6-30-2016

Tumor Suppressor p53 Response To UV Light In Normal Human Keratinocyte Strains From Different Individuals

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by

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Doctor of Medicine
University of Mosul-Iraq, 2010

Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Science in

Biomedical Science

School of Medicine

University of South Carolina

2016

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ACKNOWLEDGEMENTS

First and foremost, I owe the greatest gratitude to my Lord who gave me a lot of blessings that made all this work feasible and come true. Then I am grateful to my country Iraq and my sponsor organization The Higher Committee of Education Development in Iraq for providing me the opportunity to achieve my goal in continuing my studies.

Also, I would like to thank my mentor Dr. Lucia Pirisi-Creek, who inspired me, showed me leniency and offered me her didactic experience to do my research and thesis. I do appreciate all the efforts of my affable lab members: Nella Delva, Maria Hosseinipour, Yvon Woappi and Christian Graves. Thanks for their valuable advice and instructive teaching to do my research.

Great thanks to my wonderful family for their support, praying and guidance through different stages of my life. For them, I wish the best and I am pleased to show them the fruit of their efforts.

Lastly but not the least, I would like to thank my wife who was a real support for me during my study and showed me all the patience, empathy and motivation during my hard times. With her and our son, I will celebrate getting my Master's degree.

ABSTRACT

Human Papillomavirus (HPV) is a causative factor in variety of diseases ranging from simple pathologic conditions to malignant ones like cervical cancer in which HPV is the primary cause in more than 90% of cases. HPV is also associated with other tumors like head and neck squamous cell carcinoma (HNSCC) and the percentage of association varies according to the anatomical site, reaching its highest level (65%) in the oropharyngeal region. HPV proteins E6 and E7 are responsible for the transforming potential of the virus. The principal action of E6 is targeting the tumor suppressor protein p53 for degradation, while E7 inactivates the Retinoblastoma protein (Rb). It is widely believed that E6/E7 expression in HPV-positive cancers is necessary to maintain the cancer cell phenotype, and this appears to be the case in most cervical cancers, in which HPV sequences are present and expressed. However, in HPV-positive HNSCC, HPV E6/E7 may be actively expressed (HPV-active), or not (HPV-inactive). Interestingly, the gene expression profile of HPV-inactive tumor has no 'HPV signature' and at the same time it is similar to but distinct from that of HPV-negative tumors. Further, HPV positive primary tumors tend to be HPV-active while recurring HPV positive tumors usually are HPV-inactive.

Based on the previous information, we developed the hypothesis that ***HPV-positive inactive tumors arise as HPV-active lesions. We postulate that the virus acts as initiator inducing the early transformation changes. HPV-transformed cells may then progress***

to malignancy in the presence of cancer promoters like smoking and alcohol, in an HPV oncoprotein-independent pathway, giving rise to HPV-positive inactive tumors. Based upon what is known about the transforming activities of HPV E6/E7, we made the educated guess that HPV-positive tumors may progress to become inactive if p53 becomes mutated: p53 mutations are very common in HPV-negative tumors, but comparatively rare in HPV positive cancers, where inactivation of p53 is accomplished by E6.

To begin investigating this hypothesis, it was important to further investigate the behavior of p53 in normal and HPV16-immortalized cells. A previous MS student in the laboratory established that p53 levels rise in response to UV treatment not only in normal human keratinocytes (HKc) but also in HKc immortalized by HPV16 (HKc/HPV16) despite the fact that in the immortalized cells both baseline and UV-induced p53 levels are much lower than in normal HKc controls.

In this work, we found that the rise in p53 level in response to UV-induced DNA damage varies in normal HKc from different individuals in terms of both magnitude and duration. The magnitude of the response could be classified as low, moderate, and high (1, 2-3 and 5-6) folds, respectively. In addition, some samples p53 protein levels peaked at 24 h to then decrease, while others kept increasing at 48 h from ultraviolet exposure. In addition, RNA levels of p53 are induced in UV-treated cells. qRT-PCR results have demonstrated a higher p53 mRNA level in most UV-treated samples which suggests that the increase in p53 protein level is due in part to increased p53 gene expression. Morphologically, there were no noticeable differentiating features that allowed for prediction of the cells' p53 behavior from microscopic examination. Also, it was demonstrated that even if p53 expression was suppressed at mRNA level by an shRNA

against p53, the level of p53 protein and mRNA increase in response to UV-induced DNA damage.

These results point to the fact that in HPV-transformed cells, the loss of p53 is not absolute and that p53 responses are still present, although severely reduced in magnitude.

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LIST OF ABBREVIATIONS

AA.....African American

AIS ..... adenocarcinoma in situ

ATM..... ataxia telangiectasia mutated

ATR..... ataxia telangiectasia related

BPE ..... bovine pituitary extract

BSA.....bovine serum albumin

CDK .....cyclin-dependent kinase

cDNA ..... complementary DNA

Chk2.....checkpoint kinase 2

CIN.....cervical intraepithelial neoplasia

DNA..... deoxyribonucleic acid

DR..... differentiation resistant keratinocytes

E6AP .....E6-associated protein

EA ..... European American

EGF ..... epidermal growth factor

ELISA ..... enzyme-linked immunosorbent assay

EMT ..... epithelial-mesenchymal-transition

FBS ..... fetal bovine serum

HKc ..... Human keratinocytes

HKc/DR ..... differentiation resistant, HPV16-transformed human keratinocytes

HKc/HPV16 ..... human keratinocytes immortalized with HPV16 DNA

HKc/HPV16d-1..... human keratinocytes from donor 1 immortalized with HPV16 DNA

HNC ..... head and neck cancer

HNSCC ..... head and neck squamous Carcinoma

HPV..... human papillomavirus

HPVs ..... human papillomaviruses

HR ..... high risk human papillomavirus

L1, L2..... late open reading frames of papillomavirus

LR ..... low risk human papillomavirus

MDM2..... mouse double minute 2 homolog

mRNA ..... messenger ribonucleic acid

NHKc ..... normal human keratinocytes

OPSCC..... oropharyngeal squamous cell carcinoma

P53i ..... pSuper p53 interference microRNA plasmid

PBS ..... phosphate buffered saline

PCR ..... polymerase chain reaction

PV ..... papillomavirus

Rb ..... retinoblastoma protein

qRT-PCR ..... quantitative real time polymerase chain reaction

RNA ..... ribonucleic acid

Scram ..... scrambled plasmid

URR ..... upstream regulatory region

UV ..... Ultraviolet light

VLP ..... virus-like particle

CHAPTER 1: INTRODUCTION

1.1 Human Papillomaviruses (HPVs):

Papillomaviruses (PVs) involve a large group of viruses (about 200 types in humans) that belong to Papillomaviridae family [1]. Various animals like snakes, birds and mammals including humans can harbor these viruses [2]. Papillomaviruses co-evolve with their hosts with minimal cross transmission between hosts of different species and this evolution occurs over long time which may explain the tendency of these viruses to produce long term asymptomatic infection and behave as commensals [3-5]. Human papillomaviruses (HPVs) account for more than 150 types [1] and depending on the variability in the genetic material sequence, they are subdivided into 5 genera: alpha, beta, gamma, mu and nu, with the former three genera comprising the majority group of HPVs [1-2]. All the five genera have a tropism to infect cutaneous tissues and the alpha genus has the ability to infect mucosal tissues, too [2].

All PVs share a common structure of small non-enveloped particles (50-60 nm in diameter) with icosahedral capsid containing circular double-stranded DNA genome of about 8000 base-pairs [6]. HPVs can be further classified into high risk (HR) and low risk (LR) depending on the significance of the diseases they associate with. HR HPVs which are HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 are considered oncogenic types for their association with various cancers, most notably types 16 and 18. LR HPVs

like HPV types 1, 2, 6, and 11 associate with less significant diseases, for instance, skin and genital warts, condyloma acuminatum and respiratory papillomatosis [2].

1.2 HPV genome:

The viral genome consists of three regions: the upstream non-coding regulatory region (URR), and early and late coding regions (figure 1.1) [7]. The URR is responsible for regulating early region gene expression. The early coding region contains the following important genes: E1, E2, E4, E5, E6 and E7. The E1 protein has DNA helicase activity and plays a key role in viral genome replication. E2 also plays role in replication as well as transcription of viral genome, it can extol the cellular gene products and recruit them for the benefit of the virus [2]. The E4 protein is thought to be responsible for the viral tropism because different PV types contain marked variations in E4 sequence [8]. E4 also disrupts the cytokeratin network, which facilitates virus escape from the infected epithelial surfaces and transmission [9].

E5 enhances growth factors signaling pathways which promote cell proliferation [10]. E6 and E7 proteins will be discussed thoroughly later but in general they play the core role in driving HPV infected cells to re-enter the cell cycle and their deregulation can predispose to and promote cancer development and maintenance [8-9]. Late region genes encode for L1 and L2 which are major and minor capsid proteins that are responsible for capsid assembly to package the virions. It is important to mention that E1, E2, L1 and L2 regions are highly conserved among all PV types [2]. HPV lacks polymerases essential for replication, therefore employs the host cell replication proteins for viral DNA synthesis [11].

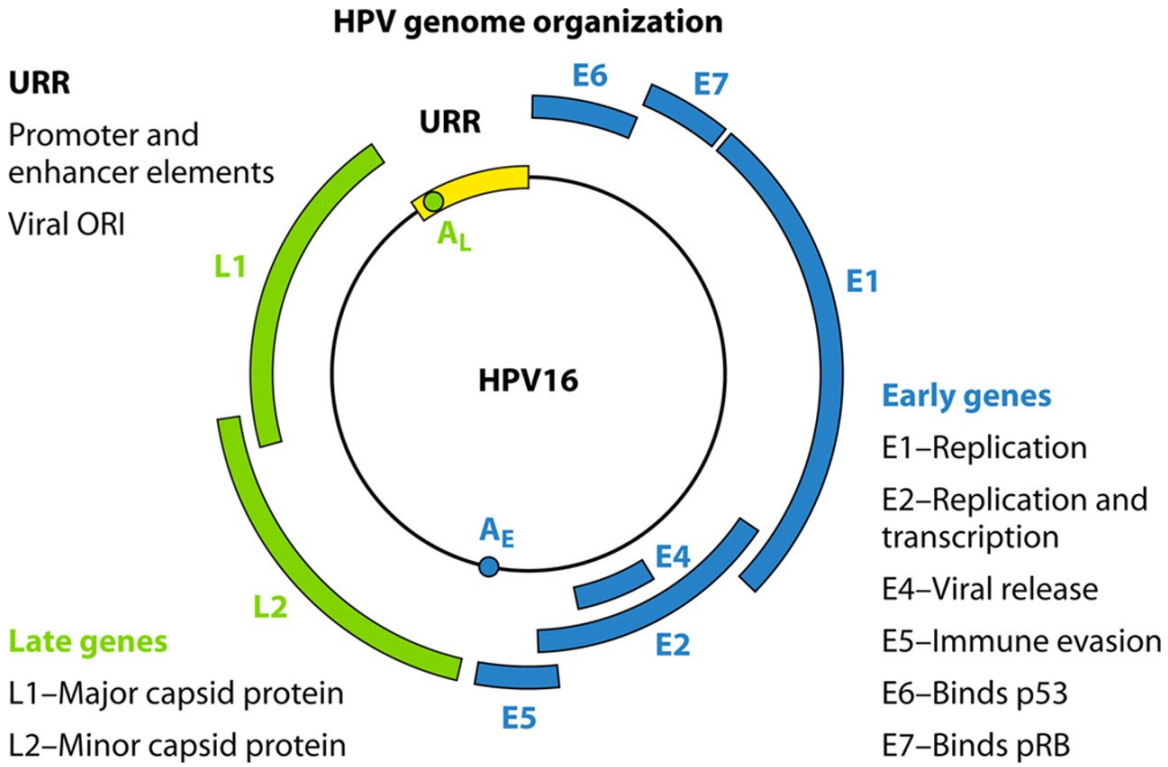

Figure 1.1: HPV 16 genome. The HPV16 genome can be considered a typical example for an HPV genome. It contains early and late regions depending on their time of expression during the viral life cycle. The early region genes E1 and E2 are involved in the viral replication and transcription. E2 also plays a role in regulating transcription of the early genes, mostly inhibitory action. In addition to being oncoproteins, the E6 and E7 gene products play other roles in the normal viral life cycle. The late region encodes 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 [12].

1.3 Mode of transmission:

The most common route of transmission is by sexual contact with carrier. HPV is regarded as the principal example of sexually transmitted infectious agent [13]. Transmission occurs through vaginal, anal and oral sexual patterns and the risk of infection increases with increasing sexual activity, getting sexually active at early age and having intercourse with multiple partners. However, nonsexual transmission of the virus has been also reported.

HPV can be transmitted from infected mother to her baby as he/she passes through the birth canal due to exposure to secretions, amniotic fluid or blood; in addition, in utero transmission could also occur [14]. Furthermore, oncogenic HPV types have been reported to be present in breast milk, but the likelihood of getting the virus by this route is low and therefore there is no reason to recommend against breast feeding. Pasteurization of the milk can abolish any chance for infection via this route [15].

1.4 HPV infection and life cycle:

HPVs have tropism for squamous epithelial tissues. Skin is considered the largest organ and contains a basal layer with stem cell properties and supra-basal layers of differentiated cells that die out as they approach the surface to form a horny layer of dead cells. The basal layer cells can undergo asymmetric mitosis to produce two types of cells, one of them is similar to the original one to maintain a constant population of stem like cells and the other type is differentiating cell to replace the cells that slough off the upper layers [16]. Infection usually occurs through trivial abrasions in the contiguity of the cutaneous and mucosal surfaces with preference to infect the basal stem cells as they keep a low level of cellular replication. HPV (at least LR HPV) benefit from the cell replication driven by wound healing processes (signaling from the local microenvironment) to introduce the viral genome into the host cell nucleus with the help of the L2 protein [17-19]. For HR HPV, E6 and E7 can drive cell proliferation independent of the wound healing process [17]. Both HR and LR HPV E1 and E2 proteins are essential to initiate viral replication [20].

As it is mentioned above, the virus has no DNA amplification machinery and it depends on the cellular pathways to increase viral copy number. Infection of the basal layer

stem cell helps to establish a long term infection as it keeps low copy number that can escape the immune surveillance. Although early infection occurs in the basal layer, viral amplification occurs in the supra-basal terminally differentiated layers whose cells have exited the cell cycle with no further DNA synthesis [reviewed in 21]. Therefore, HPV needs to redirect the cellular pathways of these differentiated cells to drive cell to re-enter the cell cycle [16]. In productive HPV life cycle, viral genome amplification occurs as the cell are pushed towards the surface and the viral genome remains as episome without integration [22-23]. Then, as viral late genes L1 and L2 are expressed, capsid proteins will be formed and assembled into capsids to produce new progeny that shed from the surface and spread to infect other cells [2]. The productive cycle can produce non-cancerous lesions like warts. HR HPV have an alternative life cycle, where HR HPV genome integrates into the host cell genome at sites of genetic damage [24-25].

