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Targeting EGFR/ERK/FOS signaling as a novel approach for HPV-positive Head and Neck cancer treatment

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List of abbreviations in alphabetical order

AGW	Anogenital warts
AML	Acute myeloid leukemia
AP-1	Activating protein-1
ATF	Activating transcription factors
ATF/CREB	Activating transcription factor, cyclic AMP-responsive element-binding
ATG	Autophagy related gene
ATM	ataxia-telangiectasia mutated
ATR	ataxia-telangiectasia and Rad3-related protein
BPE	Bovine pituitary extract
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin dependent kinase inhibitor 2A
ChIP	Chromatine immunoprecipitation
CNA	Copy number alteration
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9
DDR	DNA damage response
DEG	Differential gene expression
Dlg1	Drosophila disc large tumor suppressor 1
DMEM	Dulbecco's Modified Eagle Medium
E2F	E2 transcription factor
E6AP	E6-associated protein
ECL	Enhanced chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
EFG	Epidermal growth factor
EFGR	Epidermal growth factor receptor
eIF4E	Eukaryotic transcription factor 4E
Elk1	ETS Like-1 protein
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GPCR	G protein-coupled receptor

gRNA	Guide RNA
GSEA	Gene Set Enrichment Analysis
HIF-1a	Hypoxia inducible factor 1a
HK	Primary human keratinocytes
HNSCC	Squamous cell carcinoma of the Head and Neck
HPSG	Heparan sulfate proteoglycans
HPV	Human Papillomavirus
HPV-	HPV-negative
HPV+	HPV-positive
HR	High Risk
IC50	Half maximal nhibitory concentration
JNK	Jun N-terminal kinase
KSFM	Keratinocyte serum free medium
LC3	Light chain 3
LR	Low Risk
Maf	musculoaponeurotic fibrosarcoma
MAML	mastermind-like
MAP3K	MAPKK kinase
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
NEAA	Non-essential aminoacids
NF-kB	Nuclear Factor kappa-light chain-enhancer of activated B cells
OPSCC	Oropharyngeal squamous cell carcinoma
ORF	Open reading frame
p-ERK	Phosphorylated ERK
PCR	Polymerase chain reaction
PDGFR-b	Platelet derived growth factor receptor b
PDX	Patient-derived xenograft
PDZ	PSD95, Dlg-1, zo-1
PI	Propidium iodide
PI3,4P2	Phosphatidylinositol-3,4-bisphosphate

PI3K	Phosphoinositide 3-kinase
PI3K	Phosphoinositide 3-kinase
PI3KCA	Class I PI3K catalytic subunit
PI4,5P2	Phosphatidylinositol-4,5-bisphosphate
pRb	Retinoblastoma protein
PSD95	Post synaptic density protein
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
Rab7	Ras-associated protein-7
Ras	Rat sarcoma
RPA	Replication Protein A
RPKM	Reads per kilobase million
RTK	Receptor tyrosine kinase
SCC	Squamous cell carcinoma
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SMAD	small mother against decapentaplegic
TALEN	Transcription activator-like effector nuclease
TBS-T	Tris buffered saline plus tween-20
TCGA	The cancer genome atlas
TGF-a	transforming growth factor a
TGF-b	transforming growth factor b
TP53INP1	Tumor protein p53-induced nuclear protein 1
ULK	Unc-51 like autophagy activating kinase
UVRAG	UV radiation resistance-associated gene
VEFG	Vasculr endotelial growth factor
VEFGR	Vasculr endotelial growth factor receptor
VLP	Virus-like particle
VPS15	Vacuolar protein sorting 15
VPS34	Vacuolar protein sorting 34
Wnt	Wingless Int-1
Zo-1	Zonula occludens-1

