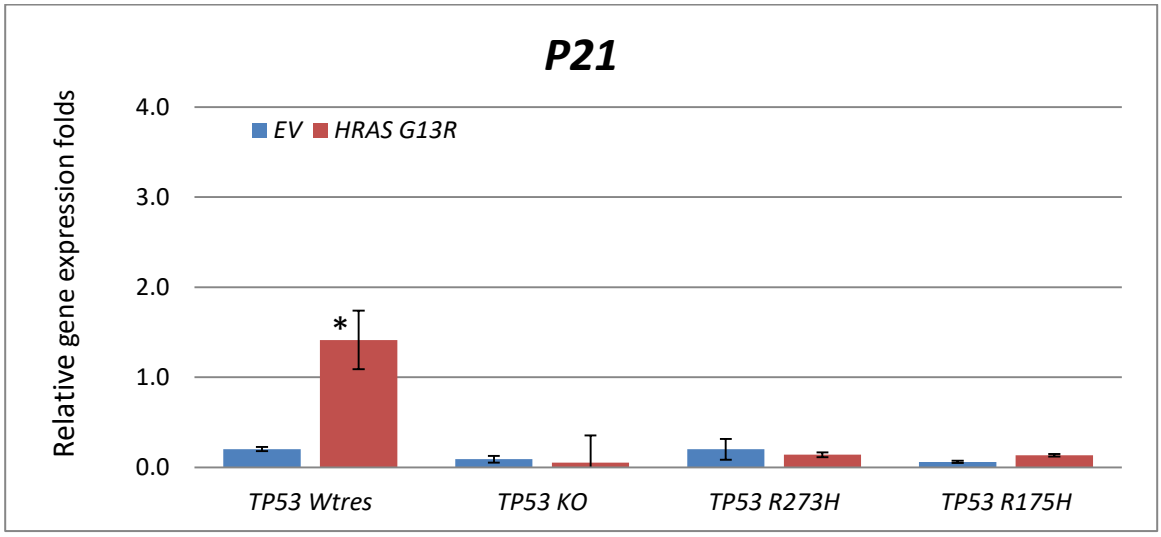
between different generated clones (Figure 3.9). This result was expected since *WNT5A* expression is correlated EMT transition (Gujral et al., 2014). This overexpression of *WNT5A* could be with great value in therapeutic field of breast cancer because previous study proposed *WNT5A* as a therapeutic target (Prasad et al., 2018). *TP63* is down regulated in all *HRAS G13R* infected MCF10A cell lines. This downregulation corresponds with previous study suggest that increased EMT in *HRAS* transformed cells is mediated by *RAS* down regulation of *TP63* (Yoh et al., 2016). Other EMT related gene is *TIMP2* which found to be increased in all *HRAS G13R* infected MCF10A cells except the one with *TP53 Wtres*. This is thought to be expressed as a defense mechanism against migration and invasion since previous study proved the ability of *TIMP2* overexpression to inhabit *HRAS* induced migration in dose dependent way.(Ahn et al., 2004). Overall different *TP53* variant can induce different invasive phenotypic changes with *HRAS G13R* overexpression combined with different EMT genes expression pattern (Figure 3.9).

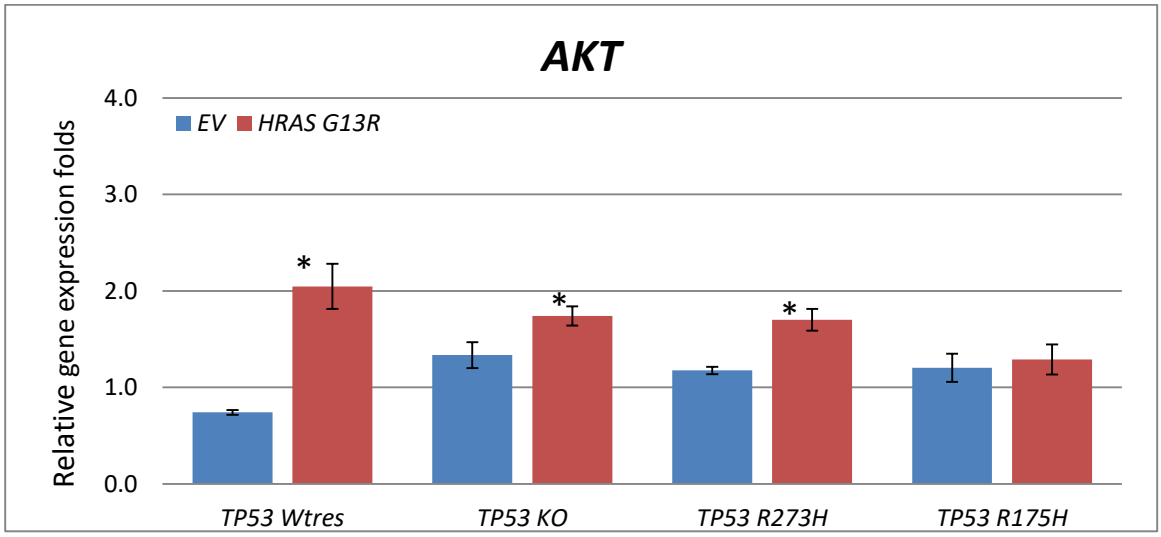
We also investigated cell death and survival group of genes to elucidate molecular mechanism of transformed cell ability to overcome anchorage-dependent cell death (anoikis) and colony formation in soft agar. It's well known that wild-type *TP53* increase normal cells sensitivity to anoikis while mutated *TP53* induce breast cancer survival and anoikis resistance (Lim et al., 2009, Tan et al., 2015). To test the balance between pro-apoptotic and anti-apoptotic genes we investigated two main player in apoptosis; pro-apoptotic gene *BAD* and anti-apoptotic gene *BCL2* (Chipuk et al., 2004). *BCL2* increased in all cells with highest folds showed by MCF10A *TP53 Wtres* (7 folds) (figure 3.9). *BAD* is *BCL2* antagonist pro-apoptotic gene. Previous report suggested that *BAD* is not only downregulated in breast cancer but also its upregulation can help in anti-invasiveness effect in addition to promotion of mitochondrial-mediated apoptosis(Cekanova et al., 2015). Here the *BAD* expression almost not affected by *HRAS G13R* overexpression in MCF10A cell with different *TP53* variant which suggest that *HRAS* overexpressed cell avoid anoikis is not mediated by regulation of *BAD* gene except for MCF10A R175H which shows *BAD* downregulation (Figure 3.9). The slight downregulation of *BAD* in *TP53 R175H* cell can be more investigated to indicate if mutated *TP53* interact or regulate *BAD* since previous study demonstrate *TP53 BAD* interaction(Jiang et al., 2007). Finally, we investigated expression of *PDL-1* gene. Several studies reported that high expression of *PDL-1* is with clinical benefits(Sun et al., 2018). Fortunately, in all MCF10A with different *TP53* variants *HRAS G13R* overexpression increased *PDL1* in a significant way (Figure 3.9). With at least 4 folds increase in *PDL-1* in MCF10A with *HRAS G13R* overexpression compared to *EV*. This gene was previously reported that *HRAS* increases its expression through stabilization of *PDL1* mRNA, and could be a therapeutic target (Coelho et al., 2017).

