Molecular Mechanisms of Loss of E7 Expression in HPV16 – Transformed Human Keratinocytes

Fadi Farooq Abboodi

Follow this and additional works at: https://scholarcommons.sc.edu/etd

Recommended Citation

This Open Access Dissertation is brought to you by Scholar Commons. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact dillarda@mailbox.sc.edu.
Molecular Mechanisms of Loss of E7 Expression in HPV16 – Transformed Human Keratinocytes

by

Fadi Farooq Abboodi

Doctor of Medicine
University of Mosul, 2010

Master of Science
University of South Carolina, 2016

Submitted in Partial Fulfillment of the Requirements
For the Degree of Doctor of Philosophy in
Biomedical Science
School of Medicine
University of South Carolina
2020

Accepted by:

Lucia Pirisi-Creek, Major Professor
Ioulia Chatzistamou, Committee Member
Kim Creek, Committee Member
Gregorio Gomez, Committee Member
Wayne Carver, Committee Member
Cheryl L. Addy, Vice Provost and Dean of the Graduate School
DEDICATION

I dedicate this work to my lovely wife, Sarah W. Abboodi, who supported and encouraged me in every single step of my study. She made a lot of sacrifices to help me achieve the PhD degree.
ACKNOWLEDGMENT

First and foremost, I owe the greatest gratitude to my Lord Jesus Christ who gave me a lot of blessings that made all this work possible and opened a door for me whenever I thought that all the doors were closed.

I would like to thank my mentor, Dr. Lucia Pirisi-Creek, who showed me all guidance and support to do my research. I was the last graduate student in her lab and I wish for her a very happy and relaxing retirement. I am also grateful to my committee members (Dr. Ioulia Chatzistamou, Dr. Kim Creek, Dr. Gregorio Gomez, and Dr. Wayne Carver) for the valuable instructions and contribution to my work. I appreciate my lab members: Maria Hosseinipour, Yvon Woappi, Nella Delva, Justin Vercellino, Geraldine Ezeka, and Christian Graves for showing me the basics of the work in our lab.

I am grateful to Dr. Phillip Buckhaults who welcomed me in his lab to perform the Genetics part of my research using reagents and protocols that they have already been tested to be effective. I would also extend this gratitude to the all the wonderful people I worked with in Dr. Buckhaults's and Dr. Creek's labs.

Sincere thanks would go to Dr. Edie Goldsmith, Dr. Mitzi Nagarkatti, Dr. Udai Singh, and Dr. Chandrashekhkar Patel for all the support, caring and guidance.

Thanks to every one of my instructors, office staff, and friends. Great thanks to my lovely parents and family for their praying, wishes and support to me.
ABSTRACT

Human papillomavirus (HPV) causes about 5% of all human cancers. The HPV oncoproteins E6/E7 are responsible for the transforming potential of the virus. Although continuous expression of the HPV oncogenes was considered indispensable for HPV-induced carcinogenesis, we and others have demonstrated that in a subset of HPV-positive head and neck and cervical cancers, the HPV oncogenes are not expressed (HPV-inactive cancers). Based on the observation that primary HPV-positive tumors express E6/E7, while metastases tend to be HPV-inactive, we hypothesized that HPV-inactive cancers begin as HPV-active lesions and lose their dependence on continuous E6/E7 expression during progression. This may be due to genetic and/or epigenetic modifications caused by the genomic instability and the additional carcinogens to which the tumor is exposed. We observed that HPV-inactive cancers of the cervix often have mutated p53, while HPV-active cancers do not. Therefore, we proposed that HPV positive tumors may become inactive if p53 becomes mutated. The CRISPR-Cas9 system was used to knock-out the p53 gene in differentiation-resistant HPV16 immortalized human keratinocytes (HKc-DR). The DNA deletions within the p53 gene were confirmed by PCR and gel electrophoresis and further validated by Sanger sequencing. Using qPCR, we found that HPV16 E7 expression was significantly lower (5-fold) in the p53-knocked out (KO) lines than in the p53-wild
type (WT) lines. Reduced E7 expression in p53-KO lines was reversed by using the demethylating agent 5-Aza-2'-deoxycytidine, suggesting that DNA methylation plays a role in this process. Also, we used in situ hybridization to detect HPV16 E7 mRNA in p53-WT and KO lines grown as spheroids on an agarose cushion. Interestingly, while all p53-WT lines have a uniform distribution of E7 signal, the p53-KO lines showed some spheroids that were completely lacking E7 mRNA, and some had a mixed population of E7-positive and E7-negative cells. These results indicate that the p53-KO lines are a heterogeneous population in regard to HPV16 E7 expression. We concluded that p53 loss-of-function mutation may be an important factor in driving HPV16 transformed cells to lose dependence on the continuous expression of the HPV oncogenes and become HPV-inactive. However, complete loss of p53 alone is not sufficient to suppress E7 expression entirely. We also determined that loss of E7 expression may be due, at least in part, to DNA methylation. We are currently examining HPV URR methylation in the p53-WT and KO lines, and isolating pure lines with complete loss of HPV16 E7 expression for further studies of the molecular mechanisms that may lead to the HPV-inactive phenotype.
TABLE OF CONTENTS

Dedication ......................................................................................................................... iii

Acknowledgment ................................................................................................................. iv

Abstract ................................................................................................................................. v

List of figures ......................................................................................................................... x

List of abbreviations ........................................................................................................... xii

Chapter 1: Introduction .......................................................................................................... 1
  1.1 Human Papillomaviruses (HPVs) .................................................................................. 1
  1.2 HPV life cycle ................................................................................................................... 1
  1.3 HPV clearance and persistence ....................................................................................... 4
  1.4 The HPV genome and its products ............................................................................... 5
  1.5 HPV-associated cancers ............................................................................................... 10
  1.6 Tumor suppressor p53 .................................................................................................... 14
  1.7 HPV and DNA methylation ............................................................................................ 17
  1.8 HPV and SIX1 ............................................................................................................... 18
  1.9 Hypothesis and rationale ............................................................................................... 20
1.10 Objectives ........................................................................................................... 21

Chapter 2: Materials and Methods ............................................................................. 22

2.1 Cell culture ........................................................................................................... 22
2.2 CRISPR-Cas9 system ............................................................................................. 23
2.3 Fluorescence activated cell sorting (FACS) ......................................................... 23
2.4 Characterization of the p53-KO and WT clones .................................................. 24
2.5 DNA/RNA isolation .............................................................................................. 26
2.6 cDNA synthesis and quantification ....................................................................... 26
2.7 cDNA quantification ............................................................................................. 26
2.8 Real time qPCR .................................................................................................... 27
2.9 Spheroid assay ...................................................................................................... 27
2.10 Paraffin-embedded spheroid block formation ..................................................... 28
2.11 Preparing chamber slides with adherent cells .................................................... 28
2.12 HPV16 E6/E7 mRNA in situ hybridization (RNAscope) ................................... 29
2.13 DNA-demethylation treatment .......................................................................... 29

Chapter 3: Results ...................................................................................................... 31

Chapter 4: Discussion ................................................................................................ 39

4.1 Conclusion ............................................................................................................. 39
4.2 Significance ........................................................................................................40

4.3 Future work .......................................................................................................42

References ...............................................................................................................43
LIST OF FIGURES

Figure 1.1 The HPV life cycle ..................................................................................3

Figure 1.2 The natural history of cervical cancer ....................................................6

Figure 1.3 The HPV 16 genome ...........................................................................8

Figure 1.4 Molecular pathways affected by HPV-oncoproteins .........................11

Figure 1.5 Gene ontology, HPV-active versus HPV-negative OPSCC samples .16

Figure 1.6: Gene ontology analysis of gene expression changes in HPV-active,  
-inactive and -negative tumors from European Americans (EA) ............17

Figure 1.7: Difference between HPV-inactive and HPV-active in age  
at the time of diagnosis (A) and survival (B) ...........................................22