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1 Abstract

Squamous cell carcinoma of the head and neck (HNSCC) is the 6th most common cancer worldwide. It accounts for 600.000 new cases and 350.000 deaths every year. HNSCC can be subdivided into two distinct subgroups: HPV-positive and HPV-negative. They present very different features including etiological agent, age, socioeconomic status, prognosis, genetic landscape, and tissue differentiation. Nevertheless, all HNSCC patients are treated with the same therapies, comprising chemo- and radiotherapy, and/or surgery. Specific therapies for HPV-positive HNSCC are needed. HPV oncoproteins E6/E7 are the drivers of oncogenesis in HPV-related cancers and they can be used as targets for therapy. To this end, we studied the effects of the knock down of E6/E7 in HNSCC-positive cell lines and we looked for pharmacological treatments to downregulate E6/E7 levels. We found that targeting the EGFR/ERK/FOS signaling pathway induced a transcriptional reduction of E6/E7 and that the ERK inhibitor Ravoxertinib was effective in killing HPV-positive HNSCC cell lines. Thus, this is a potential novel approach to address HPV+ HNSCC cancer.

2 Introduction

2.1 Human Papillomaviruses

2.1.1 Classification

Human Papillomaviruses (HPVs) are small DNA viruses that belong to *Papillomaviridae* family. Until now, 205 different HPV types have been classified, subdivided into five genera: 65 *Alphapapillomaviruses*, 51 *Betapapillomaviruses*, 84 *Gammapapillomaviruses*, 4 *Mupapillomaviruses* and one *Nupapillomavirus* (described in Table 1). There are at least 19 additional types that have not been classified yet (Van Doorslaer et al. 2013).

Papillomaviruses (PVs) that are part of a genus sharing 60–70% L1 sequence identity are termed a species; PVs within a species with 71–89% L1 sequence identity are considered a type; PVs with 90–98% L1 sequence identity are termed subtypes and those with >98% L1 sequence identity are considered variants (Bernard 2005; De Villiers 1997).

HPVs can be subdivided in two subgroups: high risk (HR) and low risk (LR) HPVs. Among the low-risk types, non-oncogenic HPVs are associated with anogenital warts (AGWs), some types of cutaneous warts, recurrent respiratory papillomatosis and Heck's disease, a rare benign mucosal proliferation strongly associated with HPV infection (Said et al. 2013; Brianti, De Flammineis, and Mercuri, 2017). High-risk types are oncogenic and associated with cervical, penile, anal, vaginal, vulvar, non-melanoma skin cancers and oropharyngeal cancers. The greatest importance is given to HPVs in the alpha genus (*Alphapapillomaviruses)*, as they are associated with the aforementioned kinds of cancer.

Table 1 - *Main HPV genotypes and their associated diseases.*

Genus	Species	Representative HPV types	Tropism	Associated Diseases
Alpha-PV	**α1**	32	mucosal	Heck's disease
	α2	3, 10, 28	cutaneous	Flat warts
	α4	2, 27, 57	cutaneous	Common warts
	α7	18, 39, 45, 59, 68	mucosal	Intraepithelial neoplasia, invasive carcinoma
	α9	16, 31, 33, 35, 52, 58	mucosal	Intraepithelial neoplasia, invasive carcinoma
	α10	6, 11	mucosal	Condylomata acuminate
		13		Heck's disease
Beta-PV	**β1c**	5, 8, 12, 14, 19, 20, 21, 24, 25, 36, 47	cutaneous	Epidermodysplasia verruciformis
	β2	9, 15, 17, 22, 23, 37, 38	cutaneous	Epidermodysplasia verruciformis
	β3	49	cutaneous	Epidermodysplasia verruciformis
Gamma-PV	**γ1**	4, 65	cutaneous	Warts
	γ4	60	cutaneous	Warts
Mu-PV	**μ1**	1	cutaneous	Plantar warts
	μ2	63	cutaneous	Warts
Nu-PV	**ν**	41	cutaneous	Warts

This table summarizes information on the main HPV genotypes, their tropism and associated diseases. Information in this table was gathered from several sources including pave.niaid.nih.gov (Van Doorslaer et al. 2013; De Villiers 1997; Cubie 2013). Adapted from Harden and Munger 2017 "Human papillomavirus molecular biology". Mutat Res Rev Mutat Res. 2017;772:3-12. doi:10.1016/j.mrrev.2016.07.002.