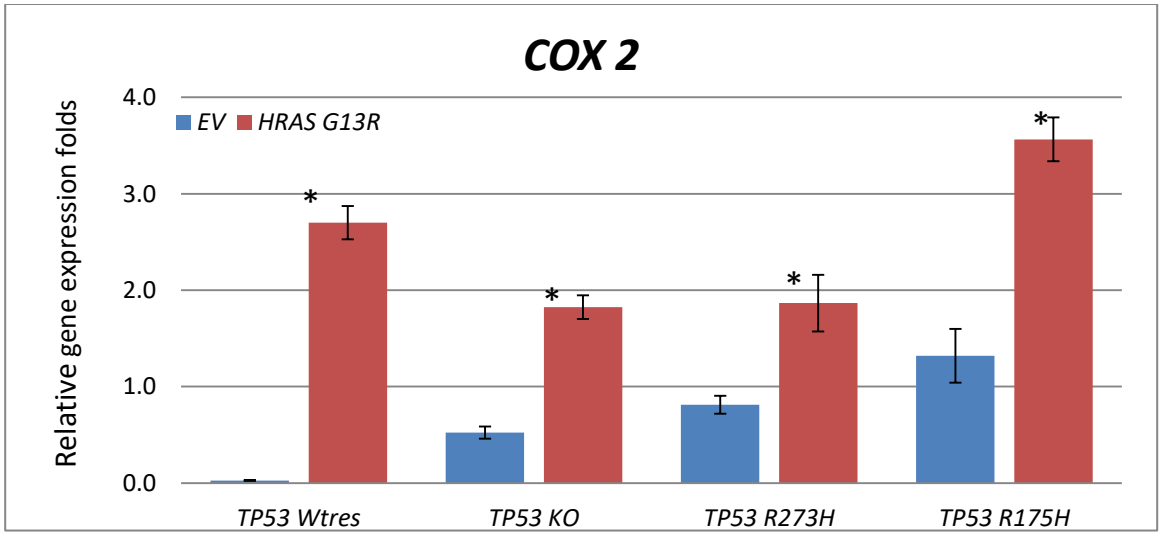
Altogether, in this study our results show that different combinations between different mutations in cancer will rise into different phenotypes that underlined by different gene expression patterns. One limitation of this study is the rarity of *G13R* mutation in breast cancer which makes it hard to study this mutation real human breast tissue. Also, to our knowledge, there are no previous studies on this mutation in breast cancer. So in order to further investigate the effect of these combinations in vivo we need to generate mouse models. But if we look at this model as a part of bigger project that aim to identify common transcriptional changes, this model is one of many models that we are going to generate in this large project and the use of *HRAS G13R* mutated gene in this study; despite that its not common in breast cancer; can be explained since it comprises one of many models that we are going to generate. The presence of even rare type of breast cancers will help in detection of more universal changes in breast cancer. In other words the heterogeneity of breast cancer can be covered by including rare types of cancer.

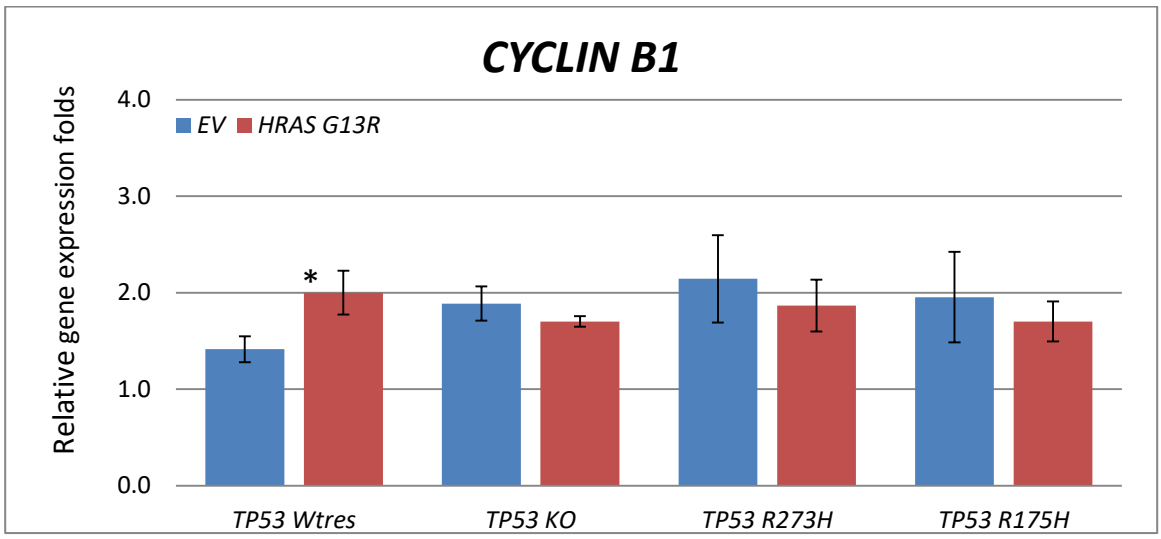
4. Conclusion and future outlook.