Figure 1.8: The p53 network .................................................................................22

Figure 1.9: In vitro model of HPV16 mediated transformation of human  
keratinocytes ....................................................................................................36

Figure 2.1: Gel electrophoresis of p53 KO/WT qPCR products .......................37

Figure 2.2: Sanger sequencing data ....................................................................39

Figure 2.3: Schematic representation of the spheroid assay ..........................40

Figure 3.1: RT-qPCR HPV16 E7 of p53-KO and WT HKc/DR at early passage  
number .............................................................................................................11

Figure 3.2: RT-qPCR of p53-KO and WT HKc/DR cells at passage number  
(10-14) .............................................................................................................42
Figure 3.3: Reversal of E7 expression upon DNA demethylation .................43

Figure 3.4: HPV16 E6/E7 mRNA in situ hybridization of spheroid grown p53-KO/WT HKc/DR cells .................................................................45

Figure 3.5: HPV16 E6/E7 mRNA in situ hybridization of adherent p53-KO/WT HKc/DR cells ........................................................................45

Figure 3.6: Dispersed and coherent colony pattern ..............................................46

Figure 3.7: Effect of SIX1 overexpression on E7 expression in p53-KO cells.....46
LIST OF ABBREVIATIONS

BPE ................................................................. bovine pituitary extract

Cas-9 .......................................................... CRISPR associated protein 9

CDK ............................................................ cyclin-dependent kinase

cDNA ........................................................... complementary DNA

CIN ............................................................. cervical intraepithelial neoplasia

CpG ............................................................. cytosine guanine island

CRISPR ...................................................... clustered regularly interspaced short palindromic repeats

DMSO .......................................................... dimethylsulfoxide

DNA ........................................................... deoxyribonucleic acid

DR .............................................................. differentiation resistant keratinocytes

E2BS .......................................................... E2 binding site

E6AP ........................................................... E6-associated protein

EA .............................................................. European American

ELISA ......................................................... enzyme-linked immunosorbent assay

EMT ........................................................... epithelial-mesenchymal-transition
FBS ..................................................................................................fetal bovine serum
FFPE ..............................................................................................formalin fixed paraffin embedded
GFP .................................................................................................. green fluorescent protein
gRNA ................................................................................................guide RNA
HKc ............................................................................................... human keratinocytes
HKc/DR ........... differentiation resistant, HPV16-transformed human keratinocytes
HKc/DR-SIX1 ................................................................. SIX1-overespressing HKc/DR
HKc/HPV16 ................. human keratinocytes immortalized with HPV16 DNA
HNC ..............................................................................................head and neck cancer
HNSCC .................................................................................. head and neck squamous carcinoma
HPV .................................................................................................. human papillomavirus
HPV16 ........................................................................................ human papillomavirus type 16
HR ...............................................................................................high-risk human papillomavirus
hTERT ................................................................. human telomerase reverse transcriptase
ISH ................................................................................................... in situ hybridization
Kb .............................................................................................kilo base
KO ..........................................................................................knocked-out
LCR ...........................................................................................long coding region
LR ............................................................... low-risk human papillomavirus

MDM2 .......................................................... mouse double minute 2 homolog

mRNA .............................................................. messenger ribonucleic acid

NHKc ............................................................. normal human keratinocytes

OPSCC .......................................................... oropharyngeal squamous cell carcinoma

ORF .............................................................. open reading frame

PBS ................................................................. phosphate buffered saline

PCR ................................................................. polymerase chain reaction

PFA ................................................................. paraformaldehyde

PV ................................................................. papillomavirus

Rb ................................................................. retinoblastoma protein

RNA ............................................................... ribonucleic acid

RT-qPCR ......................................................... real time quantitative polymerase chain reaction

SIX1 ............................................................... sineoculis homeobox homolog 1

TGF-β ............................................................ transforming growth factor β

URR ............................................................... upstream regulatory region

UV ................................................................. ultraviolet light

WT ................................................................. wild-type
CHAPTER 1: INTRODUCTION

1.1 Human papillomaviruses (HPVs):

Human papillomavirus is a small non-enveloped virus of about 50-60 nm in diameter. The virus has an icosahedral capsid and a circular double-stranded DNA genome of about 8000 base pairs [1]. HPV belongs to the Papillomaviridae family. More than 200 types of HPV can infect humans and the remaining types have been found in other animals [2,3]. HPV types have a predilection to infect cutaneous tissues and some of them can infect mucosal tissues, too [3].

According to the severity of the underlying conditions caused by these viruses, HPVs have been classified into low risk (LR) and high risk (HR) types. The LR types, like HPV types 6 and 11, have been associated with non-malignant conditions like skin and genital warts; respiratory papillomatosis; or other non-cancerous lesions. The HR types, like types 16 and 18, are found to be linked to cancer development especially cancers of the cervix and other anogenital as well as head and neck regions [3].

1.2 HPV life cycle:

HPV is considered the most common sexually transmitted virus, and sexual contact is the most frequent route of transmission [4]. The location of the HPV-associated cancers may be related to the type of sexual activity. Oral sexual
contact pattern may increase the risk of developing HPV-positive head and neck cancer (HNC). A similar correlation can be proposed for cervical and other anogenital cancers. Increasing the sexual activity; starting sexual life at early age; and/or having multiple sexual partners all are factors that increase the risk of getting infected. However, transmission from infected mother to fetus/baby during pregnancy/delivery or through lactation has also been documented but the risk and the frequency are much lower than the sexual route [5,6].

HPV has a tropism to infect epithelial tissue with preference to the stratified epithelium. The multilayer architecture and the polarity of the tissue are essential components of the viral productive life cycle [Reviewed in 7]. Infection is usually acquired through micro-wounds in the epithelium with a predilection to infect the basal layer as it keeps a low rate of divisions and this will help the virus escape the immune surveillance and to produce long term infection [Reviewed in 8]. HPV binds to heparan sulfate proteoglycans found in the cell membrane [9,10]. This will be followed by conformational alterations in the capsid protein to deliver the virus into the cell then introducing its genome into the nucleus [11]. Unlike other viral infections whose viral amplification occurs in the same infected cell, HPV infects cells of the basal layer, but its amplification occurs in the suprabasal layer depending on host cell DNA synthesis to produce its progeny [12]. HPV reprograms the suprabasal differentiated cells to re-enter the S phase of the cell cycle to keep it under proliferation then the new virions shed from the epithelial surface [3,13]. This process is called the productive life cycle of the virus where its genome is maintained as extrachromosomal episome rather than integrating into
the host cell genome and it is responsible for the development of non-cancerous lesions [14,15] (figure 1.1).

However, sometimes HPV especially the HR types integrate into the genome of basal layer cells. The integration disrupts the viral genome resulting in increased expression of the viral oncogenes and stabilization of their oncoproteins which drive transformational changes in the host cell [17-19]. Sometimes, the presence of episomal genome in the same cell that has an integrated viral genome enhances the expression of the integrated genome which could further push forward the transformational potential [20]. Although the productive life cycle of the virus prefers an infection of a multilayered differentiated epithelium, HPV-associated cancerous lesions are usually seen in areas with an atypical organization that are more vulnerable to transformation changes. Examples of

*Figure 1.1: The HPV life cycle.* The model on the left represents the productive life cycle of HPV. Infection occurs through microabrasions in the epithelium to the basal layer cells. Then, through timed and leveled gene expression switching among the viral genes, a new progeny of viruses will be formed that sheds from the apical layer. The picture on the right side shows the stages of HPV-induced malignant transformation. There is no productive pattern, but oncogenes expression throughout the epithelium with resultant dysplasia/invasive carcinoma. Figure reproduced from Thomas et al, *Oncogene* 2008, 27, 7020 [16].
these regions are the transitional zone of the cervix; the dentate line of the anus where the squamous and columnar epithelia meet; and the reticulated epithelium in of the palatine tonsillar crypts [21-23]. However, HPV HR types can cause cancer in other sites like the endocervix proper, nasopharynx, larynx, and others.