2.1.2 Structure and organization

HPVs are non-enveloped viruses consisting of an icosahedral capsid of about 60 nm in diameter. They are DNA viruses with a single molecule of double stranded circular DNA of approximately 8,000 base pairs (Knipe and Howley 2020). HPVs contain three genomic regions, including approximately ten open reading frames (ORFs). Polycistronic mRNAs generate many of the viral proteins (Favre et al. 1975; Zheng and Baker 2006).

The viral genome can be subdivided into three regions:

1. The early region (E), consisting of up to seven ORFs encoding viral regulatory proteins;
2. The late region (L), encoding two viral capsid proteins;
3. The long control region (LCR), also called the upstream regulatory region (URR), containing the origin of replication and transcription control sequences.

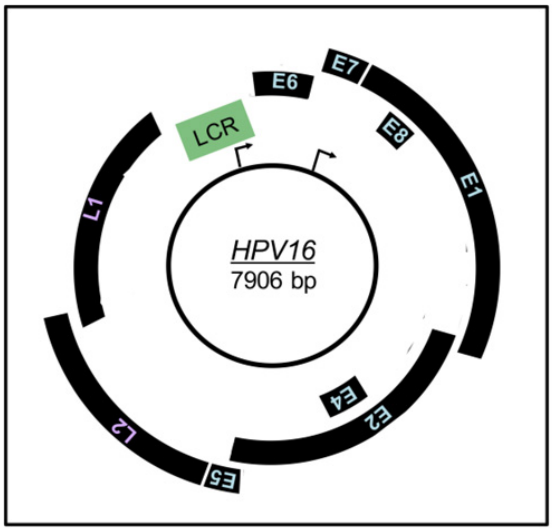
2.1.2.1 Viral Proteins

The early proteins include E1, E2, E4, E5, E6, E7 and E8 (Zheng and Baker 2006; J. Doorbar et al. 1986) (figure 1). E1 codes for an adenosine triphosphate (ATP)-dependent viral DNA helicase that can bind to the AT-rich origin of replication, the only HPV enzyme (Bergvall, Melendy, and Archambault 2013).

E2 protein functions in viral transcription, replication and genome partitioning (Bergvall, Melendy, and Archambault 2013). E2 protein can exist in a full length form, encoding a transcriptional activator, or a truncated form, transcribed from an internal ATG sequence, encoding a transcriptional repressor, as well as the E8^E2 fusion protein (McBride 2013).

E4 is embedded within the E2 gene and is primarily expressed during the late stages of the viral life cycle as an E1^E4 fusion protein. E4 is able to bind and disrupt cytokeratin filaments, and is thought to play a role in viral escape from cornified epithelial layers (John Doorbar 2013).

E5 is a small multi pass transmembrane protein, that forms dimers, interacts with and activates receptors tyrosine kinase (RTK), playing a role in proliferation and in evasion of the immune response (DiMaio and Petti 2013).



EARLY REGION: proteins necessary for viral replication

LATE REGION: viral capsid proteins

LONG CONTROL REGION: sequences controlling viral replication & transcription

ORF	HPV16 PROTEIN FUNCTION
E1	origin binding protein, ATPase-dependent helicase involved in genome replication
E2	regulator of viral gene transcription, association with E1 (origin binding), viral genome partitioning
E4	expressed abundantly as E1^E4 fusion protein, cytokeratin network destabilization, virus release and transmission
E5	small transmembrane protein, interacts with EGF receptor activating mitogenic pathways
E6	drives cell cycle allowing genome amplification in upper epithelial layers, association with E6AP and degradation of p53, PDZ-protein binding, hTert activation
E7	drives cell cycle allowing genome amplification in upper epithelial layers, association with and degradation of pRB, mitotic mutator
E8	expressed as E8^E2 fusion protein, acts as a repressor of transcription and replication during the viral life cycle
L1	major capsid protein, assembles into pentameric capsids forming the icosahedral virion (prophylactic vaccines)
L2	minor capsid protein, involved in viral DNA encapsidation, facilitates viral entry and trafficking

Figure 1 - HPV16 Genome Organization and Protein function.