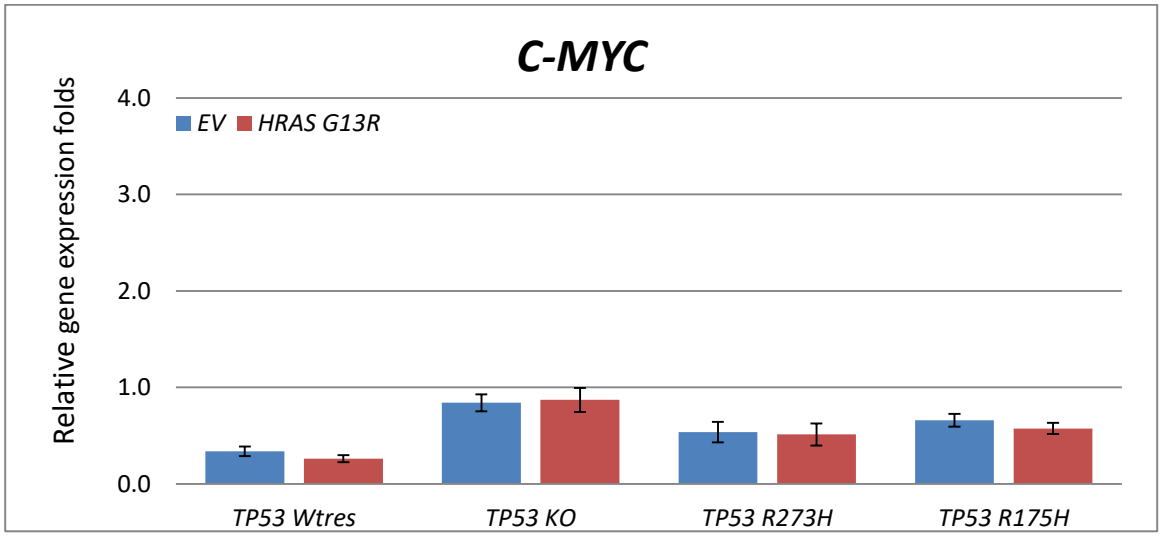
Although G13R is not an *HRAS* common mutation in breast cancer, we used it as a proof concept to show that although there is heterogeneous molecular changes in breast cancer still there are common changes that can be used as biomarker for breast cancer diagnosis, prognosis, and therapy. Overall, here we demonstrate that *HRAS G13R* interacts with different *TP53* variants in different ways to induce different tumorigenic and aggressiveness cell phenotypes. On the molecular level, we noticed that the expression pattern of some genes was variable and differential between different cell lines. On the other hand, we noticed that the expression pattern of specific genes was common between all tested cell lines. This indicate that despite the heterogeneity in breast cancer we still have common molecular changes that can be used as molecular markers in breast cancer. Our future aim is to generate more breast cancer cell models that will be used to better understand the pathogenesis of breast cancer especially transcriptional reprogramming. In specific our major goal will be to identify common changes between different transformation models in order to identify biomarker for breast cancer diagnosis, prognosis, and therapy.

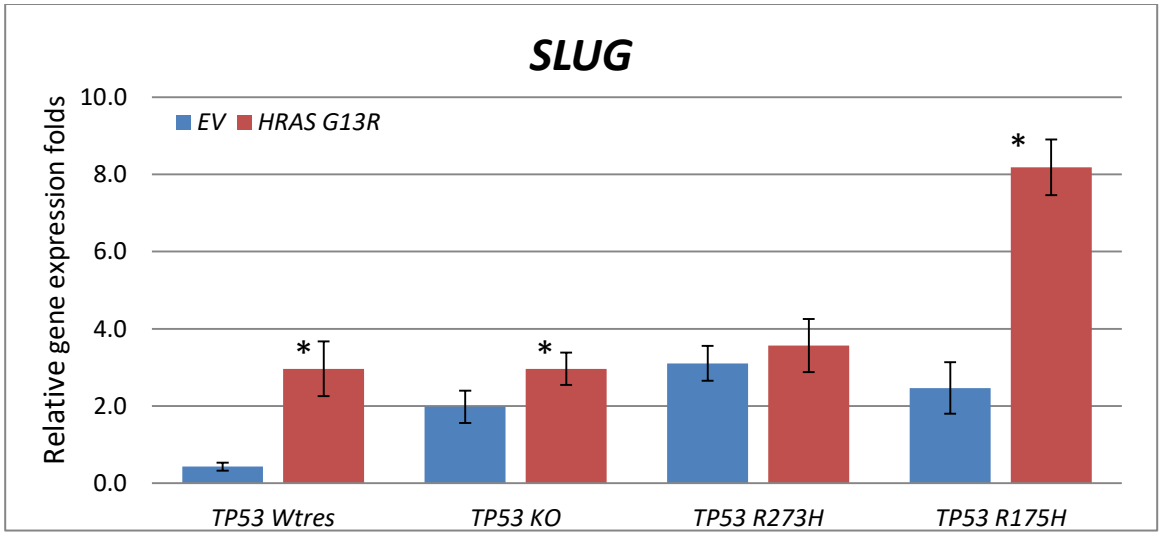

P21
Relative gene expression folds
4.0
3.0
2.0
1.0
0.0
EV
HRAS G13R
*
TP53 Wtres
TP53 KO
TP53 R273H
TP53 R175H

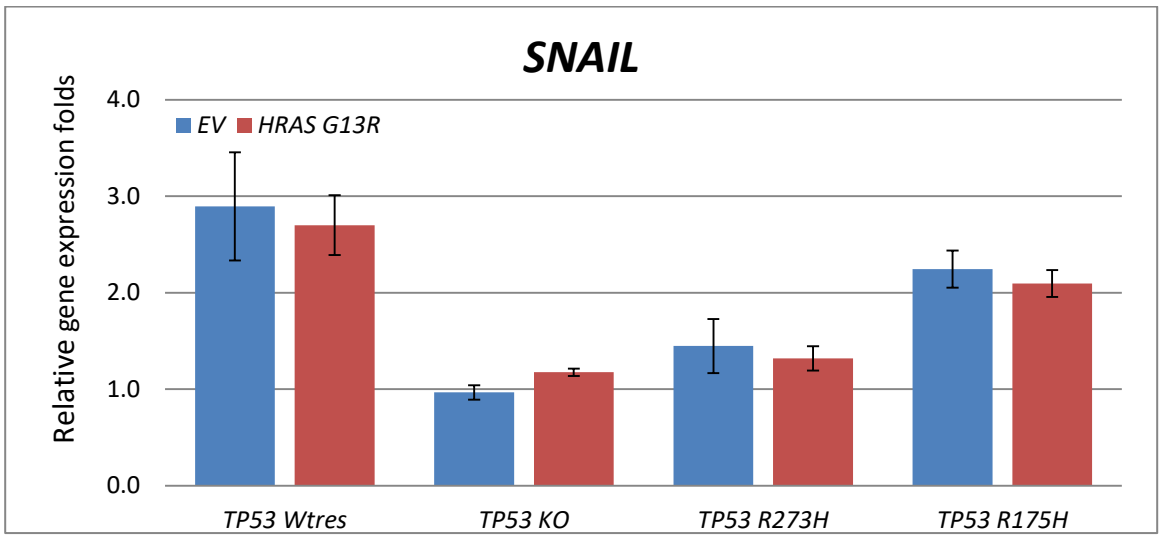

AKT
Relative gene expression folds
4.0
3.0
2.0
1.0
0.0
EV
HRAS G13R
*
TP53 Wtres
TP53 KO
TP53 R273H
TP53 R175H