1.3 HPV clearance and persistence:

There are more data collected concerning cervical cancer if compared to other HPV-associated cancers. Most infections occur around the time of puberty and the start of the sexual life though infection could happen anytime later in life [Reviewed in 24]. This could be linked to the hormonal changes and the endocervical epithelial damage caused by exposure to the acidic vaginal secretions.

Figure 1.2: The natural history of cervical cancer. Schematic representation of HPV-positive cancer as an example of HPV-associated cancers. Most of the cases undergo clearance within the first 2 years. Persistent infection may progress to low-grade dysplasia where the majority of the cases may regress. When the dysplasia develops into high grade, it is more likely to progress to invasive cancer. Figure reproduced from Pagliusi et al Vaccine 23 (2004) p572 [38].
The lifetime risk of getting infected is 80-90% [25]. About 90% of the HPV infections are asymptomatic transient infections that will clear spontaneously within a period of 2 years from the time of acquisition [26,27]. The remaining 10% will continue as a persistent infection that may develop into low-grade dysplasia. 80% of low-grade dysplasia will regress spontaneously and resolve and the remaining percentage will proceed into high-grade dysplasia [25]. Again, although high-grade dysplasia is considered a pre-cancerous condition, only less than a half of the high-grade dysplasia cases may develop into cervical cancer over a total period of 5-30 years from the time of infection [28-30].

The overall risk of developing cervical cancer from an HPV HR infection is only 0.3-1.2% [31]. At any given time, 12% of all women are HPV-infected with a much higher percentage in young age [32] (figure 1.2). Risk of HPV infection/cancer development can be reduced by prophylactic vaccination, personal hygiene, male circumcision, and better awareness [33,34]. On the other hand, immunodeficiency, smoking, oral contraceptive use, and chlamydial infections may increase the risk [31, 35-37].

1.4 The HPV genome and its products:

The HPV genome is divided into 3 functional regions: the Upstream Regulatory Region (URR), also known as Long Coding Region (LCR) (1 kb in size); and 2 coding regions designated as Early (4 kb) and Late (3 kb) which contain the Open Reading Frames (ORF) [39,40]. The URR contains the sequences that regulate the ORF gene expression. It contains the early promoter which in HPV16 is located at p97, while the late promoter is contained within the E7 ORF sequence.
at p670 [41]. The Early (E) and Late (L) genes have been numbered according to the size of the ORF with number 1 referring to the largest one [40]. HPV encodes 6 Early and 2 Late gene products. E1 has a DNA helicase activity, and along with E2, it plays an important role in viral genome replication. Additionally, E2 regulates transcription of other genes. E2 represses E6/E7 gene transcription and a release of this repression occurs during integration that disrupts E2 yielding to the transformational potential of the virus [3,18,42]. Actually, E2 can cause senescence in HPV-positive cancer cells if it is re-introduced into cells with only integrated viral genome [43]. There is no E3 in the HPV16 genome.

Figure 1.3: The HPV 16 genome. The HPV16 genome can be considered a typical example of an HPV genome. It consists of early and late regions and the URR. E1 and E2 are involved in viral replication and transcription. E2 also plays a role in regulating transcription of the early genes, mostly inhibitory action. E6 and E7 are oncogenes and play other roles in the normal viral life cycle. The late region encodes for L1 and L2 proteins which are respectively the major and minor capsid proteins. The Upstream regulatory region (URR) contains promoter and enhancer elements and the origin of replication Figure reproduced from Stanley M, Clinical Microbiology Reviews Apr 2012, 25 (2) 216; DOI: 10.1128/CMR. 05028-11 [81].
The E4 protein disintegrates the cellular cytoskeleton to release the virus from infected cells [44] (Figure 1.3). The E4 sequence varies considerably among different papillomaviruses which is thought to be involved in viral tropism [45]. E5 protein augments the proliferative action of E6/E7 protein with a possible role in driving the malignant transformation [46]. However, some resources say that E5 sequence is often deleted during integration which may mean that E5 is not important in late-stage carcinogenesis [47].

The E6 and E7 oncoproteins have a long list of actions that are important in the context of HPV-mediated transformation. E6 and E7 are small proteins of 21 and 17 kDa, respectively [48,49]. The hallmark action of E6 is its interference with the tumor suppressor protein p53 with a subsequent abrogation of apoptosis; release of cell-cycle arrest; and accumulation of DNA damage [50]. E6 can ubiquitinate and degrade p53 with the help of E6-associated protein and E3-ubiquitin ligase [51]. E6 can also export p53 from the nucleus or disrupt its nuclear localization signal, making it unavailable to exert its role [52]. Moreover, E6 manipulates the p53-DNA binding sites to decrease p53 transcriptional activity [53,54]. However, not all of the p53 is degraded in an E6-expressing cell [55-58].

Our lab has also shown that p53 level still increases in response to UV radiation of HPV16 transformed cells [59]. Furthermore, E6 promotes proliferation and immortalization by enhancing the transcription of telomerase reverse transcriptase (hTERT) which adds hexamer repeats to the telomeric ends of the chromosomes [49, 60]. This action in addition to E7 role on Retinoblastoma protein allows for an unlimited proliferative potential of the HPV HR infected cells [61,62]. E6 can induce
chromosomal abnormalities through inactivation and degradation of human minichromosome maintenance 7 (hMCM7) whose function is to prevent more than one round of DNA replication during a single cycle [63,64]. Loss of p53 will result in loss of the G1 and G2 checkpoints that result in chromosomal aneuploidy driven by continuous proliferation [65] (figure 1.4).

On the other hand, the E7 protein has another set of interactions. The hallmark role of E7 is inactivation and degradation of the tumor suppressor retinoblastoma protein (Rb) and its family members p105, p107 and p130 [66]. E7 has no DNA binding capacity, but it phosphorylates/inactivates Rb releasing the transcriptional factor E2F that will enter the nucleus to mediate E7 proliferative functions and drive the cell cycle [67-69]. Additionally, E7 suppresses the activity of p21/p27 releasing CDK2 and stabilizes it, which also drives the G1-S phase of the cell cycle [70,71]. E7 contributes to genomic instability by enhancing centrioles amplification and chromosomal aneuploidy resulting in further accumulation of genetic damage [72]. These DNA damages and stress stimuli would activate p53; however, E6 shuts down this effect by keeping p53 levels low and by inhibiting p53 activity [73-75]. HR E7 also has a telomere lengthening activity that further contributes to the immortalization potential [76] (figure 1.4).

E7 alone has some transforming capacity while E6 doesn’t have it when it is alone; however, when they are together, their effects are synergistic [77]. In an experiment to show the effect of E6, E7 and both E6/E7 in transgenic mice model, E6 transgenic mice exhibited tongue and esophageal cancer at a very low rate. E7 and E6/7 transgenic mice developed cancer in 95% of the cases, but the E6/E7
mice had high-grade carcinomas and multiple invasive lesions [78]. Despite their widespread interactions, E6/E7 proteins are essential, but not sufficient for HPV-associated cancer development and require other events to promote their transformational actions [78-80].

Lastly, the Late coding genes, L1 and L2, encode for major and minor capsid proteins [3]. Every viral coat consists of 360 L1 molecules arranged in repetitive groups of 5 molecules called capsomeres [81]. All of E1, E2, L1, L2 are well conserved among all papillomaviruses [3] (figure 1.3).