Integration is thought to be crucial for the carcinogenic transformation of HR HPV infected cells as it was shown that most HPV18 and half of HPV16 produce integrated genome [11]. This can be explained by the fact that integration disrupts E2 expression which usually inhibits the expression of other early region gene products like E6 and E7 to keep copy number under control [26]. Hence, E6 and E7 oncoproteins levels will increase with increasing the transformation potential. Furthermore, E6 and E7 messenger RNAs produced by integrated genomes are more stable than those produced by episomal genome [27]. Lastly, there are studies state that presence of both integrated and episomal genome in the same cell may enhance carcinogenesis as episomal E1 and E2 can promote integrated HPV gene expression [28]. The HPV life cycle is recapitulated and schematically illustrated in Figure (1.2).

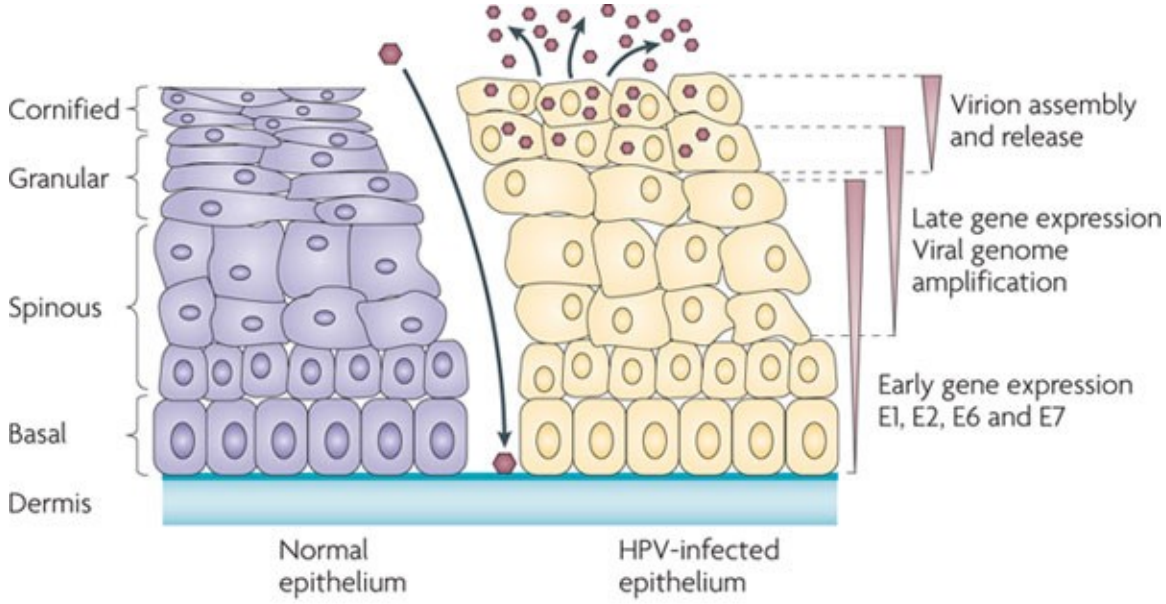

Figure 1.2: Human papillomavirus productive life cycle. The productive life cycle of HPV starts when the virus infects epithelial basal layer keratinocytes through microwounds. As the infected keratinocyte proliferates and differentiates as it migrates towards the surface, the virus initiates the expression of the early and late genes with the expression of E6 and E7 which drive the differentiating cell to go through S phase to replicate its DNA. The cell's DNA replication machinery will be used by the virus to produce more viral genomes, and ultimately more virions that shed from the surface [11].

1.5 Tumor suppressor protein p53:

Tumor suppressor gene's function is to keep the cells under control. These genes become activated when there is stress imposed upon cells that de-regulate normal cellular activities. Tumor suppressor gene products may prevent cell cycle progression to arrest cell growth and proliferation until the repair system can fix the error, or direct the cell to programmed cell death (apoptosis) if the insult is severe and can't be repaired [29]. p53 was the first discovered tumor suppressor gene. Initially, it was thought to be an oncogene rather than tumor suppressor gene [29].

p53 can be activated by three independent pathways: 1) by DNA damage which elicit activation of two kinases: the ataxia telangiectasia mutated (ATM) that is activated by double-strands breaks and in turn activates Checkpoint Kinase 2 (Chk2) [30]. 2) $p14^{ARF}$ dependent activation of p53 that happens in response to the expression of oncogenes [31-32]. 3) Ataxia telangiectasia related (ATR) dependent pathway that become activated by protein kinase inhibitors, ultraviolet rays and chemotherapeutic agents [33]. p53 activation depends on its stabilization more than increased production [29]. p53 degradation occurs by ubiquitination that is facilitated by the MDM2 protein. Normally, p53 has a feedback activation of the MDM2 gene whose product binds to p53 and label it with ubiquitin flag for degradation [29]. All three pathways of p53 activation involve inhibition of MDM2-p53 binding through modifications of the binding site, or sequestration of MDM2 and making it unavailable for the reaction [33-34].

P53 functions through different pathways: 1) Inhibition of cyclin dependent kinases (CDKs) by stimulation of the transcription of p21, resulting in inhibition of G1-S and G2-M transitions of the cell cycle [29]. 2) Directing the cell to apoptosis by activation of the Bax gene whose product is the prototype of apoptosis-inducing proteins [35] or through activated production of mitochondrial highly toxic reactive oxygen species [29]. 3) Stimulation of DNA repair mechanism by the activation of repair genes [29]. 4) Inhibition of angiogenesis necessary for tumor development by activation of anti-angiogenesis genes [36].

Abnormalities in p53 pathways have been detected in about half of the common tumors and these abnormalities are either due to mutation in p53 gene; de-regulation of

genes whose product interfere or interact with p53; or inactivation of p53 by proteins produced by viruses like HPV (figure 1.3) [29].

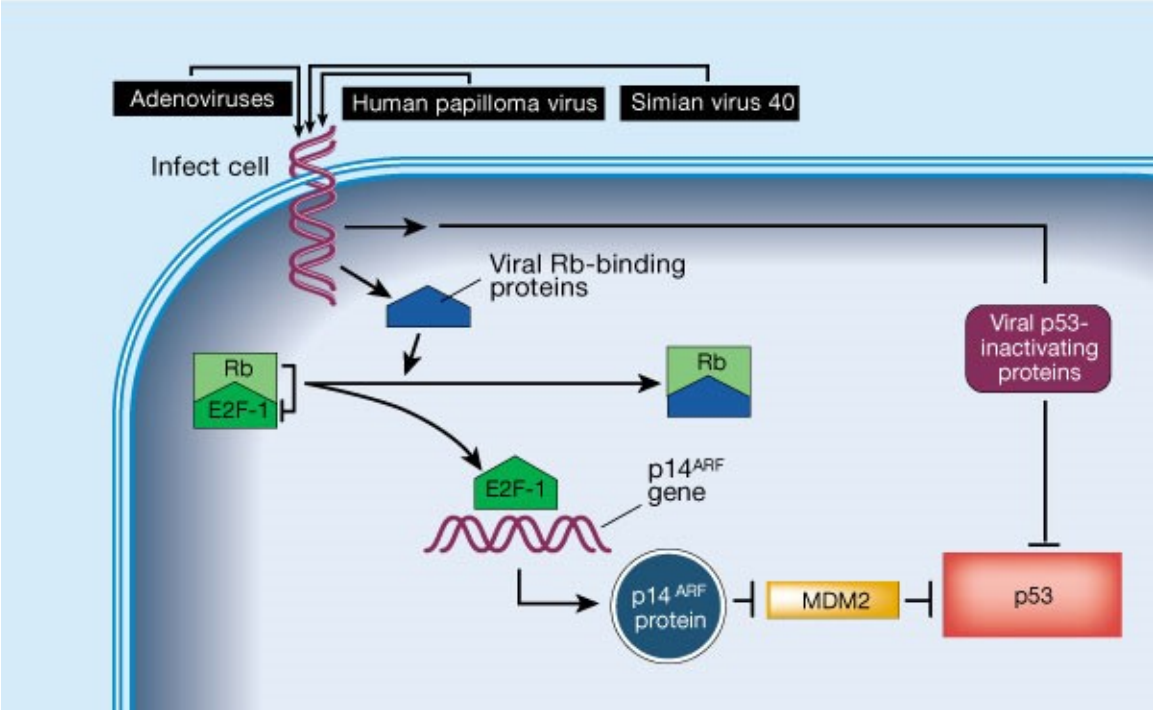

Figure 1.3: HPV interactions with p53 and Rb proteins. HPV is one of a number of viruses that interfere with p53 and Rb proteins. Viral Rb-binding proteins release E2F family members from Rb. In addition to having cellular proliferation genes inducing activity, activated E2F-1 activates $p14^{ARF}$ protein which inhibits MDM2 protein (a negative regulator of p53) and thus leads to stabilization of p53. To overcome this interference, HPV produces E6 which directly inactivates p53 to permit carcinogenesis [29].

1.6 The HPV E6 protein:

HPV E6 is small protein of 18 kDa that resides in the nucleus but also can be detected in the cytoplasm [37]. E6 plays an important role in HPV pathogenesis, especially for HR HPV [2]. Its best known and probably most important effect is its interaction with p53 protein, an action that was discovered more than 25 years ago [38]. E6 abolishes p53 functions by different mechanisms: 1) E6 forms a trimeric complex with cellular E3 ubiquitin ligase E6-associated protein (E6AP) and p53 to direct the latter to ubiquitination

and proteasomal degradation [39]. Both HR and LR HPV E6 can bind to C-terminus site of p53, however, HR E6 can also bind the core site of p53 which is essential for its degradation while the LR E6 binding is weak and doesn't result in p53 degradation [40]. 2) Both HR and LR HPV E6 inhibit p53-mediated transcriptional activity by changing p53 attachment or inducing post-translational modifications [40]. The degree of p53-DNA binding inhibition depends on the affinity of E6 to p53 which is highest in HPV16, intermediate in HPV18, HPV31 and least in HPV11 [41]. 3) Both HR and LR HPV E6 can sequester p53 in the cytoplasm by either exporting it outside the nucleus or by hiding the nuclear localization signal that is necessary to transport the protein to the nucleus [42]. 4) HR HPV E6 interferes with p53 acetylation required for DNA binding [43].

HR HPV E6 has a PDZ (PSD95/Dlg/ZO-1) binding motif (PBM) which enables the E6 protein to interact with variety of PDZ domain-containing proteins leading to proteasome-mediated degradation of these proteins [44]. The PDZ-binding capacity of HR E6 is important for HPV carcinogenesis and abnormalities in this association result in abnormal genome amplification and S-phase re-entry [45].

HR HPV E6 activates telomerase reverse transcriptase (TERT) transcription which results in cellular immortalization in conjunction with E7 inhibition of retinoblastoma protein (Rb) [46-47]. In addition, E6 has other targets of action including modulation of the immune response, G-protein signaling, mediating genetic instability and disruption of cell adhesion and polarity [2].

1.7 The HPV E7 protein:

Like E6, the E7 protein is small (13 kDa) and localized in the nucleus and probably in the cytoplasm too [37,46]. E7 is important to support DNA synthesis in differentiating cells [48]. E7 plays a comprehensive role in HPV pathogenesis as it functions on different levels of enhancing cell cycle progress. The hallmark role of E7 is its association with tumor suppressor retinoblastoma protein (Rb) and its related family members p105, p107 and p130. Rb represses G1-S transition of the cell cycle by binding to and inactivating the transcription factor E2F [49]. When it is hypo- or de-phosphorylated, Rb binds E2F and prevents its binding to its DNA binding sites which are located in the promoter regions of genes that regulate cell cycle, differentiation, mitosis and programmed cell death, thus impeding E2F action [50]. During normal cell cycle, cyclin-dependent kinases 4, 6 and 2 (CDK4/6 and CDK2) induce Rb phosphorylation (inactivation) releasing E2F which then can translocate to the nucleus to exert its transcriptional activity of S phase entry genes [51].

HPV E7 has no DNA binding activity, but it preferably binds to Rb-E2F complex to destabilize Rb by ubiquitin-dependent proteasomal degradation and weaken the complex association with uncontrolled release of E2F [52-53]. HR HPV E7 has much higher binding affinity for Rb than LR HPV E7 [54].

The E2F family contains eight members (E2F1-E2F8), E2F1-E2F5 act as transcriptional activators and E2F6-E2F8 act as repressors [55]. Activation of E2F1 by E7 induces feedback E2F6 overexpression which aborts E2F1 action and direct the cell to exit cell cycle and adopt differentiation [56]. E7 can abolish this effect and prevents E2F6 repression of E2F1 which provides further cell cycle enhancement [57].

HR HPV E7 enhances CDK2 activity which is important for G1-S phase transition and progression through different mechanisms: 1) HR E7 can bind and inactivate p21 and p27 CDK inhibitors whose function is to repress cell cycle progression by targeting CDK2. Hereby, CDK2 level remains elevated despite high level of p21 which is inactive; on the other hand, LR HPV E7 has low p21-binding affinity and less inhibitory effect [58-59]. 2) Both HR and LR E7 can bind directly to CDK2 maintaining constant activity [60]. 3) HR E7 can promote CDK2 activity by enhancing its de-phosphorylation through the CDC25A tyrosine phosphatase enzyme [61].

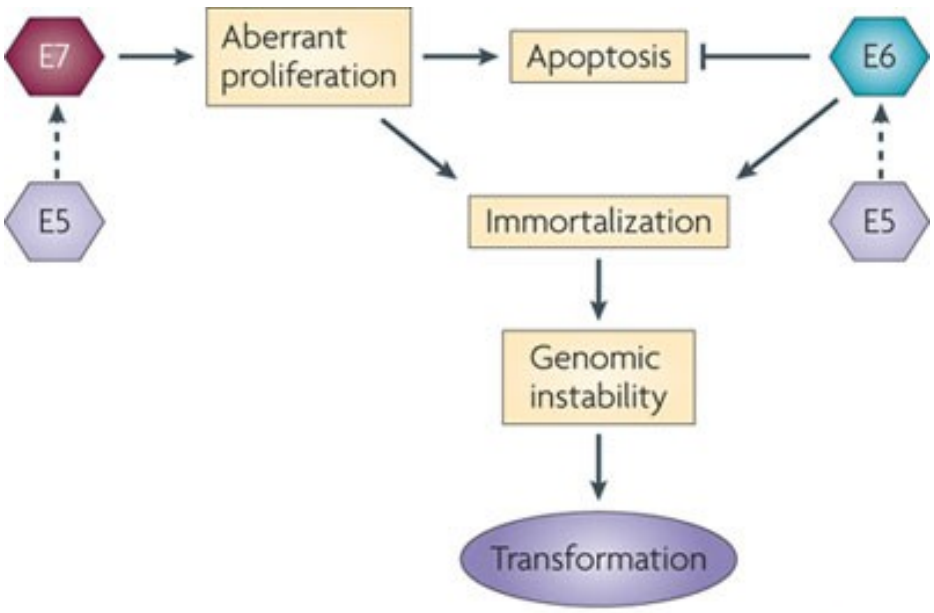

Figure 1.4: The carcinogenic mechanisms mediated by HPV oncoproteins. E7-induced inappropriate cellular proliferation triggers apoptosis that is counteracted by E6 which inhibits apoptosis. The combined roles of E6 and E7 are sufficient to induce immortalization of the infected cell. Inhibition of apoptosis and induced immortalization can lead to accumulation DNA damages with subsequent genomic instability that is under the absent role of cell cycle checkpoints can transform the affected cell to cancerous cell. E5 role (presented as dotted line) is thought to be supplementary to E6 and E7 [11].