On the left, the HPV16 genomic map containing 7906 base pairs, only the coding strand is shown. Arrows indicate promoters. The early region is depicted in light blue and contains proteins necessary for viral replication including E1, E2, E3, E4, E5, E6 and E7. The late region is shown in purple and contains the viral capsid proteins L1 and L2. The LCR is shown in green and contains sequences controlling viral replication and transcription. Viral oncoproteins are briefly described in the table on the right. Adapted from Harden ME, Munger K. "Human papillomavirus molecular biology". Mutat Res Rev Mutat Res. 2017;772:3-12. doi:10.1016/j.mrrev.2016.07.002.

HPV E6 and E7 allow genome amplification driving cell cycle progression in upper epithelial layers. E6 and E7 belonging to High Risk Human Papillomaviruses (HR HPVs) are oncogenic.

HR HPV E6 induces the degradation of p53, a tumor suppressor, fundamental to prevent cancer occurrence. p53 has many different functions ranging from induction of DNA damage response (DDR), to cell cycle arrest and apoptosis (Zilfou and Lowe 2009). Its importance in preventing tumors is given by the fact that it is mutated in about 50% of cancers (Muller and Vousden 2013). HR HPV E6 interacts with p53 and with the ubiquitin E3 ligase E6-associated protein (E6AP) in a ternary complex, inducing the ubiquitination of p53, followed by its degradation by the

proteasome system (Figure 2a). Only HR HPVs are able to induce p53 degradation (Scheffner et al. 1990; Oh, Longworth, and Laimins 2004).

This mechanism keeps p53 at low levels, so that infected cells can escape from apoptotic processes and cell cycle arrest. p53 downregulation causes chromosomal instability, eventually leading to carcinogenesis (J. T. Thomas and Laimins 1998; Thompson et al. 1997). HR HPV E6 protein can also activate telomerase, a ribonucleoprotein that elongate telomeres to protect chromosomes from DNA damage and from fusion with neighbor chromosomes, usually overexpressed in cancer (Klingelhutz, Foster, and McDougall 1996). Moreover, HR HPV E6 targets cellular proteins containing a Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and Zonula occludens-1 protein (zo-1) (PDZ) domain, important for cell contact and signaling pathways (Kiyono et al. 1997; Lee, Weiss, and Javier 1997).

LR HPV E6 protein does not activate telomerase and is not able to directly interact with p53 and to send it to degradation, but it can downregulate its transcriptional activity by binding to p300 or histone acetyltransferase Tip60 (M. C. Thomas and Chiang 2005; Jha et al. 2010). E6 proteins of beta HPVs do not target these pathways. Instead, they inhibit Notch and transforming growth factor β (TGFβ) signaling by associating with mastermind-like (MAML) and small mother against decapentaplegic (SMAD) proteins, respectively (Brimer et al. 2012; Tan et al. 2012; Rozenblatt-Rosen et al. 2012; Mendoza et al. 2006).

HR HPV E7 protein binds and degrades the retinoblastoma tumor suppressor (pRB) (Roman and Munger 2013; Dyson et al. 1989a). pRb is a member of the so-called *pocket proteins* family together with p107 and p130 (Dyson et al. 1989b; T. Hu et al. 1995; Davies et al. 1993; Smith-Mccune et al. 1999). Pocket proteins are involved in cell cycle regulation: they interact with E2F (from E2 transcription factor, for the ability to bind the adenoviral protein E2), a family of transcription factors in higher eukaryotes (reviewed in Kouzarides 1995). E2Fs have emerged as fundamental transcriptional regulators of cell cycle-dependent gene expression. Expression of E2F target genes, given by E2F activity, is high in all cancers, often due to inactivation of its main binding partner and key regulator pRB, overexpression of cyclin-dependent kinases (CDKs) or inactivation of CDK inhibitors (Kent and Leone 2019). pRb, in a hypophosphorylated form, negatively regulates E2F, by interacting with it and causing a quiescent state of the cell. D-cyclins are transcribed

in the presence of mitogenic signals and associate with CDKs, specifically CDK4 and CDK6, which phosphorylate pRb. Hyperphosphorylated pRb induces the release of E2F that exerts its transcriptional function expressing a family of genes involved in cell cycle progression (Sherr and Roberts 1999).