Figure 1.4: Molecular pathways affected by HPV-oncoproteins. This diagram is applicable to all HPV-associated carcinogenesis. High-risk HPV E6 inhibits apoptosis; deregulates proliferation and differentiation; and induces immortalization. High-risk E7 enhances cellular proliferation by abrogating cell cycle checkpoints and induces genomic instability. Both E6/E7 can inhibit tumor suppressor genes, and drive transformation and carcinogenesis. Figure reproduced from Gupta et al Journal of Biomedical Science (2019) 26:28 P11[83].
1.5 HPV-associated cancers:

1.5.1 Head and neck cancer:

Head and neck cancers make up 3.5% of all cancers in the United States and Europe [84], and the 6th most common cancer worldwide [85], with a global annual incidence of 600,000 [86,87]. Tobacco smoking and alcohol consumption are the 2 classical risk factors for head and neck squamous cell carcinoma (HNSCC). In the last 30 years, there was an increasing trend for oropharyngeal squamous cell carcinoma (OPSCC) with the discovery of HPV as an evolving risk factor [88-90]. HPV association with head and neck cancers varies according to the anatomical site with the highest contribution (90%) in the oropharynx (tonsillar bed) [91-93]. Moreover, HPV16 prevalence in HPV-positive OPSCC is about 90% followed by HPV18 (3%) [94,95].

There are marked differences between HPV-positive and HPV-negative tumors in various aspects. Morphologically, HPV-positive cancers tend to be non-differentiated non-keratinizing basaloid carcinoma [82,96,97], with regional lymph nodes involvement [82,98]. In contrast with HPV negative tumors, HPV-positive tumors occur more commonly in younger age patient with no history of smoking or alcohol consumption [99,100]. There is no field cancerization in the tissues that surround the HPV transformed lesion [82] which may be linked to the younger age of the patients at the time of diagnosis that means less accumulation of mutations. The HPV-positive patients have a better prognosis [98,101] that could be linked to their younger age with less comorbidity; better immune reaction [102]; better
sensitivity to chemo-radiotherapy [103]; and lower incidence of p53 mutations and field cancerization [104].

Recently, our lab and others found that not all HPV-positive head and neck cancers are expressing the HPV-oncogenes E6/E7. This group was designated as HPV-inactive (HPV DNA + | E6/E7 mRNA -), to distinguish it from the HPV-active (HPV DNA + | E6/E7 mRNA +); and HPV negative tumors (HPV DNA - | E6/E7 mRNA -) [105-107] (table 1.1). Interestingly, the gene expression profile varies among these 3 cancer types. In HPV-active tumors, there is an increased expression of genes involved in cell cycle progression, cell division, proliferation, DNA replication, and DNA repair giving a picture of “proliferative profile”. In HPV-negative cancers, the genes that are involved in motility, migration, epithelial-mesenchymal transition, and angiogenesis are usually overexpressed which give an invasive and metastatic signature. Distinctly, HPV-inactive cancers vary from the HPV-active ones and they have a gene expression profile that is close to, but not identical, HPV-negative cancers [106] (figures 1.5, 1.6).

**Table 1.1: Updated HPV-associated tumors classification.**

<table>
<thead>
<tr>
<th>Tumor classification</th>
<th>HPV DNA</th>
<th>HPV E6/E7 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>HPV-positive active</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>HPV-positive inactive</td>
<td>Positive</td>
<td>Negative</td>
</tr>
</tbody>
</table>
Figure 1.5: Gene ontology, HPV-active versus HPV-negative OPSCC samples. The HPV-active tumors have a higher expression of genes involved in the growth cell cycle, and mitosis “proliferative signature” (red bars). This signature is absent in HPV-negative tumors, which are characterized by higher expression of genes involved in cell motility, extracellular matrix, angiogenesis, and EMT (blue bars) Reproduced from Tomar et al Head Neck. 2016 p18[106].

Figure 1.6: Gene ontology analysis of gene expression changes in HPV-active, -inactive and -negative tumors from European Americans (EA). HPV-inactive tumors lack the "HPV signature" typical of HPV-active tumors, however, they share many but not all the features of HPV-negative tumors, lacking changes in a whole class of pathways including cell projections, leading edge, adherence junctions. This finding suggests the gene expression signature of HPV-inactive tumors is intermediate between that of HPV-active and HPV-negative Reproduced from Tomar et al Head Neck. 2016 p18 [106].
1.5.2 Cervical cancer:

HPV is responsible for almost all cases of cervical cancer [108]. Although cervical cancer is a rare long term sequelae of HR HPV infection, it has a large burden on human health. Despite the decreasing incidence in the developed countries, it ranks the 2nd most common cancer in women world-wide due to the lower surveillance and the higher incidence in the developing and low-income countries [109]. The annual global incidence is over 500,000 with annual mortality that exceeds 250,000 [109]. Similar to what we found in the head and neck cancers, Banister et al have found that 8% of HPV-positive cervical cancers have only the viral DNA with no E6/E7 transcripts (HPV-inactive) [110].

Figure 1.7: Difference between HPV-inactive and HPV-active in age at the time of diagnosis (A) and survival (B). HPV-inactive cervical cancer patients (solid blue) are 9 years older at the time of diagnosis and have a shorter survival duration (6.4 years less) than HPV-active patients (dashed pink line) Figure reproduced from Banister et al. Oncotarget, 2017, Vol. 8, (No. 8), pp: 13375-13386 [110].
Also, there is an obvious discrepancy in terms of clinical feature, gene expression, mutations, and epigenetics. HPV-inactive cervical cancers occurred in older women and had poorer survival (figure 1.7). Morphologically, adenocarcinomas and adenosquamous carcinomas were more common in the HPV-inactive lesions. The HPV-active tumors showed higher expression of E2F target genes, and increased AKT/mTOR pathway. Interestingly, the gene expression profile of the HPV-inactive tumors revealed an increased expression of WNT/β-catenin pathway. On the other hand, HPV-inactive tumors contained a significant amount of somatic mutations compared to HPV active tumors especially involving p53, WNT, and PI3K signaling [110].

1.6 Tumor suppressor p53:

p53 was the first identified tumor suppressor protein [111]. It is estimated that 50% of tumors have loss-of-function mutations in p53 gene with the remaining half has a certain level of p53 functional inactivation produced by viral proteins or changes in the interacting pathways [111-113]. WT p53 is required for the proposed action of chemotherapeutic drug and p53 mutation plays a role in developing chemoresistance [114,115]. p53 is normally inactive and becomes active when the cell is stressed or damaged. DNA damage, abnormal oncogenes activity, or exposure to chemotherapeutic drugs and UV light are examples of these stressful events [111]. p53 activation occurs by stabilizing the protein and releasing it from MDM2 inhibitor either by modifying p53- binding site, or by sequestering MDM2 and making it unavailable for p53 binding [116,117] (figure 1.8). This activation results in various downstream pathway interactions. It can
arrest the cell cycle by halting the G1-S and G2-M transitions by stimulating the expression of p21 which is an inhibitor of cyclin-dependent kinases [118].

p53 plays an important role in the DNA repair mechanisms through the induction of genes that are involved in nucleotide excision repair and chromosomal recombination, hence, it protects the cell from the accumulation of genetic errors and maintains the genetic stability [121,122]. If the damage is un-repairable, p53 will direct the cell into apoptosis through several mediators like Bax protein, a prototype of pro-apoptotic proteins [123], or by stimulating the production of the mitochondrial highly toxic reactive oxygen species [111]. Furthermore, p53 can hinder tumorigenesis by stimulating the expression of genes that interfere with this process [118, 124]. Therefore, we see in the HPV-negative and HPV-inactive tumors where p53 is often mutated, the genes that are involved in angiogenesis are over-expressed compared to that of HPV-active tumors where p53 mutation is an infrequent event [106]. For all these actions, p53 was named the “guardian of the genome” [119,120] (figure 1.8).