However, E7 abrogation of the checkpoints in the cell cycle results in p53 stabilization which tries to turn the cell back under control, but this point of weakness in E7 action is easily counterbalanced by the effect of HR E6 on p53 [62]. HR HPV E7 alone has some transforming ability while E6 has not, however, the combined effect of HR E7 and E6 has synergistic value in potentiating the transforming capacity of HR HPV [63].

In addition, HR E7 also has telomere lengthening activity even in the absence of E6 [64] and contributes to genomic instability, especially, mitotic aberrations [65]. The combined roles of E6 and E7 is illustrated in figure 1.4.

1.8 HPV associated carcinogenesis:

HPV is regarded as oncovirus and its association with cancer development is well-established, especially for the HR types. HPV is responsible for 5% of all human malignancies [66]. HPV16 ability to transform human keratinocytes and fibroblasts in vitro has been demonstrated almost 3 decades ago [67]. Nowadays, HPV as carcinogenic infection can be detected in many cancers mainly cervical, head and neck and anogenital cancers in addition to other cancers. The intensity of association varies from merely been discovered to have HPV infection element to HPV being the culprit of the carcinogenesis.

1.8.1 Cervical cancer:

Although the incidence of cervical cancer is decreasing in the United States and Europe due to screening for early lesions, cervical cancer incidence is still high in the developing countries in Sub-Saharan Africa, Asia and Central and South America. Cervical cancer ranks the fourth most common cancer worldwide and the second most common in women. Globally, the annual incidence exceeds 500,000 cases and almost half of this

number represents the annual mortality rate [68]. Nowadays, HPV is regarded as the principal causative agent in cervical cancer. HPV infection of the cervical transformation zone could occur early in life during adolescence especially with early onset of sexual activity. The life-time risk of exposure to the virus is 80% and the risk decreases with increasing age [69]. At any time, HPV presence in the absence of clinical lesion has been detected in 11-12% of all women. This prevalence increases with increasing the severity of the underlying lesion to range from 50-70% in low-grade neoplasia to 90-100% in invasive cervical cancer, HPV 16 and HPV 18 comprise the majority of cases [70].

HR HPVs produce subclinical transient infection that usually clear out in about 80% of cases within 1-2 years. Persistent infection may give rise to low-grade cervical intraepithelial neoplasia grade 1 and 2 (CIN-1 and CIN-2) within a couple of years. However, in the majority of cases there is spontaneous resolution for the low-grade lesions. If lesions persist for longer period, high-grade lesions cervical intraepithelial neoplasia grade 3 (CIN-3) or adenocarcinoma in situ (AIS) may develop which can progress to cervical cancer in half of cases [69]. However, the ratio of HPV induced lesions that progress to cancer to the total number of HPV induced lesions is very low and this process could take anytime between 5 and 30 years [71-72].

1.8.2 Head and neck cancer (HNC):

Head and neck squamous cell carcinoma (HNSCC) comprises more than 90% of HNC with global incidence of 600,000 new cases per year which accounts for about 20 per 100,000 people [73]. HNSCC is responsible for 3.5% of cancer cases in the United States and Europe [74], however, it is the sixth most common malignancy worldwide due to the high prevalence of the disease in India, Southeast Asia and Latin America [75]. Although

HNSCC incidence has decreased in the United States, the incidence of oropharyngeal squamous cell carcinoma (OPSCC) has increased mainly in younger age group and especially in regions of the tonsils and the base of tongue accounting for 90% of OPSCC cases [76-77].

HPV association with HNSCC nowadays is widely accepted as a causative agent besides tobacco smoking and alcohol consumption. About 25% of HNSCC and 60% of OPSCC are positive for HPV (HPV+) and in 90% of the cases is HPV16 [78]. Depending on the presence and absence of HPV, HNSCC has been classified as HPV-positive (HPV+) and HPV-negative (HPV-) with differences in the molecular, etiological, clinical and behavioral characteristics between the two groups [79]. Molecular analysis showed obviously distinct gene expression profiles with higher expression for the genes involved in cell cycle progression, mitosis and proliferation forming what can be described as HPV signature [80-81]. On the other hand, HPV (-) tumors lack the HPV signature and have higher expression for the genes involved in cell motility, epithelial to mesenchymal transition and angiogenesis [Reviewed in 82].

Figure 5 shows the results of gene ontology analysis conducted on microarray data from oral and oropharyngeal cancer cases, demonstrating the differences in gene expression between HPV+ and HPV- HNC.

HPV(+) tumors occur more commonly in younger age group who are usually non-smoker and non-alcoholic, HIV infected, marijuana users and more in men than women especially homosexual men. In contrast, HPV(-) tumors are more likely to affect older, smokers and/or alcoholics [83-84]. HPV(+) HNSCC tends to be poorly differentiated, non-keratinizing, with basaloid morphology, without field cancerization and usually present

with nodal metastasis, features that are opposite to what is seen in HPV(-) HNSCC [12]. Several studies have shown the favorable prognosis for HPV induced HNSCC with a 3-year disease free survival and overall survival in HPV(+) compared to HPV(-) 85% versus 49% and 90% versus 65% respectively, considering HPV positivity as an independent good prognostic factor [85]. Although some have pointed to the probable more intensified treatment that patients with HPV (+) tumors might receive as a reason for the more favorable outcome, others showed that HPV (+) tumors have favorable outcome regardless of the treatment [86].

The better prognosis in HPV (+) cancer is thought to have a multifactorial explanation. HPV (+) tumors usually have wild-type p53 and Rb tumor suppressor genes activity which allow apoptotic response of cancer cells to radio- and/or chemotherapy and the lack of field cancerization which means absence of wide-spread genetic mutations [87].

The affected population usually consists of younger age group, non-smokers and non-alcoholic, so they have less comorbidities and hence are less vulnerable to the negative effects of cancer therapeutic modalities [86]. Moreover, better prognosis could be owed to body immune surveillance against HPV as it was shown that there is activation of the adaptive immune system in form of CD8 (+) T-cell lymphocytic tumor infiltration and the more infiltration associates with better outcome [88].

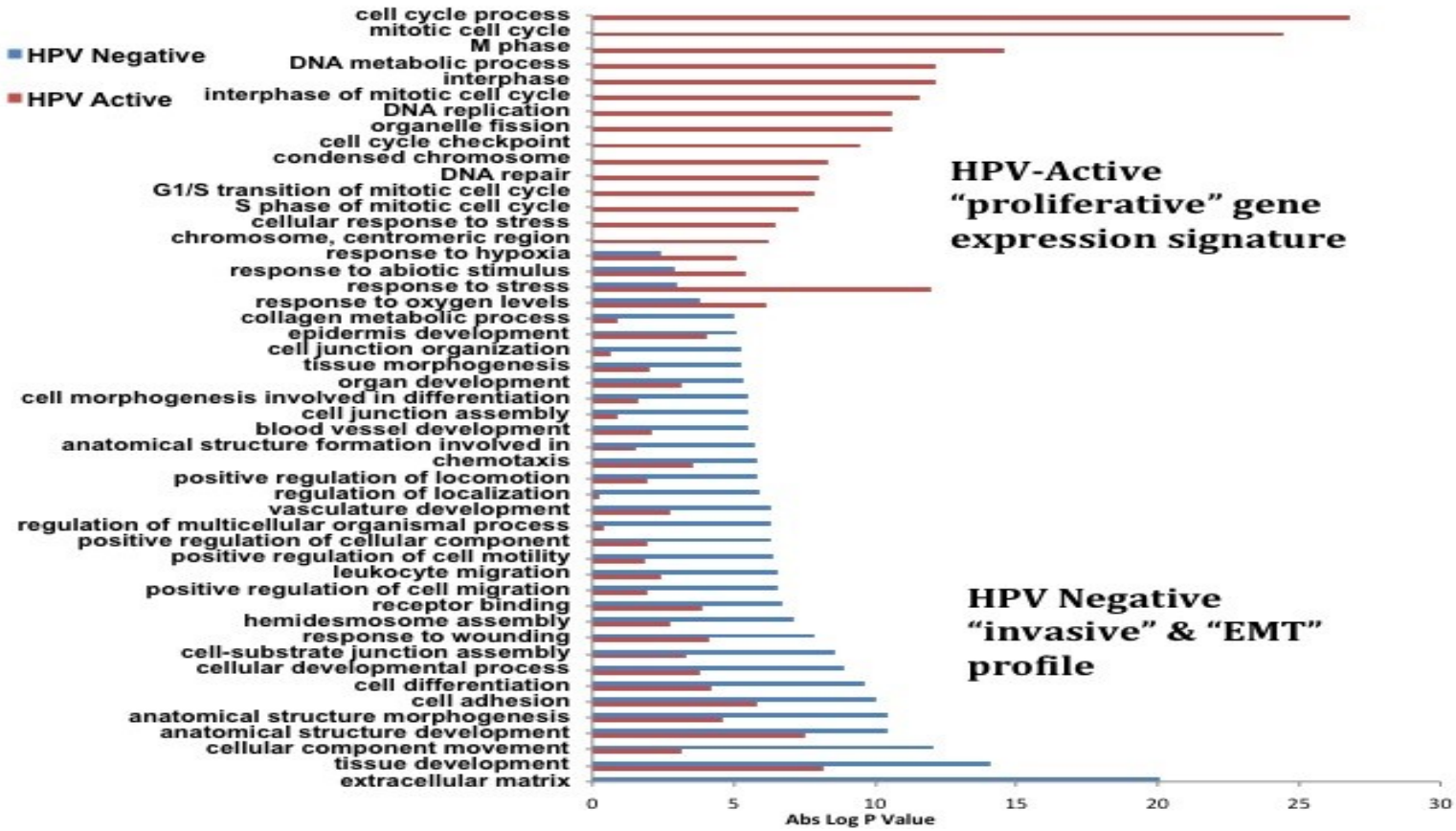

Figure 1.5: Gene ontology, HPV-active versus HPV-negative OPSCC samples. A clear "proliferation signature" can be observed in HPV-active tumors (red bars in the figure), with increased expression of genes involved in pathways that control growth, cell cycle, mitosis. This signature is absent in HPV-negative tumors, characterized by cell motility/extracellular matrix/angiogenesis/epithelial-mesenchymal transition (EMT) signature [81].

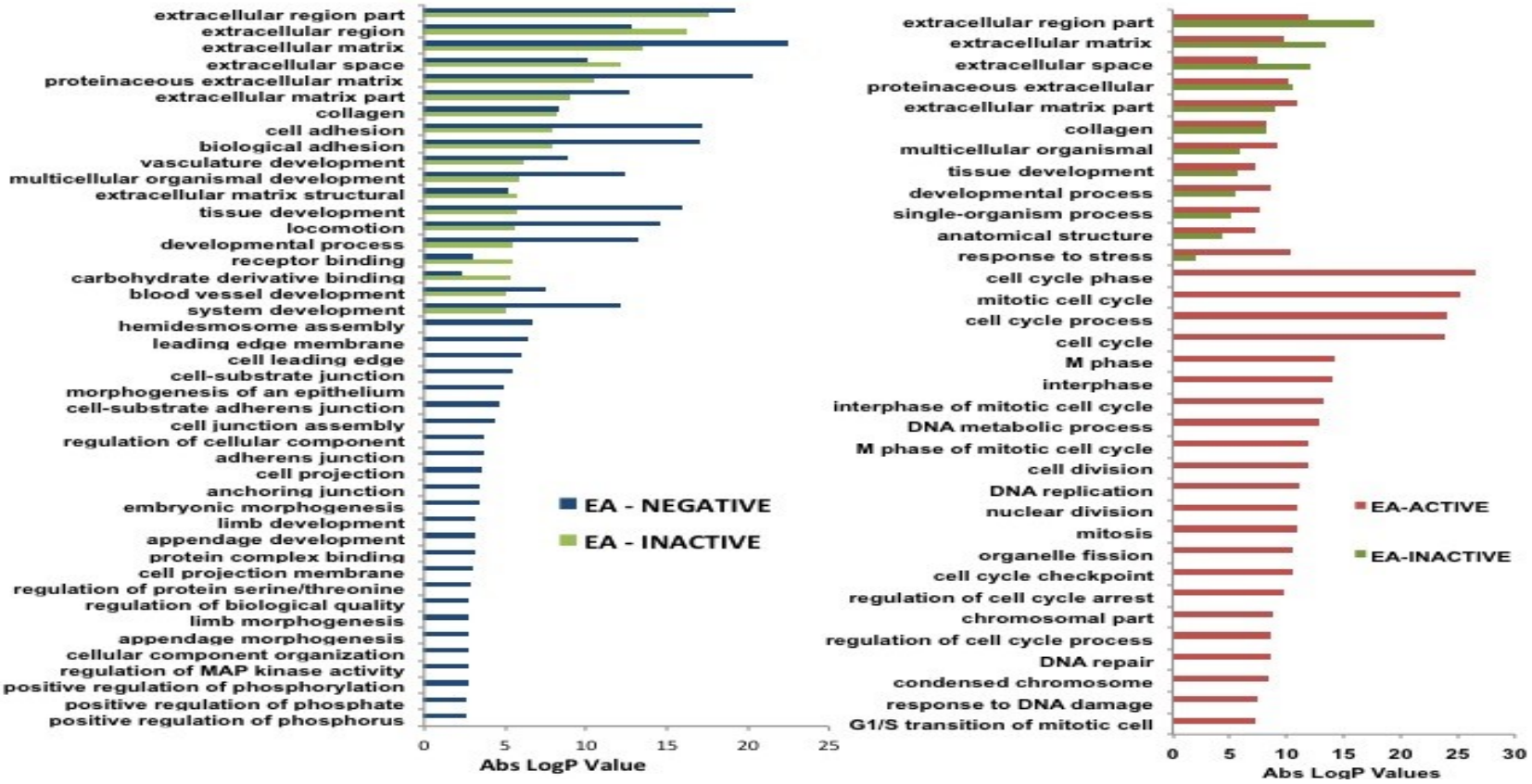

Figure 1.6: Gene ontology analysis of gene expression changes in HPV-active, -inactive and –negative tumors from European Americans (EA). Clearly, HPV-inactive tumors lack the "HPV signature" typical of HPV-active tumors, however, they share many but not all the features of HPV-negative tumors, lacking changes in a whole class of pathways including cell projections, leading edge, adherens junctions. This finding suggests gene expression signature of HPV-inactive tumors is intermediate between that of HPV-active and HPV-negative [81].