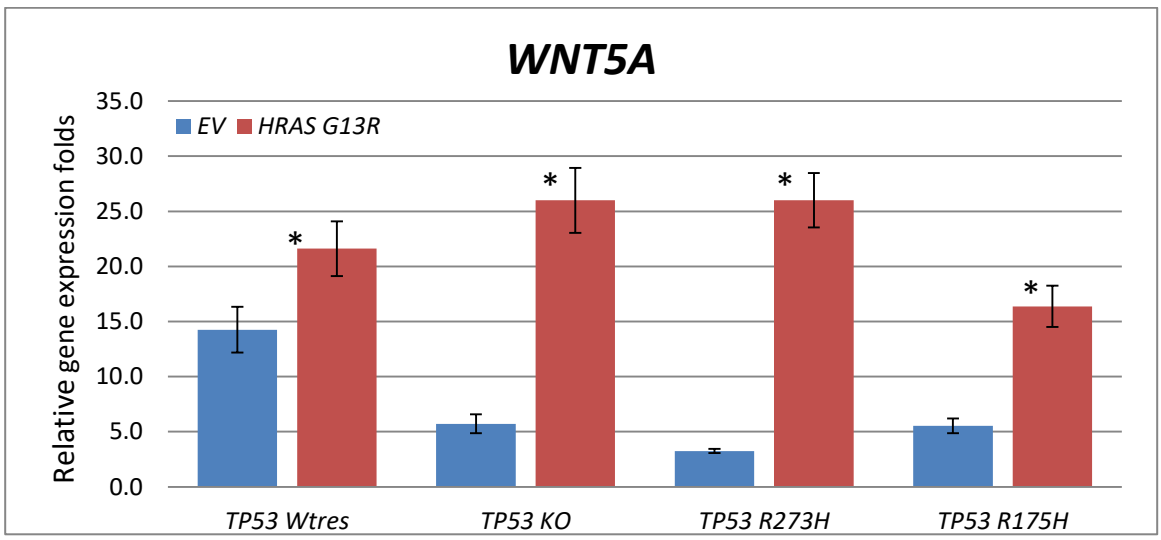

COX 2
Relative gene expression folds
4.0
3.0
2.0
1.0
0.0
EV
HRAS G13R
*
TP53 Wtres
TP53 KO
TP53 R273H
TP53 R175H

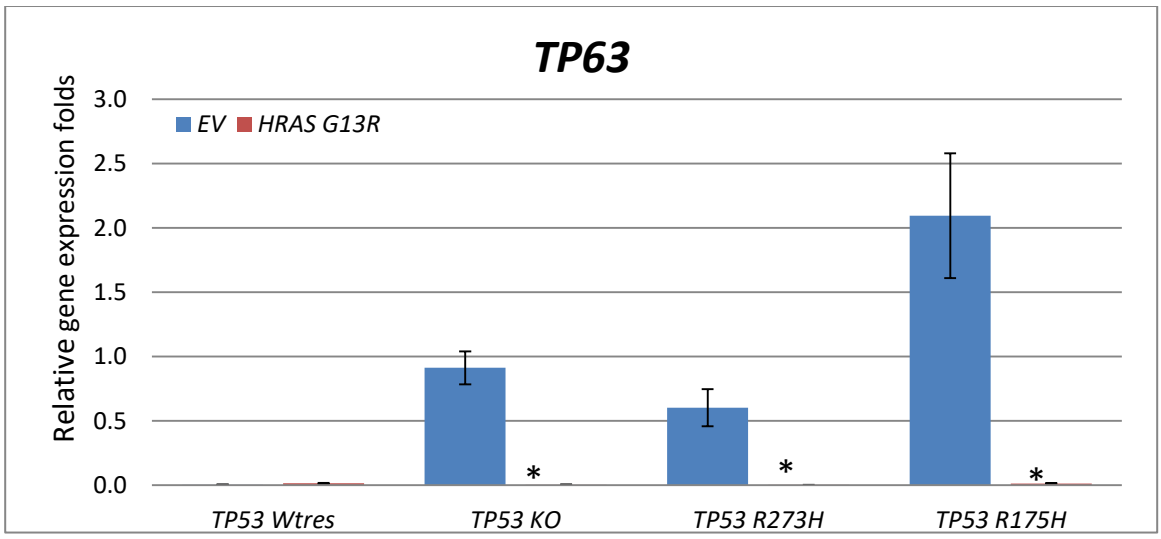

CYCLIN B1
Relative gene expression folds
4.0
3.0
2.0
1.0
0.0
EV
HRAS G13R
*
TP53 Wtres
TP53 KO
TP53 R273H
TP53 R175H