Most of the p53 mutations are missense mutations. They are usually classified as conformational mutations that are most significant when the DNA-binding site is altered resulting in the inability of p53 to bind its target gene promoters [125]. The deleterious effect of p53 mutations is not confined to the loss-of-function, but also to gain-of-function effects. Mutant p53 may sequester other tumor suppressor proteins including p53 itself, p63 and p73 which further decreases the apoptotic capacity of the cell [126]. Moreover, the gain-of-function property of mutant p53 causes the protein to acquire carcinogenic properties.
The important pathway in this context is its interaction with other transcriptional factors including NF-κB, SMAD, Sp1, and others [129]. Consequently, loss of p53 function promotes cancer cell survival, tumor growth, invasion, metastasis, and chemoresistance [130-132]. Additionally, mutant p53 is highly stable due to the conformational change that renders it misfolded which results in micro- and macro-inclusion bodies in the cells that could become toxic to the cells when they aggregate because they resist proteasomal degradation [133-136]. Targeting mutant p53 or its connections could be an important target for anticancer therapy [129].

Figure 1.8: The p53 network. Stimuli like DNA breaks, oncogenes activation, and UV exposure activate P53 either by modifying it or by inhibiting the MDM2 negative regulator. P53 activation will result in widespread downstream interactions with different pathways to induce growth arrest, apoptosis, or anti-angiogenic effect. As negative feedback, p53 activation stimulate MDM2 gene which inhibits further activation of p53 in the absence of additional stimuli. Figure reproduced from Vogelstein et al. Nature 2000; Vol. 408 [111].
1.7 HPV and DNA methylation:

Epigenetic modifications may serve as a key factor in cancer initiation and progression [137,138]. In general, DNA methylation suppresses genes expression; therefore, tumors usually have global hypomethylation with hypermethylation of CpG islands of tumor suppressor gene promoters [139,140]. Higher methylation profile in the genes of cell cycle and differentiation in the epithelial stem cell is usually observed compared to the differentiated supra-basal cells [141]. Differential methylation processes occur in viral and cellular genomes [141]. Cervical cancer often has aberrant methylation leading to suppression of tumor suppressors and activation of oncogenes [142]. As we mentioned earlier, E6/E7 expression is regulated by E2. E2 binding to E2-binding sites (E2BS) in the viral URR regulates E6/E7 expression in a differential pattern. At low levels, E2 binds to the high-affinity E2BS 1 with a subsequent increase in promoter activity and E6/E7 expression. At higher levels, E2 binds the low-affinity E2BS 3 and 4 promoting the down-regulation of URR promoter activity and decreasing E6/E7 expression [143].

Although integration of the HPV genome disrupts E2 gene and releases the inhibition of E6/E7 expression, methylation of the E2BS may substantially precede this step [144,145]. According to the literature, methylation of the E2BS 1 results in recruitment of transcriptional complex with increased promoter activity and E6/E7 expression [146]. On the other hand, methylation of E2BS 3 and 4 inhibits E2 binding, hence abrogating promoter inhibition and subsequently the E6/E7 expression will increase [143,147,148]. In other words, methylation will enhance
HPV-oncogenes expression with suppression of the cellular tumor suppressor genes by hypermethylation of their promoters [149]. However, our data show a different story that may bring to light new pathways involved in the context of HPV-inactive cancers. HPV-inactive lesions often have global DNA hypomethylation with hypermethylation of promoter-associated CpG islands [110].

1.8 HPV and SIX1:

SIX1 is a DNA-specific transcriptional factor that belongs to SIX superfamily of homeobox genes. SIX genes are master regulators of various downstream pathways that are involved in embryogenesis and organogenesis [150-153]. Aberrant activity of SIX1 might cause tumorigenesis including cancers of breast, cervix, ovary, liver, kidney as well as skeletal muscle [154-159]. SIX1 overexpression in malignant cells promotes cell proliferation, survival, and invasiveness which lead to increased cancer aggressiveness, metastasis, and poorer outcome [156,159-162]. Also, SIX1 can induce epithelial-mesenchymal transition by down-regulation of the epithelial-related genes like E-cadherin and upregulation of the mesenchymal markers like N-cadherin and vimentin [163,164]. This action is mediated through SIX1 influence on TGF-β, MAPK, WNT signaling [165,166].

SIX1 strongly correlates with HPV E7. This relationship was tested in an in vitro model of HPV16 transformed human keratinocytes (figure 1.9) [167,168]. The model starts with primary human keratinocytes (HKc) that were transfected by HPV16 DNA to produce early passage HPV16 transformed keratinocytes (HKc/HPV16) that have a high proliferative capacity. Through selection in medium
containing fetal bovine serum (5%) and high calcium (1 mM) these cells were transformed into differentiation-resistant transformed human keratinocytes (HKc/DR) which are not yet tumorigenic but have unlimited potential for proliferation. SIX1 level increases as the cell passes from HKc to HKc/HPV16, then to HKc/DR and HPV16 E7 levels increase as well. This is thought to occur because HPV E7 inhibits Rb, releasing E2F which stimulate the expression of SIX1. When SIX1 was over-expressed in HKc/HPV16, the cells were pushed to be HKc/DR [169]. Interestingly, SIX1 over-expression in the HKc/DR stage of this cell line drives the cells to become tumorigenic when they are injected into nude mice [166], a similar effect to oncogenic Ras or Herpes simplex virus 2 (HSV2) transfection [169-171].

Figure 1.9: In vitro model of HPV16 mediated transformation of human keratinocytes. A schematic representation of our cell line development with the related variation in HPV oncoproteins and SIX1 levels as well as the switching in TGF-β signaling as cells progress through these steps.
TGF-β signaling through the canonical/Smads pathway decrease Ski level which is necessary to boost the activity of URR by its interaction with NF1. This, in turn, will inhibit the URR activity, hence, it will inhibit E6/E7 expression. The decrease in Ski level enhances the HKc/HPV16 sensitivity to the growth inhibitory action of TGF-β [172]. SIX1 promotes the pro-tumorigenic function of TGF-β and inhibits its anti-proliferative role [160]. SIX1 causes switch of TGF-β signaling from the canonical pathway to the non-canonical pathway promoting E7 expression and development of TGF-β insensitivity and EMT process [165,166,173].

1.9 Hypothesis and rationale:

Human papillomavirus is responsible for 5% of all human malignancies [174]. HPV E6/E7 oncogene products are responsible for the transforming potential of the virus through a series of molecular interactions. It is widely believed that continuous E6/E7 expression in HPV-positive cancers is indispensable to maintain the cancerous cell phenotype. Surprisingly, about 8% of HPV-positive cervical cancer [110], and up to 30% of HPV-positive oropharyngeal squamous cell carcinoma (OPSCC) are HPV-inactive [106] which means they don’t express these oncogenes. As we have mentioned earlier, the gene expression profile of the HPV-inactive cancers is different from the HPV-positive active tumors (which are characterized by higher expression of the genes involved in cell proliferation) and is similar, but not identical, to that of the HPV-negative tumors, (characterized by higher expression of genes involved in cell motility, angiogenesis and EMT) [106]. Interestingly, the HPV-positive primary OPSCC tumors tend to be HPV-active while recurrent or metastatic HPV-positive tumors in the same patients
usually are HPV-inactive [105]. Contrary to the widely held belief that HPV existence in the HPV-inactive tumors is merely a coincidental infection with no influence on the carcinogenesis, we think that HPV-inactive tumors may arise from HPV-active lesions that lose E7 expression during progression.

**We hypothesize that HPV-inactive tumors start as HPV-active lesions, then lose their dependence on the continuous expression of E6/E7 oncogene.** This may be due to genetic and/or epigenetic modifications caused by the genomic instability and the additional carcinogens to which the tumor is exposed. Based on the finding that HPV-inactive cervical cancers often have mutated p53 similar to HPV-negative while HPV-active tumors don’t [110], we proposed that p53 mutation is a critical step for the development of HPV-inactive lesions. Therefore, we investigated the effect of p53 mutation on HPV16 E7 expression. We knocked out the p53 gene in HPV16-transformed differentiation-resistant human keratinocytes to simulate the loss-of-function mutation in the p53 gene. Due to the close positive bidirectional correlation between HPV E7 and SIX1 mentioned earlier, we checked the effect of SIX1 over-expression on the role of p53 mutations in driving the conversion of HPV-active to HPV-inactive lesions.