Recently, it has been shown that another distinct category could be considered in classifying HNSCC which is called HPV-positive inactive HNSCC [81,89]. Hence, the new classification of HNSCC should be in form of: 1) HPV (+) active tumors which have the viral DNA and express the oncogenic proteins E6 and E7. 2) HPV (+) inactive tumors which contain the HPV DNA but do not express E6 and E7 proteins. 3) HPV (-) tumors which are negative for the viral DNA and of course for its proteins. Gene analysis of HPV-inactive tumors show gene expression that lacks the "HPV-signature" but also differs from HPV-negative tumors which further enhances considering them as a separate identity (see figures 1.5 and 1.6).

Interestingly, HPV-positive primary HNSCC tend to be HPV-active, whereas recurring cancers of the head and neck are more often HPV-inactive [89]. Further, HPV-positive cancers are more likely to be HPV-inactive in African American (AA) than in European American (EA) patients. The suggested significance behind HPV-inactive tumors is that HPV infection in these tumors starts positive active and triggers the early carcinogenic events by the effects of E6 and E7 proteins, then the virus become inactive in terms of gene expression and the further steps of tumor development are driven by the earlier E6, E7-induced carcinogenesis.

1.8.3 Anogenital cancers:

Include cancers that affect the vulva, vagina, penis and anus. HPV16 has been detected in majority of these cancers with prevalence almost around 90% and less commonly other types like HPV18, 31, 33, 34, 52 as well as LR HPV types like HPV6, 11 [90,91]. HPV prevalence is prominent in basaloid and warty cancers and less prevalent in the keratinizing squamous cell carcinoma of these regions (like HNC) [92].

1.9 HPV vaccines:

1.9.1 Prophylactic vaccines:

The evidence that HPV is a carcinogenic agent has led to the development of prophylactic vaccine to prevent the infection and subsequently abolish cancer development.

1) Gardasil® (Quadrivalent Human Papillomavirus (HPV Types 6, 11, 16 and 18) Recombinant Vaccine) is the first quadrivalent HPV L1 protein virus-like particle (VLP) vaccine in the United States. It is produced in recombinant yeast and adsorbed on a proprietary aluminum-containing adjuvant. It is prepared from the highly purified VLPs of the recombinant major capsid (L1) protein of HPV Types 6, 11, 16, and 18 which are self-assembled into VLPs. These particles have no viral DNA, therefore, they cannot infect cells or reproduce [93]. According to the HPV types contained in GARDASIL, it provides protection against almost 70% of cervical, vulvar and vaginal cancerous and precancerous lesions as well as 90% of genital warts [94].
2) Cervarix® Bivalent recombinant HPV vaccine that provides protection against HPV16 and HPV18. It provides protection against 70% of the cervical cancer as well as grade II and III intraepithelial dysplastic lesions of the cervix, vagina and vulva, however, it doesn't protect against anogenital warts as it doesn't have the responsible HPV types [95].
3) 9vHPV vaccine (nine valent HPV vaccine) provides protection against HPV types 6, 11, 16, 18, 31, 33, 45, 52 and 58. Potentially, it protects against 90% of cervical

cancer, as well as, HPV-related vaginal, vulvar and anal cancers and precancerous lesions and 90% of genital warts [96-98].

Prophylactic HPV vaccines are highly recommended for girls before their 26th birthday. The administration regimen is either 2 doses 6-12 month apart if the girl is younger than 15 year-old mainly used in France or 3 doses with intervals of 2 month between the first two doses and 6 month between the second and the third doses if the girl age is above 15 years, HIV infected or immunocompromised which is the commonly used protocol in US [99]. Now, the importance of vaccinating both genders at youth is becoming increasingly recognized and in some countries it is mandatory, to prevent all HPV-mediated cancers, including HNSCC [100].

1.9.2 Therapeutic vaccines:

Prophylactic vaccines are ineffective in treating pre-existing infection [101]. Therefore, the current research in HPV-induced cancer is to develop immunotherapy against various HPV types making benefits from enhancing the body immune response to viral antigenicity. One of the proposed therapeutic vaccines is Pentarix which is a recombinant fusion protein-based broad spectrum vaccine and contains E7 proteins of the most common HR HPV types 16, 18, 31, 45 and 52, which collectively account for 80% of all HPV- related tumors and elicit CD8+ cytotoxic T cell reaction to E7 protein-expressing tumor cells [102]. However, these vaccines are still under study in animals and have not been proved yet for clinical uses.

1.10 Hypothesis and rationale:

This study is a corollary of our main study, based upon the hypothesis that ***HPV-positive inactive tumors arise as HPV-active lesions. We postulate that the virus acts as initiator inducing the early transformation changes. HPV-transformed cells may then progress to malignancy in the presence of cancer promoters like smoking and alcohol in an HPV oncoproteins-independent pathway, giving rise to HPV-positive inactive tumors.***

This principle was established when a former graduate student (Dr. Swati Tomar) compared, in a gene ontology analysis, the gene expression profiles of HPV-positive active, HPV-positive inactive and HPV-negative head and neck cancers [81]. As we have shown in the Introduction, Dr. Tomar found that the HPV-positive active HNSCC have gene expression profiles that exhibit a "HPV signature" which is missing in HPV-negative and HPV-inactive cancers. Furthermore, HPV-inactive and HPV-negative tumors have gene expression profiles that are similar to each other, but not entirely overlapping (figures 1.5 and 1.6) [81]. Also, previous work has shown that recurring tumors in patients with HPV-positive active primary HNSCC tend to be HPV-inactive and the prevalence of HPV-inactive tumors is higher in African American patients than in European Americans.

Therefore, the focus of the ongoing research is to discover the molecular modifications that can abolish the need for persistent HPV-oncoprotein expression in HPV-associated tumors. As p53 is the main target for HPV E6 transformation capacity, we set out to investigate in more detail p53 function in normal and HPV16-transformed cells.

Another former graduate student, Nella Delva, tested the behavior of p53 in UV-treated HPV-transformed cells both transfected with p53sh RNA and non-transfected. She

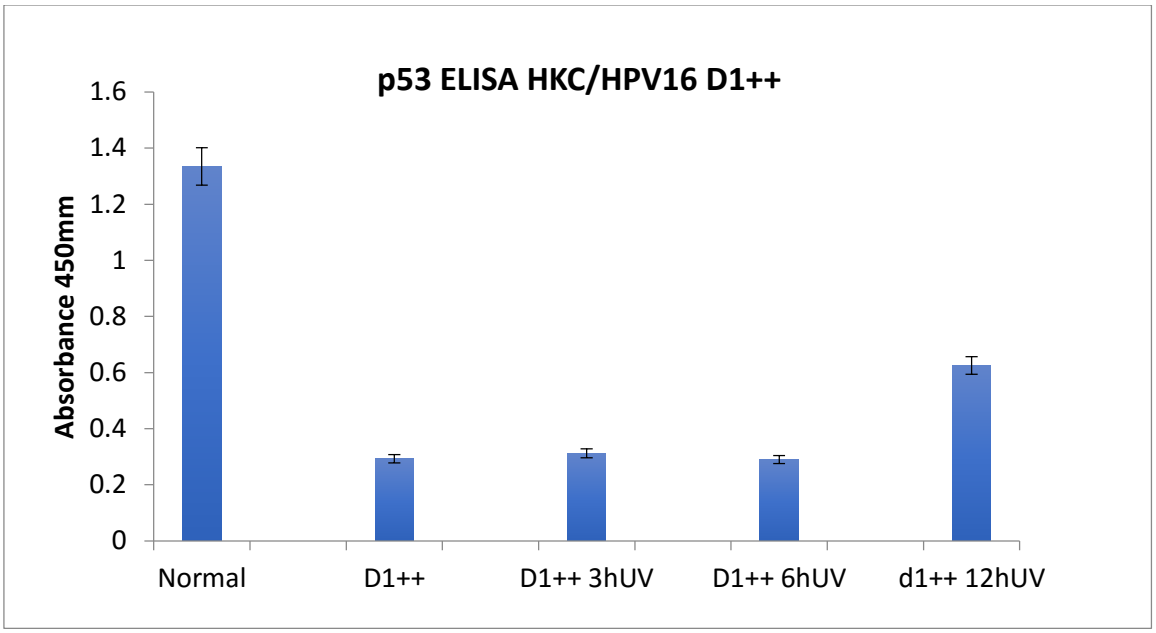

Figure 1.7: p53 ELISA results of UV-treated non-transfected HKc/HPV16 cells at passage number 35. D1++: HPV16-transformed human keratinocytes at passage number 35 display increased p53 level 12 h after UV exposure [103].

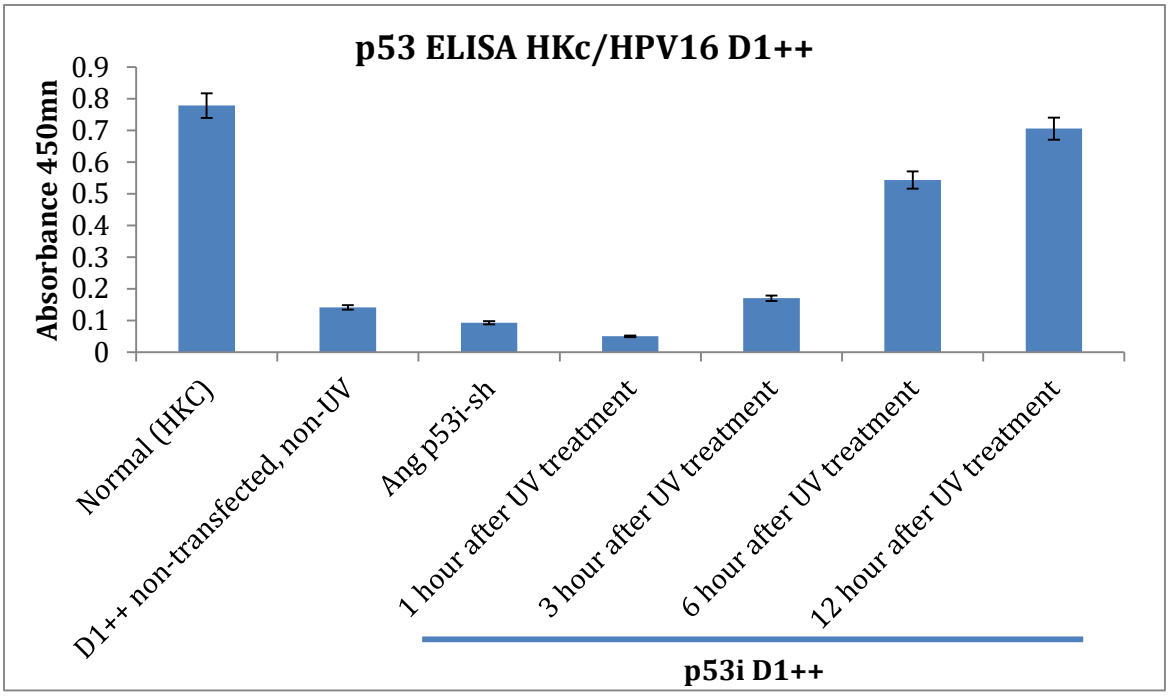

Figure 1.8: p53 ELISA results in normal HKc and the D1++ HKc/HPV16 cell line treated with UV light. From left to right normal human keratinocytes, non-transfected non-UV treated HKc/HPV16 D1++ transformed cell line, non-UV treated HKc/HPV16 D1++ transfected with p53i, HKc/HPV16 D1++ transfected with p53i 1, 3, 6, 12 h after UV exposure respectively [103].

obtained a surprising result: p53 protein level increases upon UV induced cell damage in HPV-transformed human keratinocytes (HKc/HPV16) even if p53 was knocked-down by p53i RNA transfection. This suggests that p53 functions are partially retained, despite the fact that p53 levels are low in these cells (figures 1.7, 1.8).

1.11 Aims of the project:

To confirm the proposed hypothesis, we need to further study p53 function in normal HKc. We proposed 2 specific aims:

Aim 1: To determine how p53 protein levels change in response to DNA damage in normal human keratinocytes derived from different individual donors. This will be accomplished by performing p53 ELISA and qRT-PCR in protein and RNA extracted from normal human keratinocytes collected on different time intervals after UV exposure.

Aim 2: What is the effect of knocking-down p53 on its response to cellular damage, in normal HKc? This aim will be tested by transfecting normal human keratinocytes with a plasmid expressing an shRNA against p53, and determining p53 protein and RNA levels by ELISA and qRT-PCR, respectively, after exposing the cells to UV light.

CHAPTER 2: MATERIALS AND METHODS

2.1 Samples collection:

Human foreskin samples were collected from newborn boys who underwent circumcision procedures at Palmetto Richland Hospital. The samples were placed and transported in 50ml conical tubes each contains 5ml of transport medium made by adding 50 ml fetal bovine serum (FBS) to 450 ml of MCDB153-LB basal medium [67] containing penicillin 100 IU/ml, streptomycin 100 μg/ml and gentamycin 5 μg/ml.

2.2 Foreskin processing / cells culturing:

Using aseptic technique, the foreskin samples were rinsed in glass vials contains 5ml PBS each. By the use of a scalpel and forceps, the fatty connective tissue was removed from the skin, then specimens were rinsed again in clean PBS and put in 'epidermis up' position in 6-well plate contains 2ml of 10% dispase in each well and incubated overnight at 37°C.

On the next day, with the aid of two forceps, the skin was spread on a clean petri dish cover. The epidermis was separated from the underlying dermis and cut into tiny pieces and placed into 2ml trypsin 0.25% in 15ml conical tube and incubated for 12 min at 37°C to release the keratinocytes. 1ml FBS was added to neutralize trypsin then cells were collected by centrifugation in an IEC clinical centrifuge at speed 3 for 1 min then speed 2 for 4 min. The cells were re-suspended using KSFM complete medium and plated into

60mm tissue culture plate and returned to the 37°C incubator with 5% CO_2. The medium was replaced with fresh one after 24 h then every 48 h until the plate is about 80% confluent and ready to be passaged to 100mm tissue culture plates.