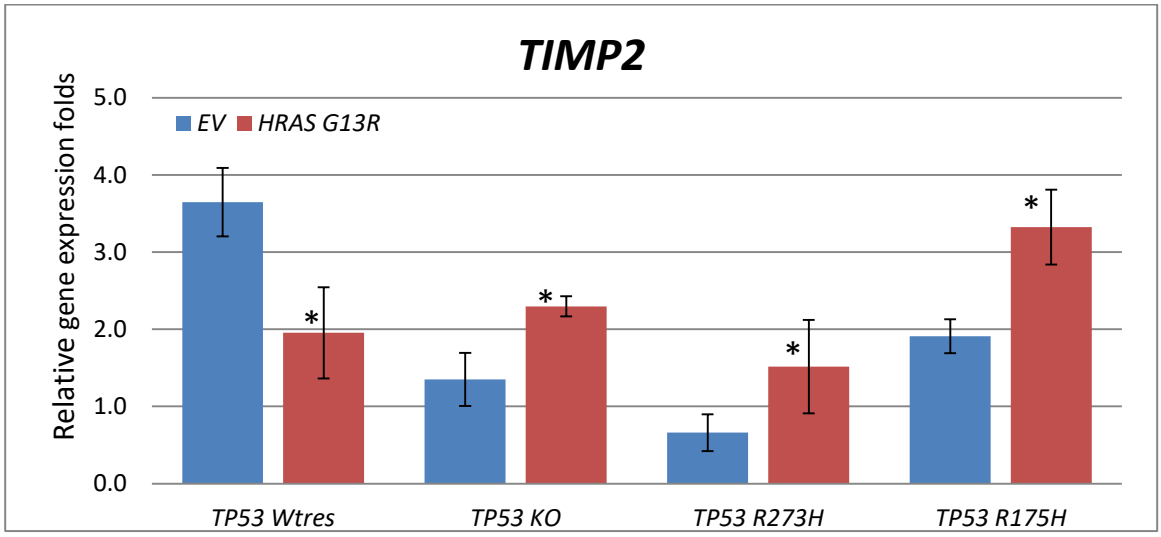

C-MYC
Relative gene expression folds
4.0
3.0
2.0
1.0
0.0
EV
HRAS G13R
TP53 Wtres
TP53 KO
TP53 R273H
TP53 R175H

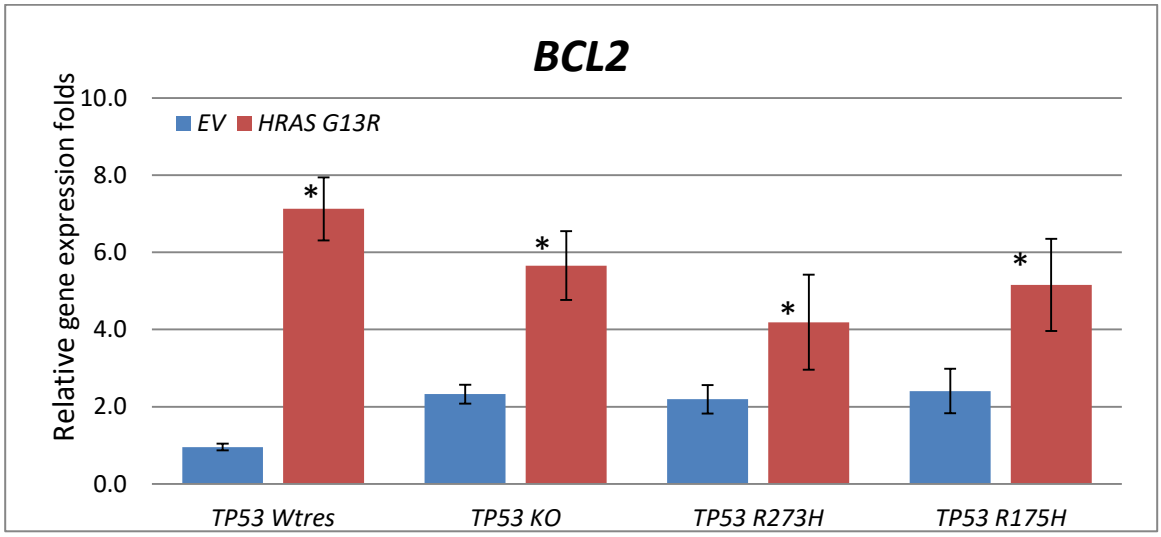

SLUG
Relative gene expression folds
10.0
8.0
6.0
4.0
2.0
0.0
EV
HRAS G13R
*
*
*
TP53 Wtres
TP53 KO
TP53 R273H
TP53 R175H

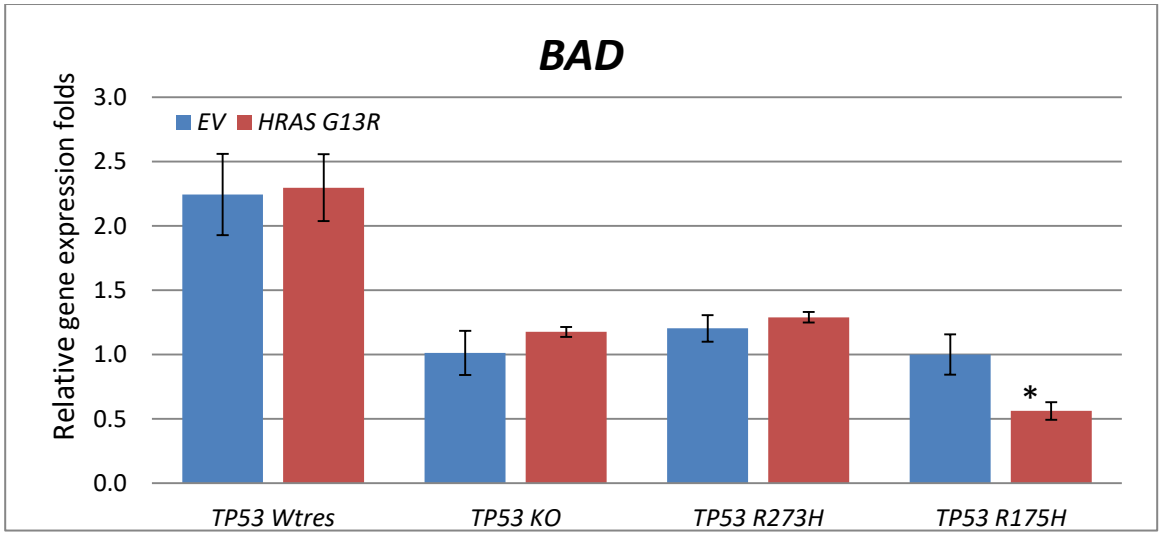

SNAIL
Relative gene expression folds
4.0
3.0
2.0
1.0
0.0
EV
HRAS G13R
TP53 Wtres
TP53 KO
TP53 R273H
TP53 R175H


WNT5A
Relative gene expression folds
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
EV
HRAS G13R
*
TP53 Wtres
TP53 KO
TP53 R273H
TP53 R175H


TP63
Relative gene expression folds
3.0
2.5
2.0
1.5
1.0
0.5
0.0
EV
HRAS G13R
*
TP53 Wtres
TP53 KO
TP53 R273H
TP53 R175H


TIMP2
Relative gene expression folds
5.0
4.0
3.0
2.0
1.0
0.0
EV
HRAS G13R
*
TP53 Wtres
TP53 KO
TP53 R273H
TP53 R175H


BCL2
Relative gene expression folds
10.0
8.0
6.0
4.0
2.0
0.0
EV
HRAS G13R
*
TP53 Wtres
TP53 KO
TP53 R273H
TP53 R175H