**1.10 Objectives:**

First, to investigate the role of p53 mutation in suppressing HPV16 E7 expression, as a critical step in the development of HPV-inactive cancers from HPV-active lesions. Second, to study the molecular mechanisms by which these mutations can suppress E7 expression. Third, to determine the significance of other mutations that may associate with the development of HPV-inactive tumors.
CHAPTER 2: MATERIALS AND METHODS

2.1 Cell culture:

Differentiation-resistant HPV16-transformed human keratinocytes (HKc/DR cell line) generated in our laboratory in 1986-87 were used in our experiments [167,168] (figure 1.9). The keratinocytes were harvested from human skin and transfected with recombinant HPV16 DNA. The resulting HPV16-immortalized human keratinocytes were cultivated in the differentiation-resistant (DR) medium that consist of keratinocyte serum-free medium 1X (KSFM) (Life Technologies, cat. 17005042) supplemented with bovine pituitary extract (BPE); epidermal growth factor 1-53 (EGF 1-53); calcium chloride to a final concentration of 1 mM; 5% fetal bovine serum (FBS); penicillin 100 IU/ml; streptomycin 100 µg/ml; and gentamycin 5 µg/ml. Unlike their original primary cells, these cells exhibit differentiation resistance and unlimited proliferative potential (figure 1.9). All the cells were grown in 5% CO₂, 37°C incubation conditions.

We also used the HKc/DR cells that were transfected with SIX1 over-expressing plasmid (HKc/DR-SIX1) to test the effect of SIX1 in our experiment for the close link between SIX1 and HPV16 E7.
2.2 CRISPR-Cas9 system:

The CRISPR-Cas9 system was used to knock-out the p53 gene in HKc/DR and HKc/DR-SIX1 cells to mimic the p53 loss-of-function mutations that are highly prevalent in HPV-inactive cancers. The CRISPR-Cas9 system was designed to excise 186 bp from exon 2 of the p53 gene. Amplification of the CRISPR-Cas9 Up and Down gRNA was done using KAPA SYBR FAST One-Step qRT-PCR Universal (cat. KK4651), Amplifying HR, p53-CRISPR-Up and Down gRNA at final concentrations of 1X, 2.5µM, and 1µg respectively in 20µl reaction volume. The PCR products were purified using Agencourt DNAAdvance kit (Beckman Coulter, cat. A48705) according to the manufacturer protocol. Lipofectamine 3000 kit (Invitrogen cat. L3000-008) was used for transfection according to the manufacturer protocol. The cells were cultured in two 6-well plates; 1 plate was transfected by CRISPR-Cas9 Up, CRISPR-Cas9 Down gRNAs of the Cas9 gene, and Green Fluorescent Protein (GFP) to final quantities of 300ng, 300ng, and 1000ng, respectively per each well. The other plate was treated with only GFP 1000ng/well as a control.

2.3 Fluorescence-Activated Cell Sorting (FACS):

FACS for GFP signal was performed 48 hours after transfection by the CRISPR-Cas9 reagents. After sorting, limited dilution of the cells (both CRISPR-Cas9/GFP treated and GFP-only treated) was achieved to get 1-2 cell/well of 96-well plate. The cells were fed by DR medium and checked over 2 weeks to look for the wells that gave a single colony growth pattern which indicates they have emerged as a clonal proliferation of single cell to get a pure clone. When the
selected wells became confluent, they were passaged into multiple 96-well rounded bottom plate and BioRad PCR plates replica.

2.4 Characterization of the p53-KO and WT clones:

A. Resazurin viability assay:

To differentiate the wells that have p53 knocked-out (KO) clones from those which still have wild-type (WT) p53, we treated one of the replica plates with nutlin to a final concentration of 20µM and treated the control plate with DMSO. 4 days later, the viability of the cells was assessed using Resazurin Viability Assay. Resazurin sodium salt (Sigma R7017 CAS 62758-13-8 M.W. 251.18) was added to both plates in a volume of 20µl/well for 4 hours at 37°C until the color started changing from blue to pink. Fluorescence was measured at 560/590nm wavelengths, and the ratio of the nutlin-treated plate reading to the DMSO-treated plate reading was calculated for each well. Then the wells were sorted according to the results into 3 groups: 1. those with a ratio of ≥0.8 were considered to be mostly KO wells; 2. those with a ratio of ≤0.3 were considered to be mostly WT, 3. the wells with a ratio whose ratios results were >0.3 and <0.8 were considered equivocal.

B. Genomic DNA qPCR and gel electrophoresis:

KAPA SYBR FAST One-Step qRT-PCR Universal (cat. KK4651); p53-KO-forward primer (5’-CTGGTAAGGACAAGGGTTGG-3’) and p53-KO-reverse primer (5’-GCCAAAGGGTGAAGAGGAAT-3’); and genomic DNA were mixed to final concentrations of 1X, 0.2µM,0.2µM, and 0.5ng/ul, respectively, per 20µl reaction.
This was followed by DNA gel electrophoresis to compare the PCR product size against p53-KO and p53-WT positive controls. Only the wells that gave a single band at the 309 bp or 495 bp length were chosen as p53-KO and WT cell lines which matched the p53-KO and p53-WT positive controls, respectively (figure 2.1).

**Figure 2.1: Gel electrophoresis of p53 KO/WT qPCR products.** Only the samples that gave a single band corresponding to one of the controls was chosen as p53-KO or p53-WT accordingly.

<table>
<thead>
<tr>
<th>p53 gene sequence from ncbi</th>
</tr>
</thead>
</table>
| GCTGGGGGCTGGACTGGCTCTGCACTGCTCTCTCTCACTTACCATCTGCTGCTGGGTGCCCTGGGCACTGTCCTGCTGCTGGGGCAATGGATTTGATCTGTCCCTGGCGGACGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
C. Sanger sequencing:

Sanger sequencing was done to further confirm the success of the CRISPR-Cas9 in deleting p53 and to check for the stability of the deletion after about 50 passage number. Using the same qPCR reagents and conditions mentioned above, p53 gene across the deleted segment was amplified for p53-KO and p53-WT lines. Then the qPCR products were analyzed by Sanger sequencing (Eton Bioscience Inc.) to confirm the deletion happened in the same intended region of exon 2. The results were compared to NCBI p53 gene sequence data (figure 2.2).

2.5 DNA/RNA isolation:

Qiagen AllPrep DNA/RNA mini kit (cat. 80208) was used to extract RNA and DNA from the cell pellets following the manufacturer protocol. The concentration and purity were measured by Implen Nanodrop Pearl.

2.6 cDNA synthesis:

Bio-Rad iScript cDNA Synthesis kit (cat. 1708891), and Bio-Rad iCycler thermal machine were used to make complementary DNA (cDNA) from the RNA template isolated from the cells. 4 µl 5x iScript reaction mix, 1 µl iScript reverse transcriptase, 750ng RNA template, and RNase free water were mixed to a total volume of 20 µl/reaction and converted using the Bio-Rad provided protocol.

2.7 cDNA quantification:

For accurate quantification of the cDNA concentration after reverse transcription, cDNA was hydrolyzed in an aliquot of each reaction by mixing 7 µl
cDNA with 2.1 µl NaOH (1M) and 1.8 µl EDTA (0.25M). The mixture was incubated in the thermal cycler at 70°C for 15 min. Then, 3.5 µl HEPES (2M) was added to it. Quanti-iT OliGreen ssDNA kit (cat. 011492) was used following the supplied protocol. A fluorescence microplate reader was used to measure the fluorescence at 480nm excitation/520nm emission.

2.8 Real-time qPCR:

BioRad iQ SybrGreen Super Mix was used to relatively quantify HPV16 E7 mRNA level in reference to B-actin as an internal control. Primers used are HPV16 E7 Forward (5’-CCGGACAGAGCCCATTACAAT-3’), HPV16 E7 Reverse (5’-ACGTGTGTGCTTTGTACGCAC-3’).