2.3 Cell passaging:

When confluent, 1.5ml trypsin 0.25% was added to each 60mm plate (3ml if using 100mm plate) and incubated at 37°C for about 5 min (until all the cells are detached from the plate). 0.5-1ml FBS then to be added and the mix was centrifuged at speed 3 for 1 min then speed 2 for 4 min. The cell pellet was re-suspended in complete KSFM medium and plated onto larger plate. Cells were re-fed after 24 h then every 48 h until they became confluent.

2.4 Transforming competent cells:

1) Making LB agar: In autoclave-safe flask we put 250 ml of distilled water and 10 g of Invitrogen LB agar powder to get final concentration of 40 g/L. The solution was autoclaved and allowed to cool down until it was cool enough to avoid destruction of Ampicillin which should be added to the agar before it turns solid to a final concentration of 100 µg/ml. Then, the agar should be stirred and gently poured into Petri plates to a thickness of about 3 mm. The plates were left at room temperature until the agar became solid, then were sealed and stored at 4 °C.
2) Making broth medium: Invitrogen Lauria broth base powder was dissolved in distilled water to achieve final concentration of 25 g/L. the solution was autoclaved, allowed to cool down, and Ampicillin was added to a final concentration of 100 µg/ml.

3) Two vials of One Shot TOP10 Chemically Competent E. coli were brought out from -80°C and left to thaw on ice. 100 ng of the required plasmid was added to each of two vials of the competent cells and mixed gently. The vials were incubated on ice for 30 min and heat-shocked by incubating them for 30 seconds at 42°C then on ice for 2 min. 250 µl of S.O.C medium were then added to each vial. They were shaken horizontally at 225 rpm for 1 h. The cells were spread over the agar plate and incubated overnight at 37°C.
4) On the next day, a small sample of a single colony was added to 150 ml of broth in a flask and the sealed flask was incubated overnight in an environmental shaker at 37ºC

2.5 Purifying plasmid DNA from transformed cell culture:

The PureLink HiPure Plasmid DNA Purification Kit from Invitrogen was used to purify the plasmids using the Maxiprep protocol. 30 ml of Equilibration Buffer (EQ1) were added to HiPure Maxi Column and allowed to drain. The cell culture was spun at 400 x g for 10 min to form sediment that was re-suspended in 10 ml Resuspension Buffer (R3) followed by adding 10 ml Lysis Buffer (L7) and a 5 min incubation at room temp. 10 ml of Precipitation Buffer (N3) were added and mixed well, then centrifuged at 12000 x g for 10 min. The supernatant was loaded onto the column and left to drain then the column was washed by adding 60 ml Wash buffer (W8). A new 50 ml tube was placed below the column and 15 ml of Elution Buffer (E4) was added and allowed to drain. 10.5 ml isopropanol were added to the eluate, mixed and spun at 12000 x g for 5 min at 4°C. Lastly, the supernatant was discarded and the pellet air-dried for 10 min before being re-suspended

in 200-500 µl TE Buffer depending on the pellet size. The plasmid DNA was stored at -20°C.

2.6 Transfection:

Using Invitrogen Lipofectamine 3000 reagent kit transfection reagent protocol manufactured by Life Technologies Inc., normal human keratinocytes (NHKc) were transfected with the p-Super anti-p53 small interfering RNA (p53i) plasmid. Control keratinocytes were transfected with p-Super encoding an inactive "scrambled" siRNA (scram), and some were left non-transfected as a control for antibiotic selection. The cells were plated on 6-well plate and the transfection was done in duplicate for each plasmid type. 1µg of p53i plasmid DNA or scram with 125µl transfection medium (basal KSFM medium) and 5µl of P3000 reagent were mixed in separate Eppendorf tubes for each well. In other tubes, 3.75µl lipofectamine reagent and 125µl transfection medium were mixed for each well. The contents of the two tubes were mixed and incubated for 5 min at room temperature then added to the wells drop by drop throughout the well. 2ml of fresh complete KSFM medium was added after 6-8 h. At 12 h after the transfection, the wells were washed and fed with fresh medium. 24-48 h after transfection, the cells were passaged to 60 mm tissue culture plates to be ready for selection. Transfection efficiency was tested by transfecting one of the scram transfected wells with GFP which has fluorescence activity that can be visualized under microscope.

2.7 Selection with puromycin:

Given another 24 h after passaging, selection with the antibiotic, puromycin, was started to kill the non-transfected cells while keeping the transfected cells because the

transfecting plasmids encode puromycin resistance. Puromycin in concentration of 0.5µg/ml of complete KSFM medium was used. The cells were observed and re-fed with puromycin containing medium every 48 h until the cells in the non-transfected wells die which can be used as indicator for selecting the cells in the transfected wells. The selected plates were allowed to become confluent to be split into 8 plates of 100mm size for the ultraviolet (UV) rays treatment.

2.8 Ultraviolet rays (UV) treatment:

When the 8 100mm plates for each of p53i, scram transfected and non-transfected cells become at least 80% confluent, it is the time to start the UV treatment assay. The purpose of UV treatment is to induce cellular insult to increase the level of stabilized p53 to measure and compared between the different groups of cells. UV treatment involves exposing the cells with removed lids and aspirated media to UV light for 30 sec inside a turned on and closed disinfected biological safety cabinet. All the plates, except for time 0 control and time 24 h control for half of the samples, were exposed to the UV light. Complete KSFM medium was added and the plates were incubated at 37°C to do cell collection at regular intervals of 12, 24 and 48 h.

2.9 Cell collection:

The medium was aspirated and 3 ml trypsin was added to each plate and incubation at 37°C until we saw the cells detached from the plate's bottom. 1.5 ml FBS then was added to each plate and spun down using IEC clinical centrifuge speed 3 for 1 min and speed 2 for 4 min. The supernatant was aspirated and the pellet was re-suspended in 1 ml PBS to

wash out the residual trypsin. Re-spinning was done followed by aspiration and the cell pellets were frozen at -80°C for later analysis.

2.10 Cell lysis for ELISA:

The cell lysis buffer 10x supplied with Pathcan®Total p53 Sandwich ELISA kit purchased from Cell Signaling Technology (catalog number 9803) was used to lyse the cell pellets to be further analyzed. The buffer was diluted 1:10 with nuclease free water. Protease inhibitor 10% was added in volume of 1:10 of the diluted buffer volume and the mix was chilled on ice before use. 200 µl of the lysis buffer mix was added to each cell pellet and mixed well by thorough pipetting to lyse the cells. Centrifuge at maximum speed for 5 min then the supernatant was pipetted into new Eppendorf tubes to be used for protein measurement and ELISA.

2.11 Bradford protein quantification assay:

The Bio-Rad Quick Start Bradford Protein Assay kit was used to measure protein concentration of the cell lysate. The Quick Start 1x Dye Bradford reagent was brought to room temp before starting the assay. Bio-Rad Bovine Serum Albumin (BSA) in concentration of 2mg/ml was used and 8 1:2 serial dilutions were used as a reading standard. Tube number 1 contains only 20 µl of BSA. Tube number 2 contains 20 µl BSA and 20 µl water. Each following tube has 20 µl of the mix in the previous tube mixed with 20 µl water up to tube number 8 which contains only 20 µl water as blank. Undiluted sample as well as 10x diluted samples were used to avoid having to repeat the experiment if the sample reading falls out of the standards range. Then, the standards and the samples were loaded onto 250 µl 96-well microplate in triplicate with each well contains 5 µl of

each standard or sample and 190 µl of 1x dye reagent and mixed well by pipetting using multichannel micropipette. A microplate reader was used to read the absorbance at 595nm within 5 min of mixing and the results for the standards were plotted to find the slope equation that was applied to determine the protein concentration in the samples.

2.12 Total p53 sandwich ELISA:

Total p53 was measured using Pathcan® Total p53 Sandwich ELISA kit manufactured by Cell Signaling Technology catalog number 7370C. The microwell strips were allowed to reach room temperature before use. The cell lysates were diluted using substrate diluent supplied with the kit to get concentration of 0.5 mg/ml which corresponds to the best absorbance response according to the experiment chart in the kit protocol. 100 µl of the diluted mix for each sample including the blank was added to each well in triplicate and the strips were sealed with tape and incubated overnight at 4°C which proven to give signal intensity better than the 2 h incubation at 37°C suggested as another option by the protocol. On the next day, the wells were washed 4 times with 1x wash buffer supplied with the kit using 200 µl/well each time and the plate was drained by inverting it and tapping it on lint-free tissue each time. 100 µl/well of reconstituted detection antibody was added and the plate re-sealed and incubated for an h at 37°C. Then the wash step was repeated and 100 µl/well of reconstituted HRP-linked secondary antibody was added and the plate was incubated for 30 min at 37°C. Again, the wash step was repeated and 100 µl/well TMB substrate was added and re-incubated for 10 min at 37°C. At the end, 100 µl/well of stop solution was added and the plate was put on a shaker for few seconds before it was read on a microplate reader at 450 nm. The results were analyzed to measure the difference in total p53 level for each experiment in response to UV radiation.

2.13 DNA and RNA isolation:

Qiagen AllPrep DNA/RNA mini kit (catalog number 80208) was used to extract RNA and DNA from the collected cells using the supplied protocol. The cell pellets were allowed to thaw on ice. B-mercaptoethanol was added to Buffer RLT Plus in volume of 10 µl to each 1 ml of the buffer. 600 µl of the mix was added to each pellet and by vigorous pipetting and vortexing to homogenize the cells. The lysate was added to AllPrep DNA spin columns and centrifuged at 10000 rpm for 15 sec. Columns were kept at room temp for later DNA purification. 600 µl 70% ethanol was added to the flow-through and mixed well, then it was added to the RNeasy spin column and centrifuge at 10000 rpm for 15 sec. 700 µl Buffer RW1 was added to the column and spun again at 10000 rpm for 15 sec. 500 µl of Buffer RPE was added for two times and spun at 10000 rpm for 15 sec in the first time and for 2 min in the second time to wash the column and the flow-through was discarded each time. Lastly, the column was put in clean 1.5 µl tube and 50 µl RNase free water was added to the column to elute the RNA and spun at 10000 rpm for1 min and the flow-through was pipetted again into the column and spun again to get higher RNA concentration.

For DNA purification, 500 µl of each of AW1 and AW2 wash buffers was added to the AllPrep DNA spin column and the spinning at 10000 rpm for 15 sec and at full speed for 2 min, respectively. Lastly, 100 µl of preheated Elution Buffer (EB) was added and left for 2 min to elute the DNA. The eluate containing the DNA was collected by centrifugation at 10000 rpm for 1 min and the flow-through was passed again through the column to get higher DNA concentration.

2.14 RNA quantification:

RNA concentration was quantified using Implen Nanodrop Pearl. The eye of the spectrophotometer was cleaned with RNase free water before measuring each sample to obtain more accurate results.

2.15 cDNA synthesis:

Bio-Rad iScript cDNA Synthesis kit was used to produce cDNA from the RNA template obtained from the samples. 5x iScript reaction mix and iScript reverse transcriptase were allowed to thaw on ice. In 0.5 ml tube, 4 µl of the reaction mix and 1 µl of the transcriptase along with RNA and nuclease free water in volumes that makes a total volume of 20 µl and contains 700 ng RNA. The tubes are placed in the Bio-Rad iCycler and a protocol of 5 min at 25°C, 30 min at 42°C, 5 min at 85°C then hold at 4°C was used to produce cDNA template for later qPCR.

2.16 RiboGreen assay:

cDNA was hydrolyzed by mixing 7 µl of cDNA, 2.1 NaOH (1M) and 1.8 EDTA (0.25). The mix was incubated in a thermal cycler at 70°C for 15 min. Then, 3.5 µl HEPES (2M) was added to the mix. Invitrogen QuantiT Ribogreen RNA kit was used to quantify cDNA concentration. 20xTE buffer was diluted 20 times to get 1x solution. RNA standards were prepared by mixing 1000, 500, 100, 20 and 0 µl of Ribosomal RNA standard 2µg/ml with 0, 500, 900, 980 and 1000 µl of 1xTE buffer respectively which makes a concentration gradient of 1000, 500, 100, 20 and 0 ng/ml. The standards and the samples were loaded onto simple 96-well plate in triplicate. For the standards, 100 µl were loaded onto each well and for the samples 97 µl of TE 1x plus 3 µl of cDNA mix. With the help of

multichannel pipette, 100 µl QuantiT Ribogreen reagent were loaded onto each well. After incubation for 5 min in the dark, the plate was read on a microplate reader using 485 nm excitation and 530 nm emission. The results were analyzed mathematically to find cDNA concentration.

2.17 Preparing cDNA dilutions:

A dilution of the cDNA stock was made to equalize the concentration among samples by taking 5 µl of the cDNA in a new 1.7 ml Eppendorf tube and adding nuclease free water with volume sufficient to make the final concentration 0.25 ng/µl to simplify the calculation when doing PCR.

2.18 Primers reconstitution for PCR:

The forward and reverse primers for each gene were reconstituted by dissolving them in nuclease free water with volume in µL equal to the number of nanomoles of the primer (given with the primer) divided by the desired final concentration in micromoles/L to make a 50 µM stock of each primer. The solutions were incubated at room temperature for 5 min then vortexed. 50 µl of each of the forward and the reverse primers were mixed in clean tube to make a stock solution containing both primers at a concentration of 25 µM that is diluted by 1:10 to give the working concentration for PCR which is 2.5 µM.

2.19 Real-time (quantitative) reverse transcription PCR (qRT-PCR):

qRT-PCR was used to compare p53 gene expression among samples collected at different time intervals from UV exposure. 96-well Bio-Rad PCR plates were used. A master mix of iQ SybrGreen Super Mix, forward and reverse primers mix at concentration of 2.5 µM and nuclease free water in volumes ratio of 10:2:6 µl respectively were added

to each well. The total volume of the master mix should be adjusted to take into account the loss of the solution due to pipetting by making a total mix that is enough to cover the number of samples in triplicate plus 10% of them. 54 µl of the master mix was added to 0.5 µl tube and 6 µl of the cDNA dilute of each sample with vortexing and spinning down to ensure proper mixing. 20 µl of each mix was placed in each one of triplicate wells for each sample. Then, the plate should be sealed with tape and spun down for 1 min at 3000 rpm to bring the solution to the bottom of the wells. The qRT-PCR protocol was set up to steps of 95°C for 10 sec, average melting temp for the forward and reverse primers for 30 sec and this step was repeated for 40 cycles before it went to 95 °C for 4 sec. the Cq threshold value was standardized to 50 for all the experiment before analyzing the data to find the relative expression of p53 to that of the reference gene B-actin.