BAD
Relative gene expression folds
3.0
2.5
2.0
1.5
1.0
0.5
0.0
EV
HRAS G13R
*
TP53 Wtres
TP53 KO
TP53 R273H
TP53 R175H

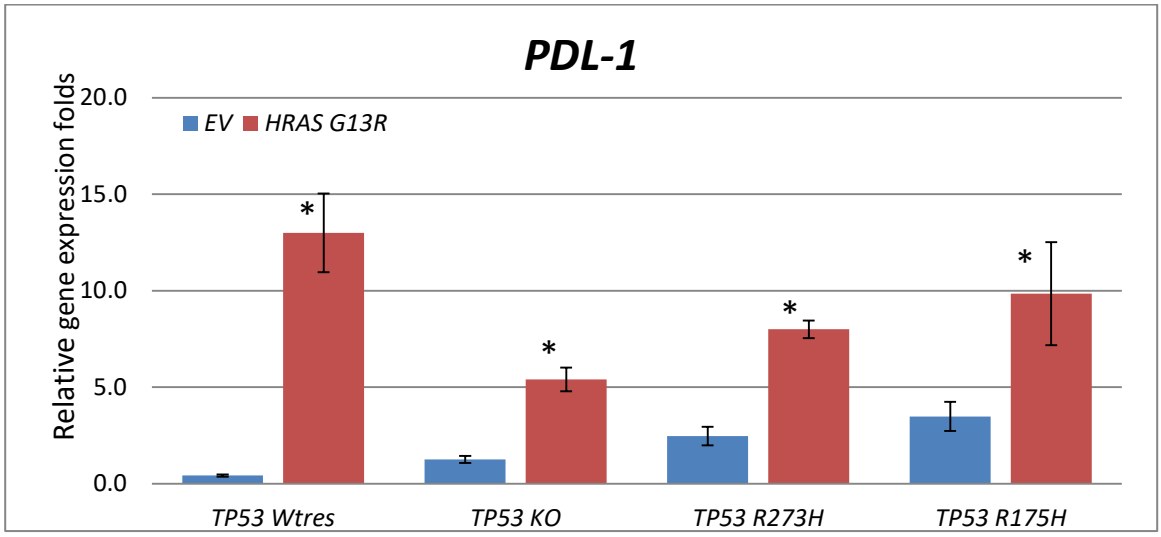


Figure 3. 9. Real time PCR results showing the relative expression level of different genes correlated to the house keeping gene *UBC*. All gene expression folds were calculated relative to the expression of level in normal MCF10A cell (parental). Bars indicate standard error mean (SEM) of three replicates. The statistical significance of the results was determined by measuring the p value. (* indicates p-value <0.05)

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6. Appendix:

6.1.Appendix 1: plasmid construction:

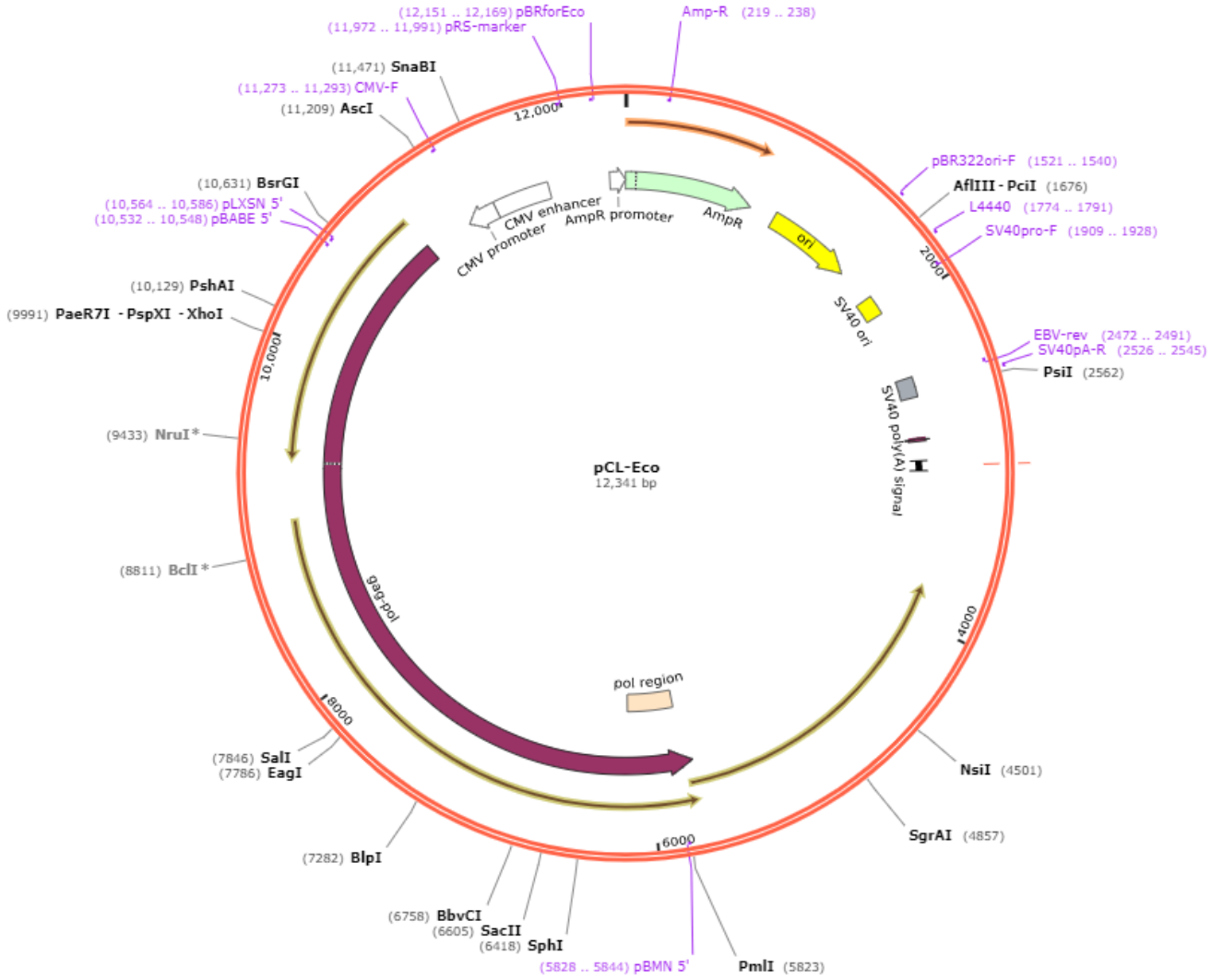

Figure 1: pCL-Eco plasmid from addgene website accessed in 14-1-2020 (https://www.addgene.org/browse/sequence/254697/)