2.9 Spheroid assay:

The cells were grown as spheroid to be analyzed later by RNAscope. The bottom of 100 mm tissue-culture plates was covered by 5ml of 0.5% agarose cushion prepared by making 1:10 dilution of 5% agarose gel mixture in DR medium.

Figure 2.3: Schematic representation of the spheroid assay. Cells were grown on agarose cushion to form a 3-dimensional-spheroids.
The cells formed floating spheroids rather than adhering colonies. After 3-4 days, the spheroids’ diameters were measured using Lumenera Infinity1 Software and when they exceeded 200µm, spheroids were collected for further examination (figure 2.3).

**2.10 Paraffin-Embedded Spheroid Block Formation:**

After collection, the cellular spheroids were fixed overnight in 1.5ml Eppendorf tube containing 1ml of 2% paraformaldehyde (PFA). Then, a dehydration step followed by replacing the fixative using a gradually increased ethanol concentration (70,80,95,95, 100 and 100% 15 min each). Using a micropipette, the spheroids were transferred to embedding molds filled with xylene for 10 min. The xylene was replaced by molten paraffin. The blocks incubated overnight at 56°C then -4°C before sectioning. Some of the sections were stained by Hematoxylin and Eosin stain and others by RNAscope *in situ* hybridization for HPV16 E6/E7 mRNA.

**2.11 Preparing chamber slides with adherent cells:**

Cells were seeded on chamber slides Nunc Lab Tek II (Thermo Scientific, cat. 154534PK). When they became about 80% confluent, they were washed in 1%PBS and fixed in 2-4% PFA for 30 min. Then a series of washing in 1%PBS with dehydration by imersing them in a gradually increased concentration of ethanol and rehydration again. Pretreatment with hydrogen peroxide and protease III followed it to be ready for ISH.
2.12 HPV16 E6/E7 mRNA in situ hybridization (RNAscope):

HPV16/18 E6/E7 mRNA in situ hybridization (ACDBio RNAscope 2.5 HD Reagent Kit-Brown cat. 322300; ACDBio RNAscope probe HPV16/18 cat.311121) to visualize the HPV16 E6/E7 mRNA expression in sections of the p53-KO and p53- WT HKc/DR derived spheroids. Although the probe can detect both HPV16 and 18, our cells contain only HPV16 genome and so any signal will pertain to it. 2 positive controls were used: RNAscope Hs-PPIB probe (ACDBio cat. 313901) applied to our cell of interest, and HPV16/18 E6/E7 probe applied to CRISPR-untreated HKc/DR cells. 2 negative controls: RNAscope DapB probe (ACD Bio cat. 310043) applied to our transformed cells, and same target probe HPV16/18 E6/E7 applied to normal human keratinocyte taken from newborn foreskin.

These cells were grown on chamber slides using the manufacturer protocol to fix and pretreat cultured adherent cells for single-plex 2.5 Chromogenic assay. Spheroids slides were pretreated using FFPE Sample Preparation and Pretreatment manual for the RNAscope 2.5 Assay Part1 Document No.322452. Then both the adherent cell slides and the cellular spheroid slides were stained using the manufacturer protocol for RNAscope 2.5 HD Detection Reagent-Brown User Manual Part 2 Document number 322310-USM.

2.13 DNA demethylation treatment:

5-Aza-2’-deoxycytidine (Sigma-Aldrich, cat. A3656) was dissolved in DMSO. Three different final concentrations were used (5,10 and 20 µM) to validate the therapeutic effect against its toxic effect. We used DMSO treatment as a
control. Fresh medium with either treatment was added at 0, 24, 48 hours and the cells were collected at 72 hours to be analyzed by Real-time qPCR for HPV16 E7 expression.
CHAPTER 3: RESULTS

We were able to isolate and grow 2 lines of each of p53-KO and p53-WT HPV16-transformed differentiation-resistant human keratinocytes (HKc/DR). At early passage number (<5), HPV16 E7 mRNA levels in the p53-KO cells decreased by 25-50% from that of p53-WT control (figure 3.1). Since we had previously observed that E7 level increases with increasing passage number in HKc/DR cells, we accelerated the process by culturing only 5000 cells in 100 mm cell-culture plates and kept passaging them when confluent. After 10 through 14 such passages, we collected cells, extracted RNA, and ran the RT-qPCR again for HPV16 E7. We noticed an increase in the level of E7 mRNA in both the p53-KO and WT lines. However, the expression was significantly lower in the p53-KO lines and the gap between the p53-KO and WT lines got increased with increasing the passage number (figure 3.2). This indicates that there is inhibition of the HPV16 E7 expression associated with knocking out p53 gene.

Figure 3.1: RT-qPCR HPV16 E7 of p53-KO and WT HKc/DR at early passage number. At low passage number (P<5), E7 mRNA in the p53-KO HKc/DR is lower than its level in the WT counterpart but the difference is not significant.
To explain the mechanism of inhibition of E7 expression in cells with p53KO, we postulated an epigenetic modification could have happened to the p53-KO HKc/DR cells. Therefore, we treated the cells with 5-Aza-2’-deoxycytidine, a DNA demethylating agent. We found that E7 expression in the p53-KO HKc/DR cells increases with increasing concentrations of 5-Aza-2’-deoxycytidine from 5 to 10 to 20 µM final concentration in the growth medium (figure 3.3). We compared the E7 expression results to that of DMSO-treated p53-KO HKc/DR cells as a negative control and all were compared to p53-WT lines at the same passage number to exclude variability error that could happen when comparing different samples at different passage number of cell growth. We noticed that the level of
E7 mRNA is directly related to the dose of 5-Aza-2'-deoxycytidine and the expression level of E7 in the 20 µM-treated cells is approaching the E7 level of the p53-WT. This means that a methylation process is affecting the HPV16 genome, inhibiting expression of the E7 gene.

RNAscope HPV16 E6/E7 *in situ* hybridization data obtained from p53-KO and WT cellular spheroids were confirmatory and explanatory to the RT-qPCR results. We found that in the p53-KO lines, some spheroids completely lost their HPV16 E6/E7 mRNA while others have a mixed pattern (figure 3.4). This finding suggests that loss of E7 expression occurs in clones of cells, not in the entire population of p53-KO cells. We didn’t see this variability in the p53-WT lines which exhibit a uniform distribution of HPV16 E7 mRNA throughout the spheroids. This
finding confirms that there is an inhibition of E7 expression, but it is a relative rather than an absolute suppression due to a heterogeneous population of cells.

Similarly, minimal and “patchy” E7 expression was noticed in p53-KO and HKc/DR cells that were grown adherent on glass slides, in contrast with the robust and almost ubiquitous signal detected in p53-WT HKc/DR (figure 3.5).

Figure 3.4: HPV16 E6/E7 mRNA in situ hybridization of spheroid grown p53-KO/WT HKc/DR cells. In the p53-KO lines (A) (B), some spheroids are completely lacking E6/E7 mRNA while others have a partial to total distribution of oncogenes mRNA. In the p53-WT lines, (C)(D) there is a uniform distribution of E6/E7 expression in all cellular spheroids.
Figure 3.5: HPV16 E6/E7 mRNA in situ hybridization of adherent p53-KO/WT HKc/DR cells. All cells are grown as 2D adherent culture. (A) p53-WT cells show robust E6/E7 mRNA expression. (B) p53-KO cells have minimal E6/E7 expression. (C) RNAscope supplied positive control Hs PPIB. (D) RNAscope supplied negative control DapB

Morphologically, we found 2 different patterns of cell growth in both the p53-KO and WT lines cultured at very low density to allow for the formation of monoclonal colonies (figure 3.6). A dispersed morphology, where the cells exhibit a sparsely scattered pattern that gives the colonies a satellite shape, and the coherent pattern, where the cell group together in a tight pattern forming round or oval colonies. We determined the number of colonies of each pattern in each line after fixing the cells in methanol and stained with Gentian violet. We found that
dispersed colonies are more common in the p53-KO lines than in the WT lines. The ratio of the average percentage was KO:WT; 70%:30%. Cells in dispersed

Figure 3.6: Dispersed and coherent colony pattern. (A) Dispersed colony pattern (B) Coherent colony pattern (C),(D),(E) and (F) HPV16 E7 mRNA in situ hybridization in the p53-KO dispersed; p53-KO coherent; p53-WT dispersed; and p53-WT coherent, respectively.
colonies are more likely to have a spindle morphology rather than the usual ovoid-polygonal shape.