CHAPTER 3: RESULTS

3.1 *Aim 1:* *To determine how p53 protein level changes in response to DNA damage in normal HKc strains from different individuals.*

P53 protein level depends on its stabilization rather than increasing gene expression. Because p53 protein level is very low in healthy normal cells, we decided to induce DNA damage by exposing the cells to UV radiation for 30 sec, in order to be able to detect p53. The cells were collected on different time intervals to show the changes in p53 level. 6 different foreskin samples were used and cultured until they yielded 8 confluent 100 mm plates at passage number 3 then were irradiated with UV. After harvesting the cells at 12 and 24 h from UV radiation and extracting protein, ELISA was run and the results were plotted using column chart presentation to compare the data collected among the samples as in figure 3.1.

Three of the normal HKc strains showed a mild increase (less than 2-fold) in p53 protein level at 12 h after UV radiation and the level decreased to almost the non-UV treated control level at 24 h. Two strains showed moderate increase (about 2-fold) at 12 h and kept increasing at 24 h. However, one of the HKc strains showed a considerable peak, reaching more than 5-fold the level of the control at 12 h and also kept increasing at 24 h to reach more than 8-fold the level in the untreated control.

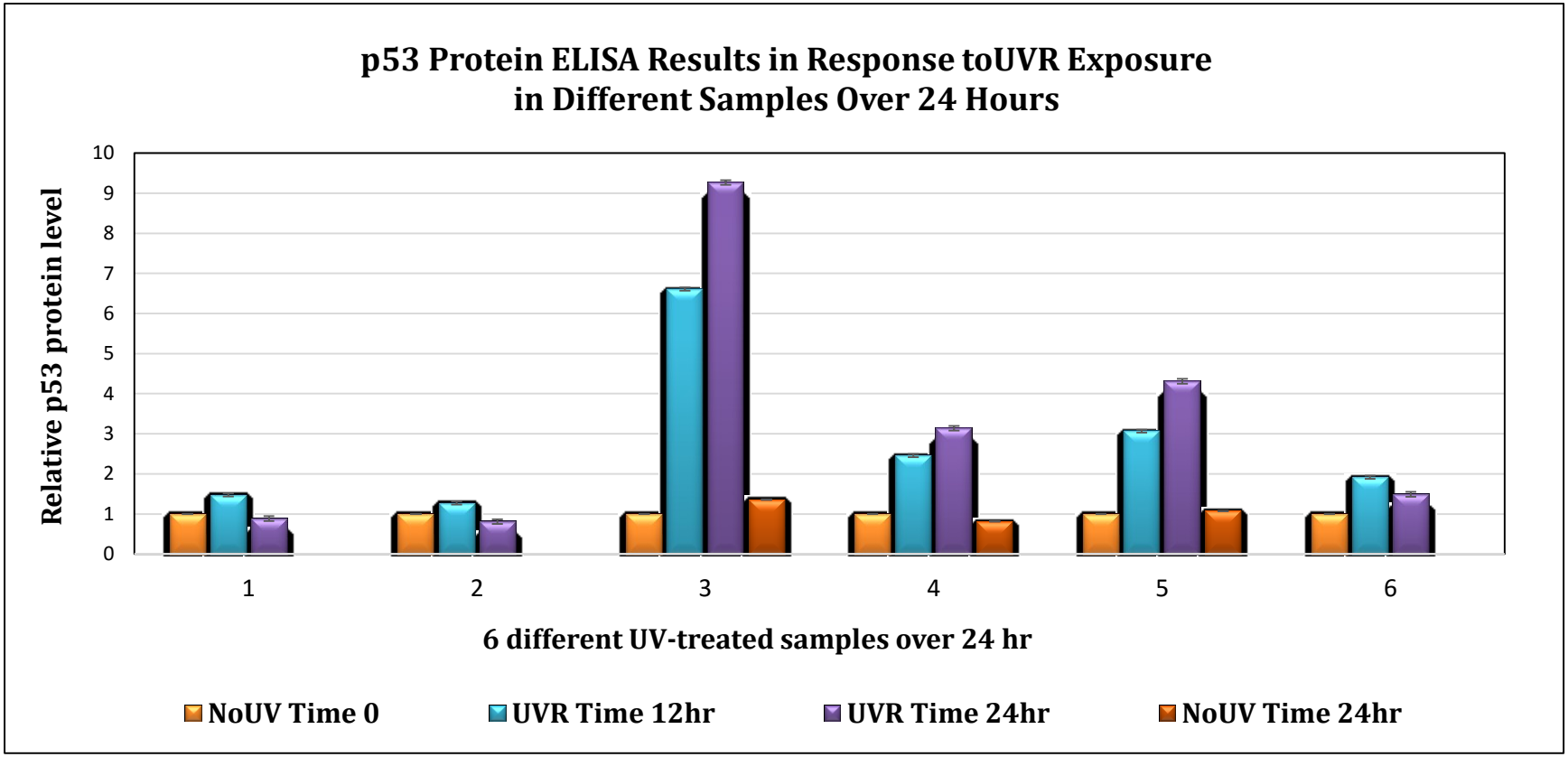

Figure 3.1: p53 protein ELISA results in response to UV light exposure in different samples over 24 h. Samples 1, 2 and 6 showed mild increase in p53 level at 12 h duration and dropped at 24 h. Sample 4 and 5 showed moderate elevation at 12 h but the levels kept increasing later on at time 24 h. Sample 3 markedly increased at 12 h and also continued to increase at 24 h. All the values have been calibrated to give the first sample of each experiment (control) a value of one to simplify the comparison among different experiments. The samples are correspondent to those in figure 3.2.

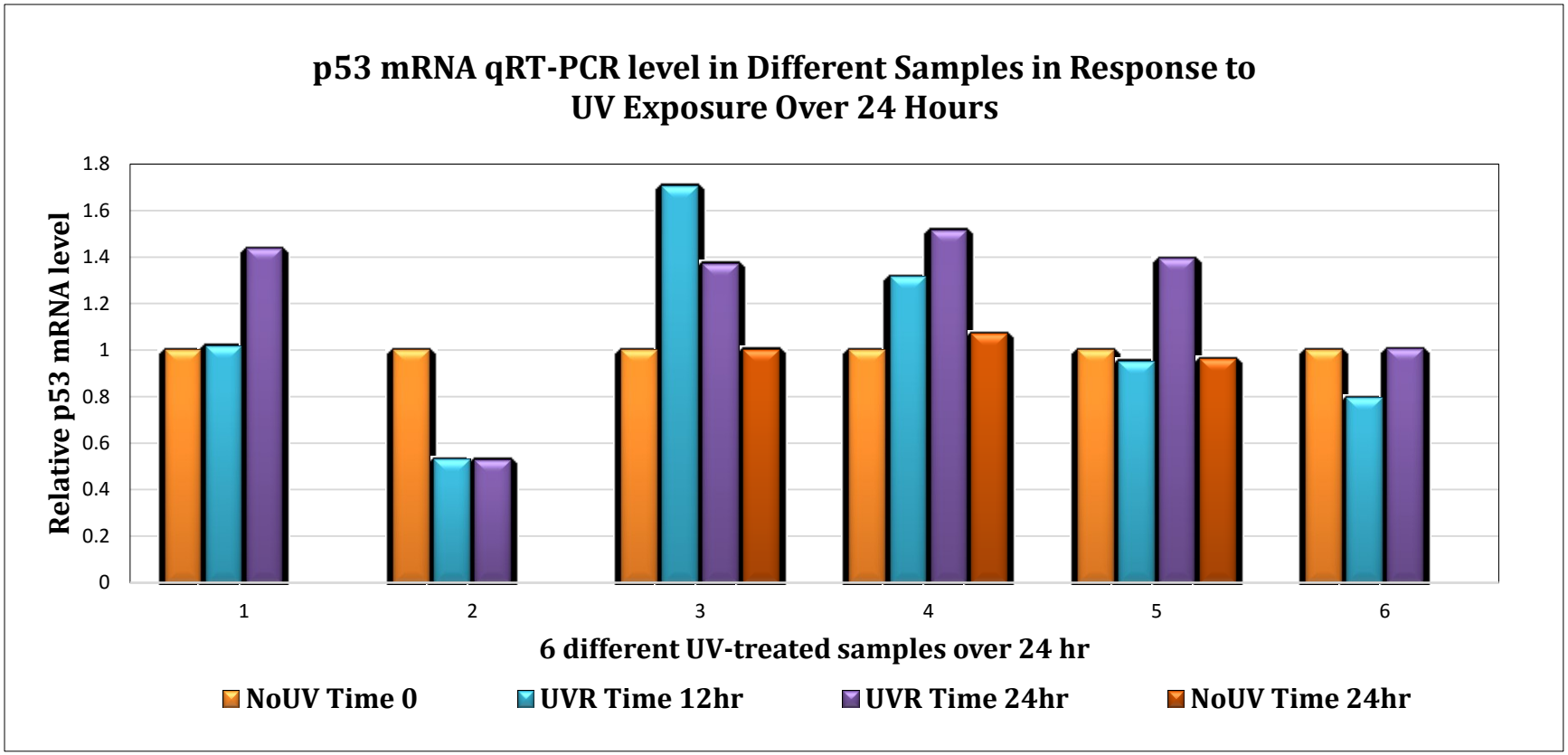

Figure 3.2: p53 qRT-PCR of 6 UV-treated human keratinocytes samples over 24 h. All the samples except the second one show increased p53 mRNA level upon UV exposure. All the values have been calibrated to give the first sample of each experiment (control) a value of one to simplify the comparison among different experiments. The samples are correspondent to those in figure 3.1.

qRT-PCR was performed on RNA isolated from the same cells and the data were plotted using column chart (figure 3.2). The results showed that there is increase in p53 mRNA level in 4 out of 6 samples. This means there is increase in p53 gene expression in these samples in response to UV-induced cellular stress.

The previous results showed there is considerable variability among different individual normal HKc strains in terms of p53 responses to cellular damage. The variability is in both the magnitude of the p53 increase and the time of the peak. Because some samples showed continuous increase at 24 h, the experiment was repeated using 4 different normal HKc strains followed for 48 h after UV treatment. p53 ELISA was performed and the results were plotted on another column chart, see figure 3.3.

In 3 of the samples, p53 level showed mild to moderate elevation at 12 and 24 h but continued going up at 48 h duration from UV exposure. On the other hand, one sample had marked increase in p53 level at 12 and 24 h durations but started to decline at 48 h.

Also, the level of p53 mRNA measured by qRT-PCR is not constant and it looks there is some increase in p53 expression in most samples when the cells are treated with UV, see figure 3.4.

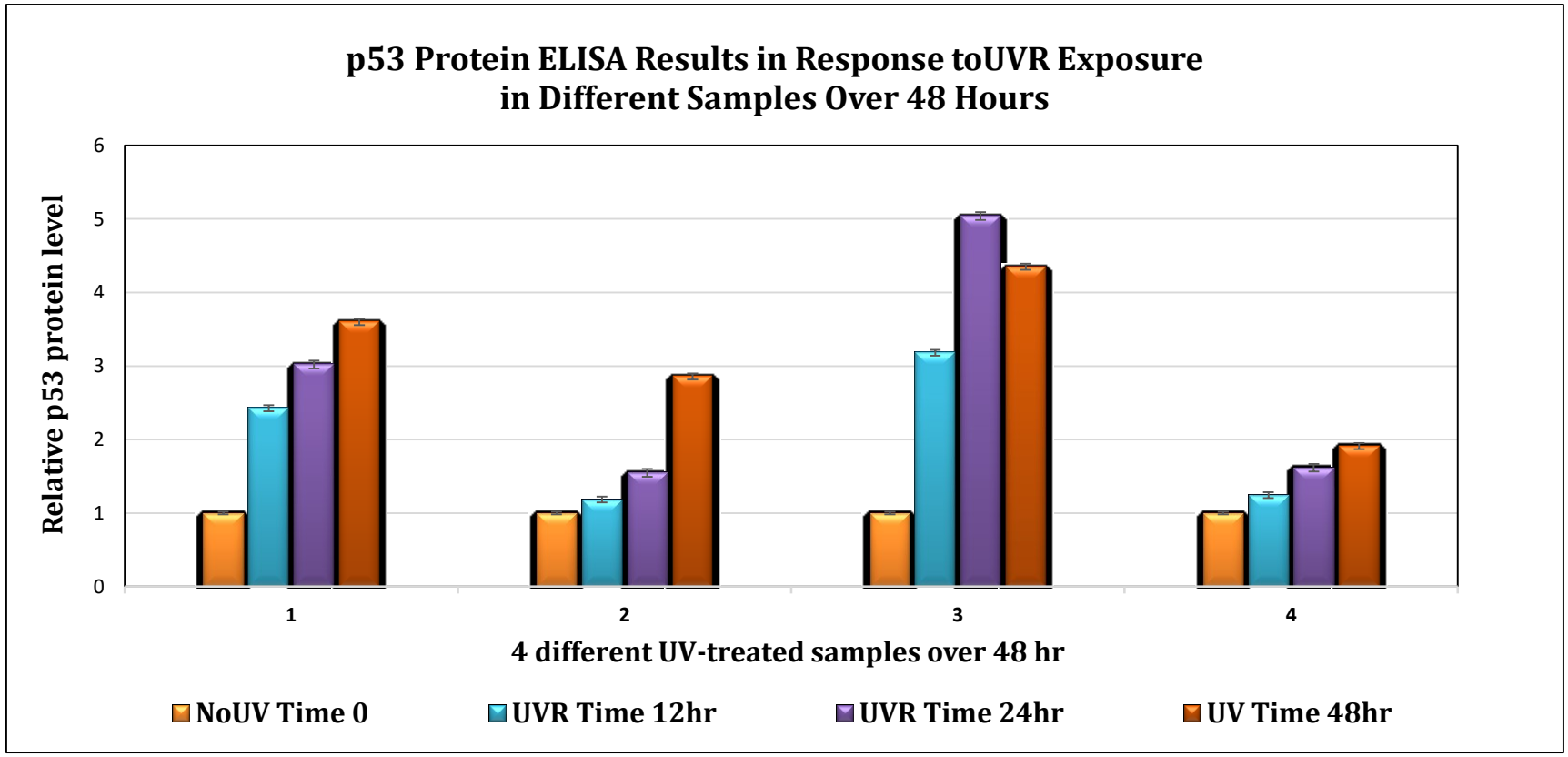

Figure 3.3: p53 protein ELISA results in response to UV light exposure in different samples over 48 h. Samples 1, 2 and 4 showed mild to moderate increase in p53 level at 12 and 24 h post UV treatment, and p53 levels continued to rise at 48 h. Sample 3 achieved marked increase in p53 protein level at 24 h but the level dropped at 48 h. All the values have been calibrated to give the first sample of each experiment (control) a value of one to simplify the comparison among different experiments. The samples are correspondent to those in figure 3.4.