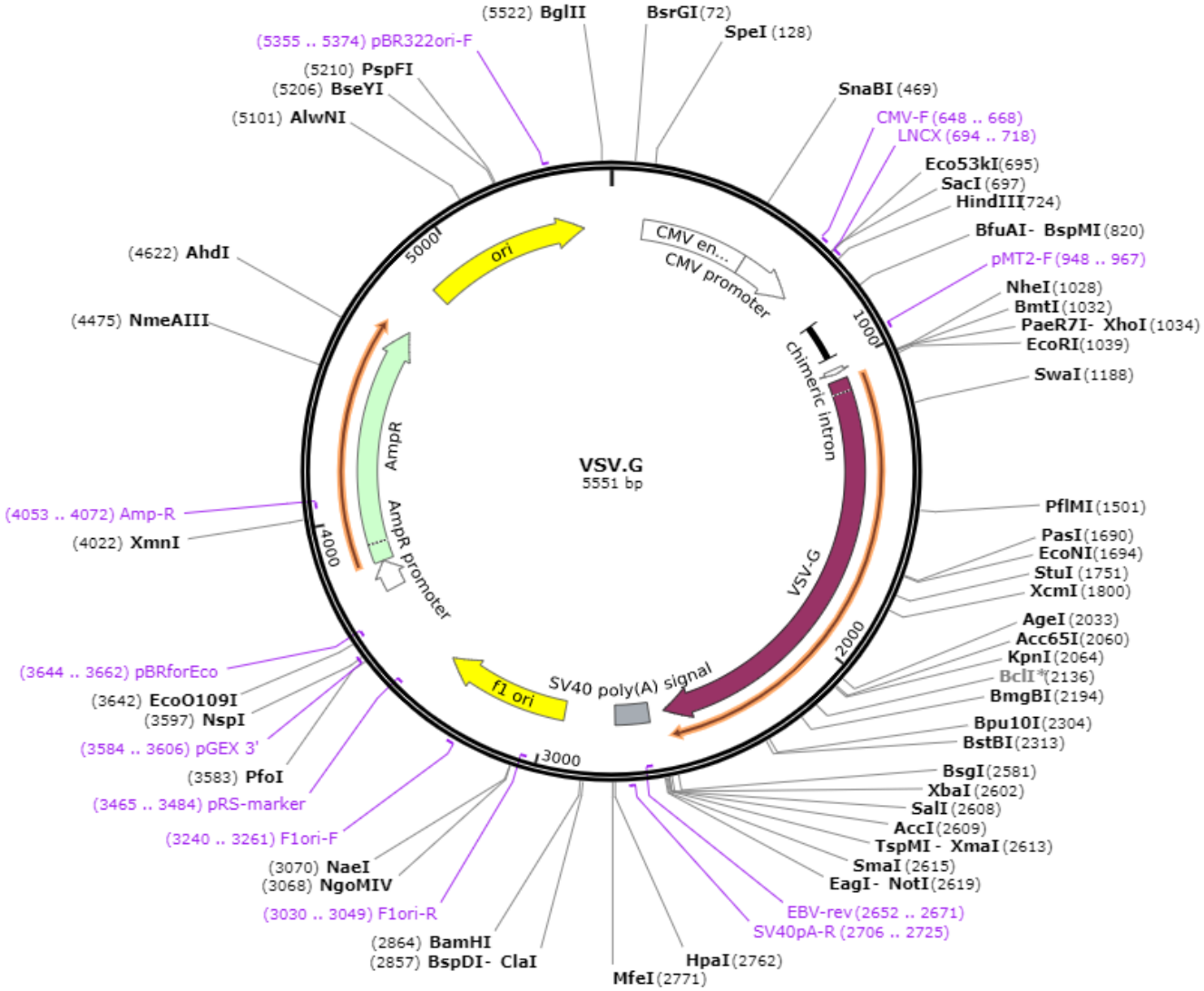

Figure 2: VSV-G plasmid from addgene website accessed in 14-1-2020 (https://www.addgene.org/browse/sequence/221993/)