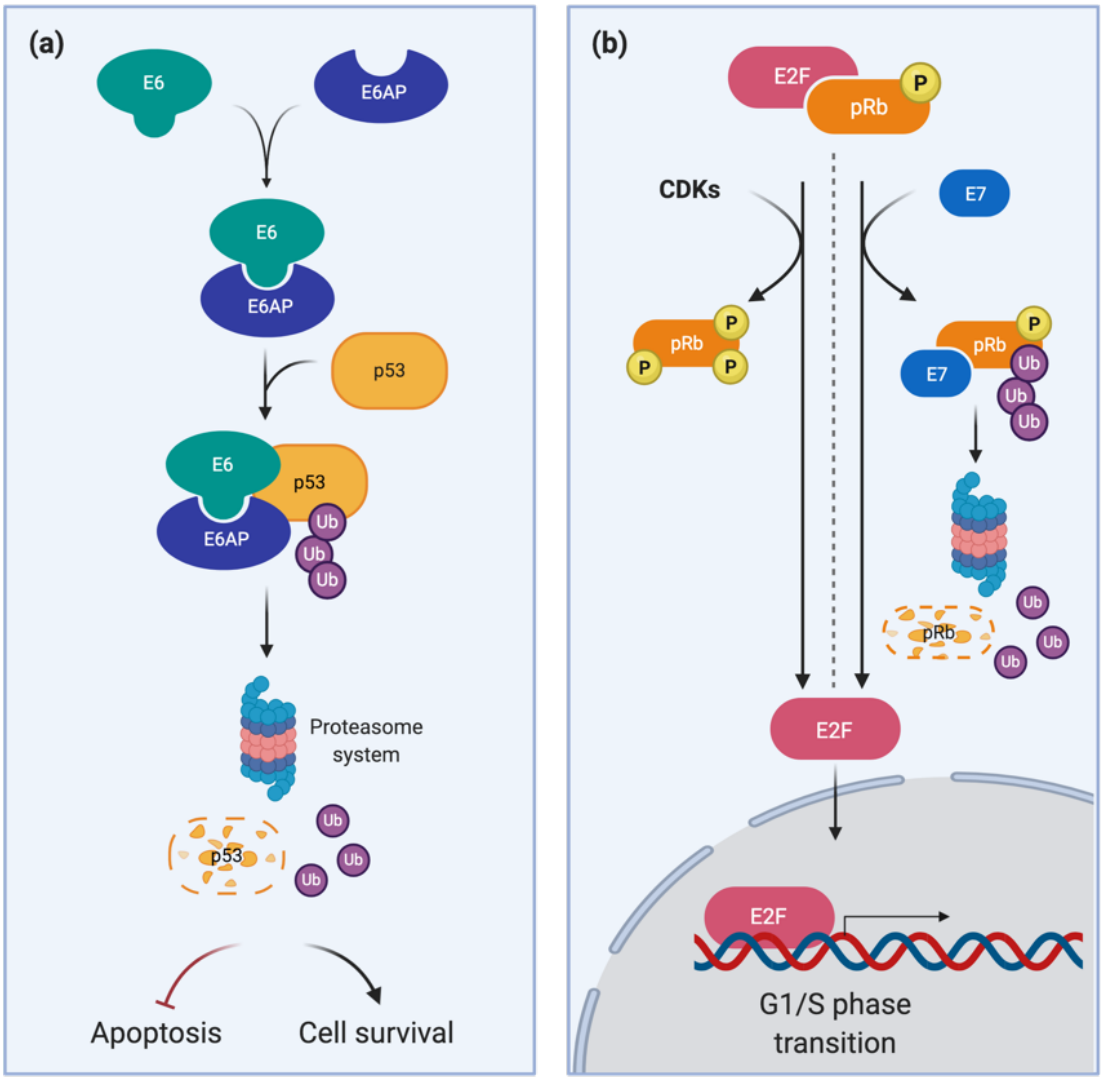


Figure 2 – E6 and E7 oncoproteins inactivate p53 and pRb pathways, respectively.
(a) E6-mediated degradation of p53 through the proteasomal system. (b) E7 induction of inactivation and degradation of pRB, leading to cell cycle progression. Adapted from Medda et al. (Submitted).