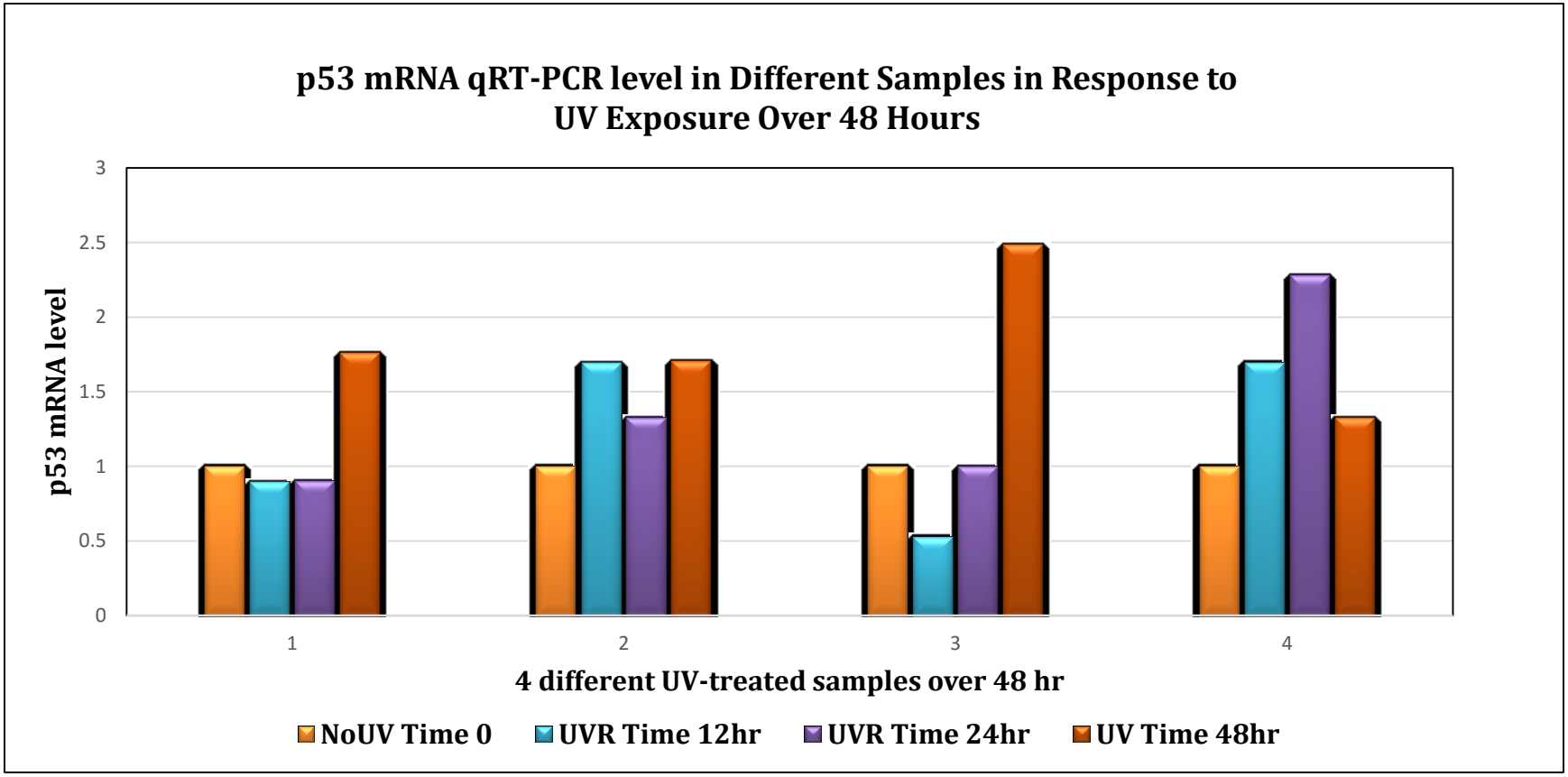

Figure 3.4: p53 qRT-PCR of 4 UV-treated human keratinocytes samples over 48 h. p53 mRNA level shows mild to moderate increases in all HKc strains treated with UV. All the values have been calibrated to give the first sample of each experiment (control) a value of one to simplify the comparison among different experiments. The samples are correspondent to those in figure 3.3.

When the results from all the experiments were combined, it became evident that p53 level behavior does not follow a constant pattern among different individuals. In some HKc strains, p53 levels increased early, to then drop at 24 h, while others continued to increase at 48 h from UV radiation. Other samples gave a considerable peak at 24 h that started to decrease at 48 h.

To determine if there are any morphological differences between the samples that associated with robust p53 stabilization and those associated with minimal changes, multiple images were taken at different time intervals as in figures 3.5 and 3.6.

On examining the UV radiated cells under the light microscope using both 4x and 10x magnification powers, there was no significant difference in terms of morphology between the cells that showed a modest response (A series) and the cells that responded with a high increase in p53 level (B series).

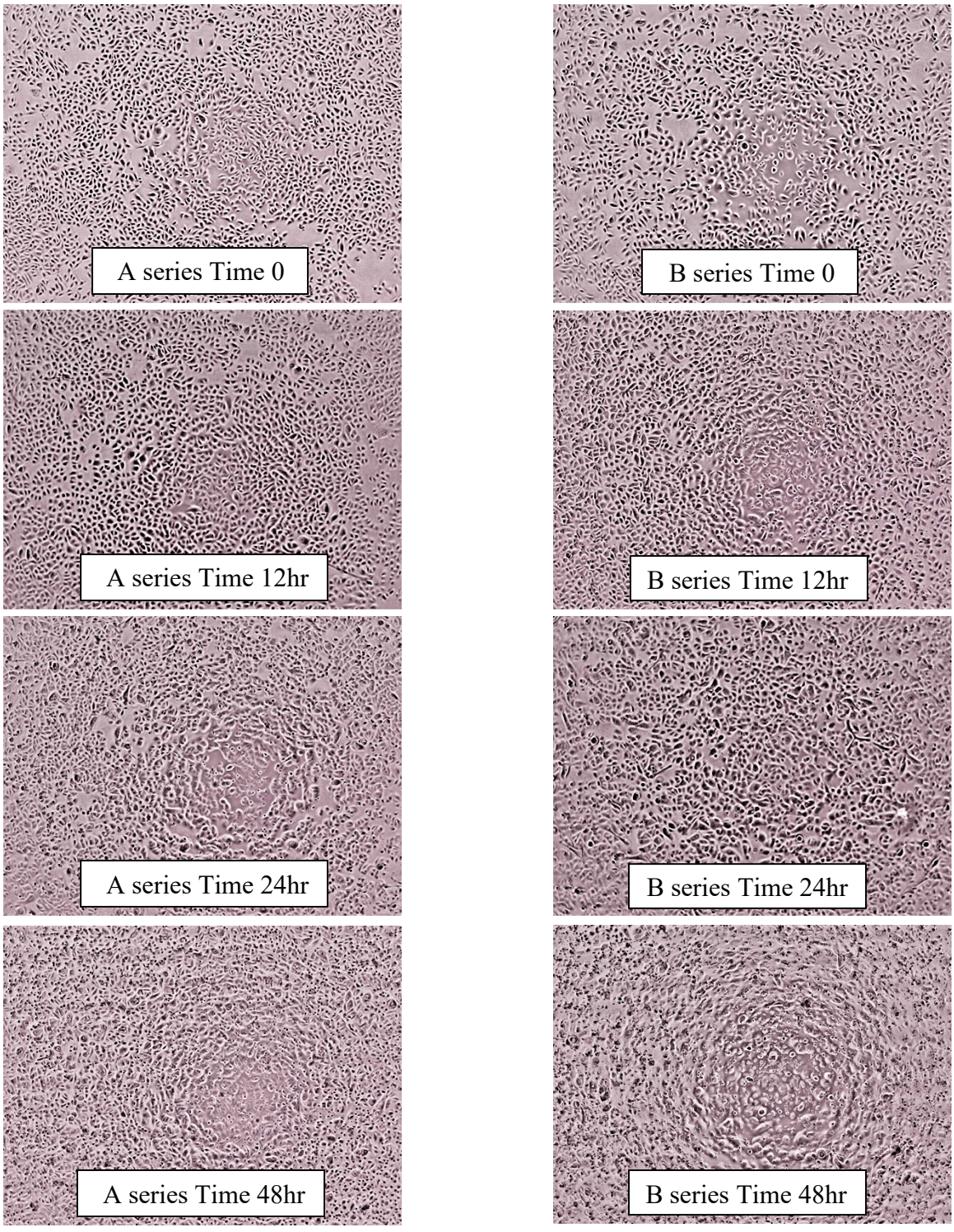

Figure 3.5: Light microscopic imaging using 4x magnification for 2 groups of cells exposed to UV. Under 4x objective lens, there was no significant variation between the cells that associate with low-peak response to UV radiation (A series) from cells that associate with high-peak response (B series).

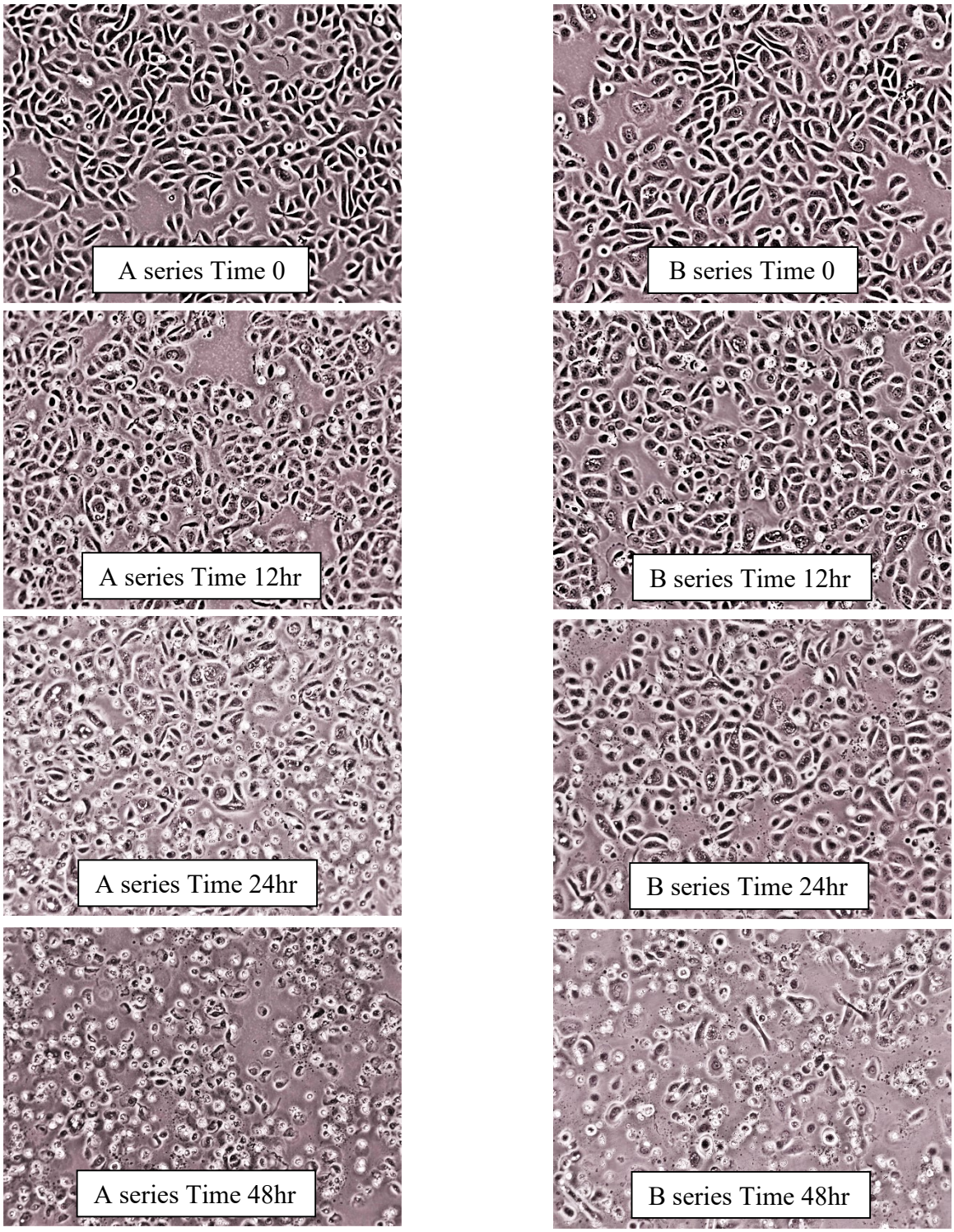

Figure 3.6: Light microscopy imaging using 10x magnification for 2 groups of cells exposed to UV. Similar to figure 11, there is no noticeable difference between the low-peak (A series) and high-peak (B series) reacting cells to UV radiation under 10x objective lens.

3.2 *Aim 2:* *What is the effect of knocking-down p53 on its response to cellular damage?*

To achieve this aim, there was a need to transfect the human keratinocytes with p53-interference plasmid (p53i) and scrambled plasmid as a control and waiting for the cell cultures to grow before exposing them to UV. Ten strains of normal HKc were used for this purpose; 5 for p53i and 5 for scrambled plasmid. However, there was a great difficulty to grow up the primary keratinocytes into 8 confluent plates after transfection with the plasmids, selection with puromycin, and trypsin based passaging. This is due to the stress imposed by each of these procedures to the primary cells.

For example, although the p53i and scrambled plasmids have puromycin resistance encoding regions, the cells transfected with these plasmids were more vulnerable to puromycin treatment than the control cells that were treated only with the transfection reagents not with the plasmids. The same results were obtained with doing different adjustments for the protocols like changing the plasmid quantity, transfection duration, puromycin concentration and plate size.

Only 1 out of the 5 p53i transfected cells and 1 out of the 5 scrambled plasmid transfected cells could be expanded to eight 60-mm plates. The cells were analyzed again by ELISA and qRT-PCR. There was mild elevation in p53 protein (figure 3.7) and mRNA levels (figure 3.8) in the p53i transfected normal human keratinocytes in response to UV despite the predicted p53 knock-down at mRNA by p53i plasmid. Scram transfected cells experienced no change in p53 protein level over 24 h from UV treatment (figure 3.9), but the p53 mRNA level showed increase in response to it (figure 3.10). However, these data can't be depended due to the small sample size which needs further experiments on different samples.