To investigate the status of HPV16 E7 expression in each colony pattern, we isolated these phenotypes by growing the cells in a limited dilution to get 1 cell/well of 96 well plates. After observing the pattern of their growth, we were able to get clonal proliferation for each colony pattern and p53 knock out status. However, the RT-qPCR results showed that the E7 expression was not markedly different between the p53-KO and WT lines in relation to colony pattern (data not shown). We also ran an HPV16 E6/E7 in situ hybridization for these colonies, but there was no significant variation in E7 mRNA distribution (figure 3.6).

Because of the close link between E7 and SIX1, we tested the effect of SIX1 over-expression on the observed difference in E7 expression between p53-KO and WT lines. We tested the effect by knocking out p53 in HKc/DR cells that were transfected with a SIX1 expressing plasmid [164]. We found that SIX1 over-expression prevents inhibition of E7 expression in p53-KO HKc/DR-SIX1 compared to p53-WT HKc/DR-SIX1 (figure 3.7).
Figure 3.7: Effect of SIX1 overexpression on E7 expression in p53-KO cells. Exogenous SIX1 over-expression in the HKc/DR cells prevents inhibition of HPV16 E7 expression in response to p53-mutation both in early passage (A) and passage number 10 (B).
CHAPTER 4: DISCUSSION

4.1 Conclusion:

Loss-of-function mutations in the p53 gene set the stage for the loss of continuous expression of HPV oncogenes in HPV16-transformed cells. When we link this research outcome with the published data about the high frequency of p53 mutations in HPV-inactive cancers, we can conclude that p53 could be a driver in the shifting process of HPV active to inactive lesions. Even though p53 activity is already at a minimum in the HPV-driven tumors due to its inhibition by HPV E6, its mutation has a more potent action. p53 inhibition is dynamic in the HPV-transformed cells and still can respond to stressing events like UV radiation [59]. Loss of p53 alone may not be sufficient, per se, to induce loss of E7 expression, but it is a necessary step.

We had difficulty in isolating cells or spheroids that are completely lacking the E7 signal for further genetic and epigenetic analysis to investigate other pathways that are affected by knocking out p53 in our cells.

We also ascertained that loss of E7 expression may be due, at least in part, to DNA methylation as we have noticed an increase in E7 expression upon demethylating the DNA with 5-aza-2'-deoxycytidine. However, a more in-depth methylation study is needed to determine the exact locations that are distinguishably different in their methylation profile between p53-KO and WT lines.
and the possible mechanism underlying these changes. These results appear to contradict what we mentioned earlier, that methylation of the HPV genome enhances its oncogenes expression [143, 146-149]. However, this discrepancy may highlight the importance of methylation of specific targets in controlling or modulating HPV oncogenes expression. Nevertheless, we still hypothesize that methylation of the HPV16 URR, which contains the sequences that control E6/E7 expression, is part of the mechanism by which cells with deleted p53 silence E7.

The finding that p53-KO HKc/DR cells are more likely to have a dispersed pattern of colony growth and a spindle-shaped morphology suggests that these cells are more likely to have an EMT and cell motility signature. EMT and motility signature is characteristic of HPV-inactive and HPV-negative tumors. Therefore, we may have already driven the HPV16-transformed cells to become independent of the expression of HPV oncogenes by knocking out p53. However, the cells need to be further studied for markers of EMT and gene expression profiles to confirm what we are hypothesizing.

The role of SIX1 in the mechanisms by which HKc/DR-SIX1 become independent of E7 also deserves additional studies, as it was intriguing to find that in HKc/DR cells that overexpress exogenous SIX1, p53-KO does not result in loss of E7 expression.

4.2 Significance:

This project expanded our understanding of the molecular mechanisms that HPV can use to transform infected normal cells into cancerous cells, whether or
not its oncogenes are expressed. HPV-positive OPSCC is more responsive to chemo-radiation therapy [103]. However, sometime chemo-radiotherapy sensitive patients turn to be resistant to it, similar to HPV-negative patients. This can be explained as being due to the emergence of a subpopulation of HPV-inactive tumor cells that have similar characteristics to HPV-negative cancers which are resistant to this treatment modalities. If this is the case, then it would be advisable to check for HPV activity status and not only HPV DNA positivity in these cancers prior to initiating standard antiproliferative therapies.

A better knowledge of the mechanisms by which HPV-inactive cancers arise is also useful in the context of therapeutic vaccines targeting HPV products. Most of the vaccines currently under investigation use the E7 protein as a target [175]. However, as we have seen, a considerable percentage of HPV-positive cervical cancer and OPSCC is HPV-inactive which means there are no E6/E7 proteins. Hence, these vaccines will be ineffective in eliminating HPV-inactive lesions and may even select for HPV-inactive clones in the tumor cell population. This issue should be considered when testing the effectiveness of the vaccine to avoid false negative results. Also, these results mandate the development of therapeutic vaccines that use a different route of interactions to eliminate HPV-inactive tumors.

Last, but not least, if HPV plays a role in the pathogenesis of HPV- inactive cancers, HPV prophylactic vaccines will have a wider range of protection especially in head and neck cancer where the percentage of HPV-inactive tumors is relatively higher and vaccination is not yet routine for males [176].
4.3 Future work:

It is important to get a pure population of p53-KO lines that have no E7 expression for further analysis. We need to study the methylation status of the p53-KO lines and to compare it to p53-WT lines. Genetic analysis and gene expression analysis will be highly valuable in detecting other possible mutations and or changes at the level of gene expression.
REFERENCES


9. Campos SK. Subcellular tracking of the Papillomavirus genome during initial infection: the remarkable abilities of minor capsid protein L2. *Viruses* 2017, 9,


61. Wise-Draper T, Wells S. Papillomavirus E6 and E7 proteins and their cellular 

62. Wentzensen N, von Knebel Doeberitz M. Biomarkers in cervical cancer 

63. Kukimoto I, Aihara S, et. al. Human papillomavirus oncoprotein E6 binds to the 
   Cterminal region of human minichromosome maintenance 7 protein. *Biochem 

64. Kuhne C, Banks L. E3-ubiquitin ligase/E6-AP links multicopy maintenance 
   protein 7 to the ubiquitination pathway by a novel motif, the L2G box. *J Biol 

65. Passalaris TM, Benanti JA, et. al. The G(2) checkpoint is maintained by 

   proteins with the retinoblastoma tumor suppressor gene product. *The EMBO 

67. Huang P, Patrick D, et. al. Protein domains governing interactions between 
   E2F, the retinoblastoma gene product, and human papillomavirus type 16 E7 

68. Boyer S, Wazer D, Band V. E7 protein of human papilloma virus-16 induces 
   degradation of retinoblastoma protein through the ubiquitin-proteasome 


81. Stanley M, Epithelial cell responses to infection with human papillomavirus

Clinical Microbiology Reviews Apr 2012, 25 (2) 215-222; DOI: 10.1128/CMR.05028-11


102. King E, Ottensmeier C, Thomas G. The immune response in HPV+ oropharyngeal cancer. *OncoImmunology* 2014; 3, e27254


175. Wick D, Webb J. A novel broad spectrum therapeutic HPV vaccine targeting the E7 proteins of HPV16,81,31,45 and 52 that elicits potent E7-specific CD8T cell immunity and regression of large, established, E7-expressing TC-1 tumors. Vaccine. 29 (2011) 7857-7866.