E7 oncoprotein binds to pRb, inducing the release of E2F and leading to uncontrolled cell cycle progression. HR HPV E7 inactivates and destabilizes pRb even in the absence of CDKs, sending it to proteasomal degradation (Figure 2b) (Tommasino and Crawford 1995; Jones and Münger 1997; Reinstein et al. 2000). E7 proteins from the LR HPV 6 and 11 have a lower pRb binding efficiency, and do not show transforming activity *in vitro* (Munger et al. 1989; Storey et al. 1988).

Moreover, E7 protein from LR HPV1 does not induce transformation of primary cells even if it shows high affinity to pRb as HR HPVs; however, it is not capable of inducing pRb degradation (Storey et al. 1988; zur Hausen 2009).

The major late gene L1 transcribes capsid proteins assembling into pentameric capsids and forms the icosahedral virion, whereas the minor late gene L2 is important for the viral DNA encapsidation. The L1 ORF is used for the phylogenetic organization and classification of HPVs, since it is the most variable gene. L1 is important for the currently available prophylactic vaccine formulation, because it spontaneously assembles into virus-like particles (VLPs) (Buck, Day, and Trus 2013)

2.1.3 HPV life cycle and infection

HPVs life cycle has been extensively studied in the last decades. Some aspects are very well defined, some others still need further elucidations.

Viral genomes enter, establish in the nucleus, and are replicated, together with cellular chromosomes, in basal cells. Infected basal cells show a low expression level of viral genes. In contrast, differentiated cells present high expression levels of viral transcripts. The process of vegetative genome replication (or amplification), as well as late gene expression and virion assembly, occurs in highly differentiated cells of the uppermost epithelial layers (Harden and Munger 2017).

2.1.3.1 Viral Attachment and Entry

The first step of viral entry is the attachment to proteins that serve as primary receptor on basal cells or exposed basement membranes: heparan sulfate proteoglycans (HSPGs) (Bienkowska-Haba, Patel, and Sapp 2009). L1 viral protein attaches to HSPGs, inducing conformational changes in the capsid. It results in loss of affinity to the primary receptor and transfer of the virus to an entry receptor. In this way, L2 amino terminus is exposed to cleavage by furin-related proteases, necessary for infections of some HPVs (Richards et al. 2006; P. M. Day and Schiller 2009; Cruz et al. 2015).

Capsid internalizes asynchronously and this step can take from two to four hours; some capsids remain on the surface for more time than others. Actin retrograde flow propels capsids by directed motion from filopodia to the cell body (Schelhaas et al. 2008; J. L. Smith, Lidke, and Ozbun 2008).

The virus is endocytosed in a clathrin, caveolin and lipid raft independent manner, then virions are trafficked and partially uncoated in acidified endosomes (Schelhaas et al. 2012; P. M. Day and Schelhaas 2014). Cyclophilin B helps the dissociation of L1 protein from the L2/viral genome complex and L1 is degraded in the lysosomes (Bienkowska-Haba et al. 2012).

L2 minor capsid protein interacts with sorting nexin 17 to facilitate the escape of L2/viral genome from late endosomal compartments. This interaction is essential for viral infection (Bergant Marušič et al. 2012; Bergant and Banks 2013). L2 associates with sorting nexin 27 to aid in viral trafficking through the retromer complex (Pim et al. 2015). The virus moves through the cytoplasm to the nucleus thanks to the motor protein dynein (L2-associated vesicles interact with dynein light chains that moves along microtubules) (Florin et al. 2006; Schneider et al. 2011). The viral genome enters into the nucleus during mitosis and L2/viral genome complex localizes at ND10 domains: this is an essential step in the establishment of infection, allowing transcription of the viral genome (P. M. Day et al. 2004).

Viral genome transcription can start with one to three days of delay from the binding to cell surface (P. M. Day, Lowy, and Schiller 2003; Roberts et al. 2007). However, infection of mitotic cells is faster, with nuclear entry and viral genome transcription occurring at earlier time points (Broniarczyk et al. 2015).