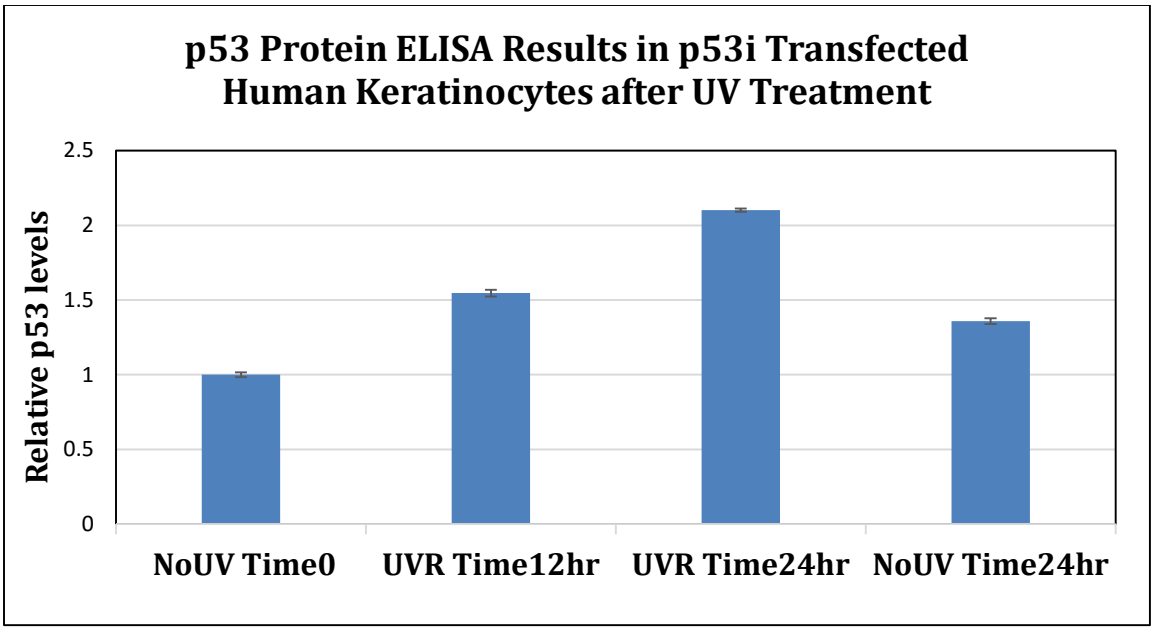

Figure 3.7: ELISA p53 protein level of UV-treated p53i transfected normal human keratinocytes. p53 protein level mildly increases over 24h from UV exposure despite the presence of p53i which knocks down p53 mRNA level.

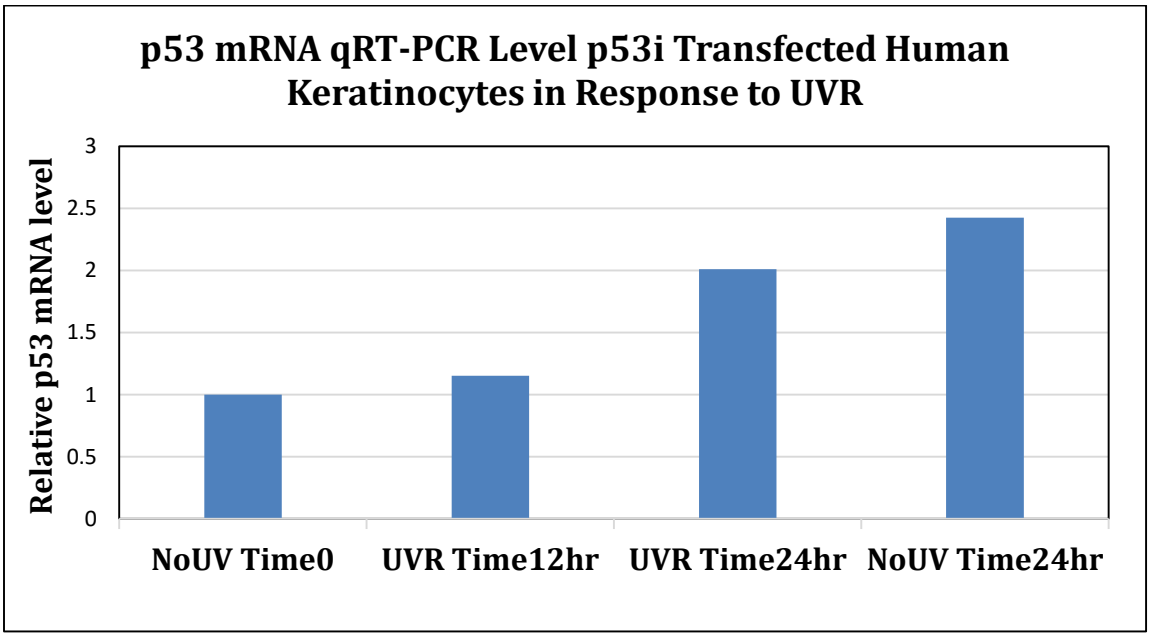

Figure 3.8: qRT-PCR p53 mRNA level of UV-treated p53i transfected human keratinocytes. Apparently, there is increase in p53 expression despite its knock-down at mRNA level by p53i. However, there is unexpected high level in the non-UV treated cells collected 24 h from the start of the experiment.

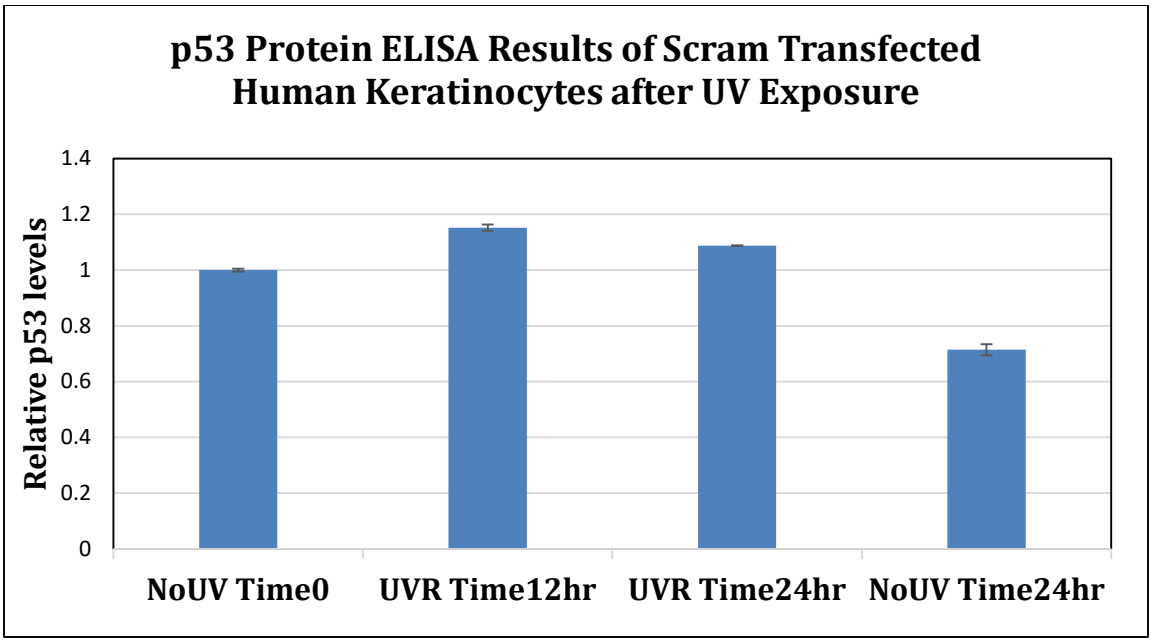

Figure 3.9: ELISA p53 protein level of scram transfected normal human keratinocytes upon UV treatment. Here with scram transfected keratinocytes, there is no detectable change in p53 level over 24 h period.

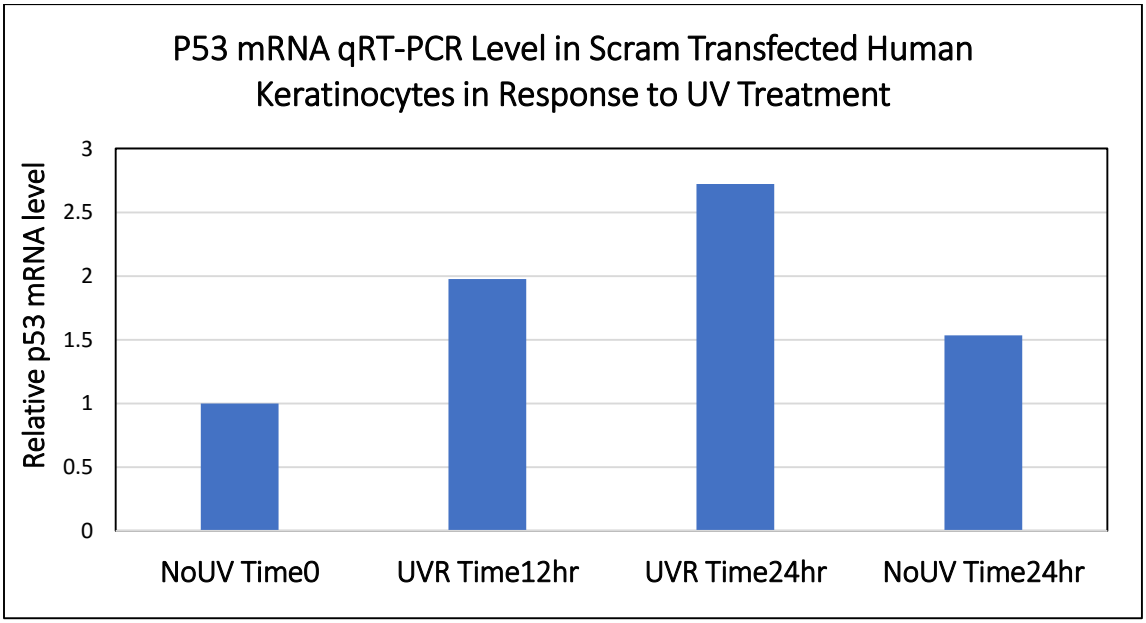

Figure 3.10: qRT-PCR p53 mRNA level in UV-treated scram human keratinocytes. P53 mRNA level increases upon UV exposure in normal human keratinocyte transfected by scrambled plasmid.

CHAPTER 4: DISCUSSION

The results presented here indicate that there is significant variability in tumor suppressor p53 protein responses to UV treatment among normal HKc strains from different individuals. As normal HKc strains were derived in the same way and expanded as soon as cells became available into the number of dishes required for the experiments, mostly at the third passage cells, it is unlikely that differences in time in culture or population doubling level may account for these responses. However, we have not made a specific correlation between population doublings and p53 responses in normal HKc, and this will have to be done.

In terms of the magnitude of p53 level increase in response to cellular stress imposed by ultraviolet ray exposure, it ranged from low to moderate to high peak response. The normal HKc strains also differed from each other in the timing of the peak of the response, some have peaked at 24 h after UV exposure while in others p53 levels keep increasing at 48 h. It is also important to mention that these differences were not due to racial variability because most of the samples were of black skin and only one of them which is the fifth sample in figure 3.1 was white skin. This was not by design but it depended on the availability and the cells' efficiency in culturing and growth which could depend on tissue freshness in terms of how long the period between circumcision and skin processing is. Therefore, p53 responses could be due to genetic variability among people or the presence of various cellular biomolecules that could alter p53 activity.

Under the light microscope, there were no noticeable morphological features to differentiate the cells that follow either response, as well as, there is no characteristic morphological variation among the groups on different time intervals after UV exposure.

Although the sample size was inadequate for dependable data, we confirmed Nella's observation that p53 mRNA and protein respond to cellular damage even if p53 was knocked-down with a p53i plasmid.

We observed an increase in basal mRNA levels for p53 in cells expressing the p53i: this is probably due to compensatory increase in p53 gene expression that may occur when protein levels of p53 are decreased by "external" factors such as E6 or p53i. The mRNA levels for p53 are higher in HPV-transformed cells both in vitro (see Nella Delva's work, shown in the introduction) and in HPV-active cancer samples [81].

The finding that tumor suppressor p53 response is not constant among different individuals could explain, at least in part, the variable tendency to develop cancer among people and the variability in prognosis because p53 is a checkpoint against the accumulation and expansion of genetic abnormalities that could potentially lead to transformation and cancer development. As we mentioned earlier, the head and neck cancers in African Americans tend to be HPV-negative and with poorer prognosis and response to treatment than in European Americans, who tend to have HPV-positive cancers with better response to treatment like chemotherapy. This probably could be explained in two points: first, HPV-negative tumors have a higher percentage of p53-loss of function mutations which abolish the functions of p53 as a checkpoint within the cells. In HPV-positive tumors, the inhibition of p53 by E6 is partly opposed by E7 and we have shown that E6 action does not lead to complete elimination of p53 function. The same appears to

be true if we simulate the action of E6 using p53i, as in our experiments. Therefore, a minimal p53 function could still be present in HPV-positive tumors trying to direct the cancerous cells to apoptosis or senescence in response to chemo- or radiation therapy. Second, according to gene ontology results, the HPV-signature in HPV-positive tumors includes an increased activity of genes involved in mitosis and proliferation. These features can make the HPV-positive tumors more vulnerable to chemotherapy which targets rapidly dividing cells.

The limitations of this study are the relatively low number of normal HKc strains analyzed, and the fact that the experiments utilizing the p53i plasmid had a poor yield of cells, and will need to be repeated.

In conclusion, tumor suppressor p53 response to UV are different in magnitude and duration among normal HKc strains derived from different normal individuals. These differences could arise from differences in various biomolecular pathways in the cellular milieu that could affect p53 function. Further, p53 level might not depend solely on its stabilization, but also on increasing gene expression. When all these findings are linked to HPV-positive inactive tumors, it can be inferred that the p53-suppressing role of HPV E6 can be replaced by other molecular pathways when they are activated by tumor promoting factors. This supports our hypothesis which states ***the HPV-positive inactive tumors arise as HPV-active and the virus as initiator induces the early transformational changes which can evolve into malignancy in the presence of carcinogenesis promoters like smoking and alcohol in an HPV oncoproteins-independent pattern in the HPV-positive inactive tumors.***

4.1 Future work:

Our future direction is to repeat the experiments to proof the correctness of the results by including more white skin samples, and to also determine whether the passage number (i.e.: the number of population doublings from primary culture to expansion for the analysis of p53 responses to UV) makes any difference in these responses. In other words, are p53 responses to UV in any way dependent on how long the cells have been in culture, and therefore on how far advanced the cells are toward natural senescence? A corollary experiment would be to analyze the stem cell-like component of each normal HKc strain and compare it with the mass population, in terms of p53 responses to UV irradiation. We also need to further explore how p53 activation by UV is linked to apoptosis in cells from different individuals.

We will try to optimize the transfection process to check the p53 activity in p53-knocked-down primary cells. We will use CRISPR/Cas 9 system for p53 to completely inactivate the gene in some cells, and then to explore the UV responses of these cells in comparison with cells from the same individual transfected with HPV16 or p53i. We will look for the activated (phosphorylated and acetylated) p53 and compare the results with that of total p53 we obtained using other ELISA p53 kits and to check if there is difference among people with different p53 activity. Gene sequencing is to be done to know the differences in p53 gene among samples with different responses. If these experiments' results will support the data, a further step will be taken in analyzing the genetic variation for the tested samples.

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