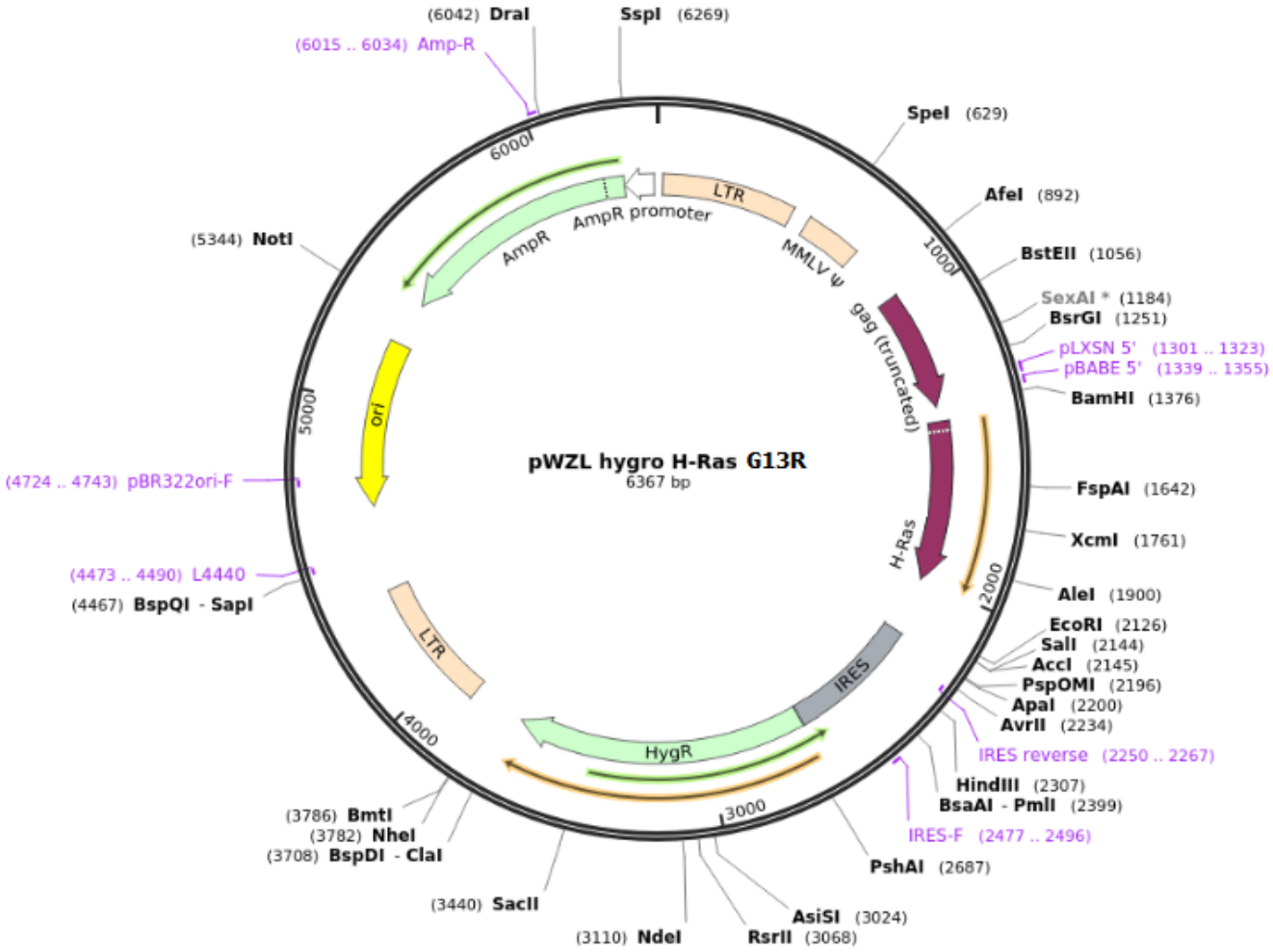

Figure 3 : pWZL hygro H-Ras G13R: pwzl hygro backbone from addgene accessed in 14-1-2020

6.2.Appendix 2: Table of used primer:

NO	Gene	Primer	Refernce
1	*HRAS*	F.P_5'- tgccatcaacaacaccaagt-3' R.P-5'- agccaggtcacacttgttcc-3'	NM_001130442.2
2	*P21*	F.P 5'- cgtcaaatcctccccttcct-3' R.P 5'- atgggttctgacggacatcc -3'	NM_001291549.1
3	*AKT*	F.P 5'- cacaaacgaggggagtacat-3' F.P 5'- tgcgccacagagaagttg-3'	NM_009652.3
4	*COX2*	F.P 5'- tcccttccttcgaaatgc-3' F.P 5'-aggttagagaaggcttcccag-3'	NM_000963.4

5	*CYCLINB1*	F.P_5'- gtcaccaggaactcgaaaat -3' R.P-5'-ttaccaatgtccccaagagc-3'	NM_031966.3
6	*C-MYC*	F.P 5'- tagtggaaaaccagcagcct-3' F.P 5'- ctcgtcgcagtagaaatacgg-3'	NM_002467.6
7	*SLUG*	F.P_5'- atacagtgattatttccccg -3' R.P-5'- agcggtagtccacacagtga-3'	NM_003068.4
8	*SNAIL*	F.P_5'- acactggcgagaagccctt -3' R.P-5'- gcctggcactggtacttctt -3'	NM_005985.3
9	*WNT5A*	F.P-5'- atgaagaagtccattggaat -3' R.P-5'- ctgggcgaaggagaaaaata -3'	NM_003392.4
10	*TP63*	F.P-5'- acaggaagacagagtgtgct-3' F.P-5'- catccctccaacacaactgc-3'	NM_003722.5
11	*TIMP2*	F.P-5'-agcagataaagatgttcaaaggg-3' R.P-5'ttctttcctccaacgtccag-3'	NM_003255.5
12	*BCL2*	F.P-5'- gccctgtggatgactgagta-3' R.P-5'- gaaatcaaacagaggccgca-3'	NM_000633.2
13	*BAD*	F.P_5'- ctcctttaagaagggacttc-3' R.P-5'- gatgtggagcgaaggtca-3'	NM_004322.3
14	*PDL-1*	F.P-5'- tgaaagtcaatgccccatac-3' F.P-5'- ttgatggtcactgcttgtcc-3'	NM_014143.4
15	*HUBC*	F.P 5'- gtcgcagttcttgtttgtgg-3' R.P 5'-gatggtgtcactgggctcaa-3'	NM_021009.6

الملخص

يعد سرطان الثدي أكثر أنواع السرطان شيوعا لدى النساء، وهو المسؤول الاول عن الوفيات لدى النساء في العالم. ان اختلال التوازن في الخلية بين الجينات المسرطنة والجينات المثبطة هو من اهم أسباب نشوء الورم السرطاني في الجسم، ويظهر هذا الخلل عادة بسبب حدوث طفرة في أحد هذه الجينات. ان التغير الذي تنشئه الطفرات في عملية التعبير الجيني لمختلف الجينات في سرطان الثدي عادة ما يكون كثير التنوع ويظهر طرزا شكلية مختلفة. من خلال العمل المبدئي لمجموعتنا تبين ان مختلف انوع الطفرات في جين *TP53* تؤدي الى تعبيرات جينية متغايرة ومتنوعة. من هذه النتيجة افترضنا ان عمل عدة نماذج لخلايا تحوي جين HRAS G13R بالإضافة الى طفرة مختلفة من*TP53* في كل نموذج سيؤدي الى ظهور طرز شكلية وتعبيرات جينية متنوعة. لاختبار هذه النظرية قمنا بإدخال جين HRAS G13R الى خلايا من نوع MCF10Aتحتوي كل منها على طفرة مختلفة في جين *TP53*. ثم قمنا بفحص علامات السرطان الأساسية (cancer hallmarks) بالإضافة لفحص التعبير الجيني لجينات محددة. أظهرت النتائج ان ادخال جين HRAS G13R أدى الى زيادة القوة السرطانية للخلايا سواء من حيث سرعة النمو والقدرة على الانتقال بالإضافة الى تخطي الخلايا لعملية الموت المبرمج بدرجات متباينة. وقد صاحب هذه التغيرات اختلاف في التعبيرات الجينية لكل خلية. بالإجمال وفرت هذه الدراسة نموذج لدراسة تأثير حدوث طفرتين في الخلية على تطور السرطان، بالإضافة الى ذلك فان هذا النموذج يمكن من خلاله دراسة الية تداخل كل من جيني HRAS و *TP53*في سرطان الثدي بما يساعد في تشخيصه وعلاجه.