2.1.3.2 Genome Replication and Gene Expression

Immediately after infection, viral genome is amplified before maintenance in the nuclei of infected basal epithelial cells. It is maintained in basal cells as stable multicopy plasmid or episome. The first viral proteins expressed are E1 and E2, required for the initial amplification of the genome (Maglennon, McIntosh, and Doorbar 2011). It is not clear which is the real number of episomal copies: in cell line studies the viral copy number was found to be approximately 200 per cell, while 50-100 copies were detected using laser capture technique on productive warts (Maglennon, McIntosh, and Doorbar 2011; John Doorbar et al. 2015). HPV genome replicates once per cell cycle during S phase, maintaining persistent infection of basal cells. However, after infection, HPV DNA can remain latent within cells even though others have entered the productive cycle. During this latent phase viral genomes can persist in the basal epithelium for years or decades.

Vegetative replication of HPV DNA occurs in differentiating cells of the squamous epithelium thanks to E1 and E2 viral proteins (Bergvall, Melendy, and Archambault 2013). E1 is required for initiation and elongation of DNA synthesis as it binds AT rich sequences at the viral origin of replication with weak affinity. This binding is stabilized by E2, which interacts with $ACC(N)_6GGT$ sequence, thus creating a high affinity binding of E1/E2 complex to the origin of replication (McBride 2013). Since no other replication enzymes are encoded by HPV, it is the host DNA synthesis machinery that replicates also viral genome. In normal conditions, differentiating cells would not be capable of DNA synthesis because they have exited cell cycle upon leaving the basal layer of epithelium. However, E1 and E2 recruit components of the cellular DNA replication machinery to the HPV replication fork, such as Replication Protein A (RPA), DNA polymerase α-primase (pol α) and topoisomerase I (topo I). E1 binds to DNA polymerase ϵ (pol ϵ) and dramatically stimulates the DNA synthesis activity of pol ϵ (Chojnacki and Melendy 2018). Moreover, E6/E7 expression leads to activation of replication machinery, allowing vegetative viral DNA synthesis.

E6 and E7 modify the cellular environment to induce genome amplification in growth arrested differentiated cells. HR E6 and E7 induce proliferation of basal and parabasal layers of the epithelium, increasing the infected area, by inhibiting apoptosis and inducing cell cycle progression, respectively.

HPV infection induces DDR in the host cells and the virus upregulates ataxia telangiectasia and Rad3-related protein (ATR) and ataxia-telangiectasia mutated (ATM) signaling pathways (S. Hong and Laimins 2013). ATM is important for differentiation-dependent genome amplification and it is activated by E1 and E7 (Sakakibara, Mitra, and McBride 2011; Fradet-Turcotte et al. 2011); ATR is important for viral replication in undifferentiated cells having a role in episomal maintenance (Reinson et al. 2013; Edwards et al. 2013).

2.1.3.3 Assembly, Maturation and Viral Release

HPV needs cell cycle exit and the presence of L1 and L2 for genome packaging, to end the viral lifecycle. This occurs in terminally differentiated keratinocytes and in particular in the nuclei of those cells that underwent viral genome replication and expression of viral proteins (Knipe and Howley 2020). L1 and L2 enter the nucleus through cellular karyopherins and L1 can assemble into

VLPs with the help of L2 that increases the efficiency, then packaging occurs with a mechanism that has not been elucidated yet (Knipe and Howley 2020; Darshan et al. 2004; Kirnbauer et al. 1993; Jian Zhou et al. 1991; Buck et al. 2004).

Maturation of the virus occurs in the upper layers of differentiated squamous epithelia. This environment exposes viral particles to oxidizing events that induce disulfide bonds accumulation between L1 proteins and capsid condensates, increasing stability and resistance to proteolysis (Buck et al. 2005).

HPVs are non-lytic viruses, and viral dispersion happens after normal loss of nuclear and cytoplasmic integrity of the terminally differentiated keratinocytes; however, also E4 may aid at virion release by binding and disrupting cytokeratin filaments structure (Knipe and Howley 2020; John Doorbar 2013).

2.1.4 Non-Productive HPV Infection and Transformation

In HPV-induced carcinogenesis the integration of the HPV genome into a host chromosome is one of the key events. HR HPV genome integration often happens near common fragile sites of the human genome, but no apparent hot spots for integration and no evidence for insertional mutagenesis have been identified (Münger et al. 2004). After integration, expression of the viral E6 and E7 genes is constitutive, while other portions of the viral DNA are deleted or their expression is disturbed. Among these, HPV E2 transcriptional repressor expression is lost, explaining a deregulated expression of HPV E6 and E7. Moreover, specifically for HPV16, E6/E7 mRNA stability is increased after integration, and specific alterations of host cellular gene expression have been detected upon HPV genome integration (Münger et al. 2004). Cells that express E6/E7 from integrated HR HPV sequences have a selective growth advantage over cells with episomal HPV genomes that still present HPV E2 expression (Jeon, Allen-Hoffmann, and Lambert 1995).

HR HPV E6 and E7 expression of these viral oncogenes confers the distinctive neoplastic phenotype of cervical cancer (reviewed in John Doorbar et al. 2015). Oncoproteins expression increases with neoplasia grade and gives an important contribution to malignant progression (Isaacson Wechsler et al. 2012; Münger et al. 2006). E6 and E7 oncoproteins induce genomic instability, thus accelerating the accumulation of mutations and the establishment and expansion of cells with tumor-promoting host cell mutations, leading to the development of malignancies (Gupta, Kumar, and Das 2018).

2.2 Head and Neck Cancer

2.2.1 Background and epidemiology of Head and Neck cancer

Head and Neck cancers are a vast group of tumors mostly (90%) originating from mutations in the squamous cells lining the mucosa of the head and neck area, for this reason they are broadly referred to as head and neck squamous cell carcinomas (HNSCC) (National Cancer Institute, 2017). According to World Health Organization statistics, the global impact of HNSCC is very high with around 650.000 new cases and 350.000 deaths recorded every year (Sabatini and Chiocca 2020). The main known risk factors for HNSCC are alcohol and tobacco use, but in the last few decades infection of high-risk human papillomaviruses, especially HPV16, was confirmed to be a major cause.

The global distribution of Head and Neck cancers is greatly heterogeneous with a high incidence in developing countries such as India, where it is a leading cause of death, and a lower incidence in other more developed countries. Recently, this situation has seen a change with a growing incidence of Head and Neck Cancers, especially related to HPV, in more developed countries such as Europe and North America, and especially in younger individuals. This seems to be due to the changes in sexual behaviors which are favoring its increase globally (Boscolo-Rizzo et al. 2018).

It is now well accepted that HNSCC can be divided into HPV-negative (HPV-) and HPV-positive (HPV+) diseases, in which the socioeconomic status of the patients as well as the clinical aspects, the molecular profiles and the prognosis of the tumors are quite different between the two subgroups (Leemans, Snijders, and Brakenhoff 2018).

The high mortality rate of this tumor, coupled with its increasing incidence, is highlighting the importance of understanding more about it and finding new treatment strategies. In fact, most Head and Neck cancers are currently treated in the same way despite their unique molecular landscape becoming increasingly clear, together with their differing causes (Dok and Nuyts 2016). However, due to their different pathogenesis, molecular landscape and responsiveness to treatment, HPV-positive HNSCC are now starting to be considered as a separate HNSCC subset which therefore require different prevention strategies and therapeutic approaches (Vigneswaran and Williams 2014).

2.2.2 Anatomy and risk factors of Head and Neck cancer

As mentioned, the term Head and Neck cancer broadly refers to cancers situated in the head and neck area and arising from squamous cells lining its mucosa. This type of cancer is further categorized based on the specific area of the aerodigestive tract from which it originates, such as the oral cavity, the pharynx and larynx, the salivary glands and the paranasal sinuses and nasal cavity (Figure 3). Specifically, several different areas of these anatomical sites can develop squamous cell carcinomas, meaning a tumor located in the oral cavity can involve any area between the palate and the mouth floor, as well as from the lips all the way to the back of the tongue. Similarly, tumors of the pharynx could affect any part starting from the nasopharynx all the way to the oropharynx and hypopharynx (Sabatini and Chiocca 2020).

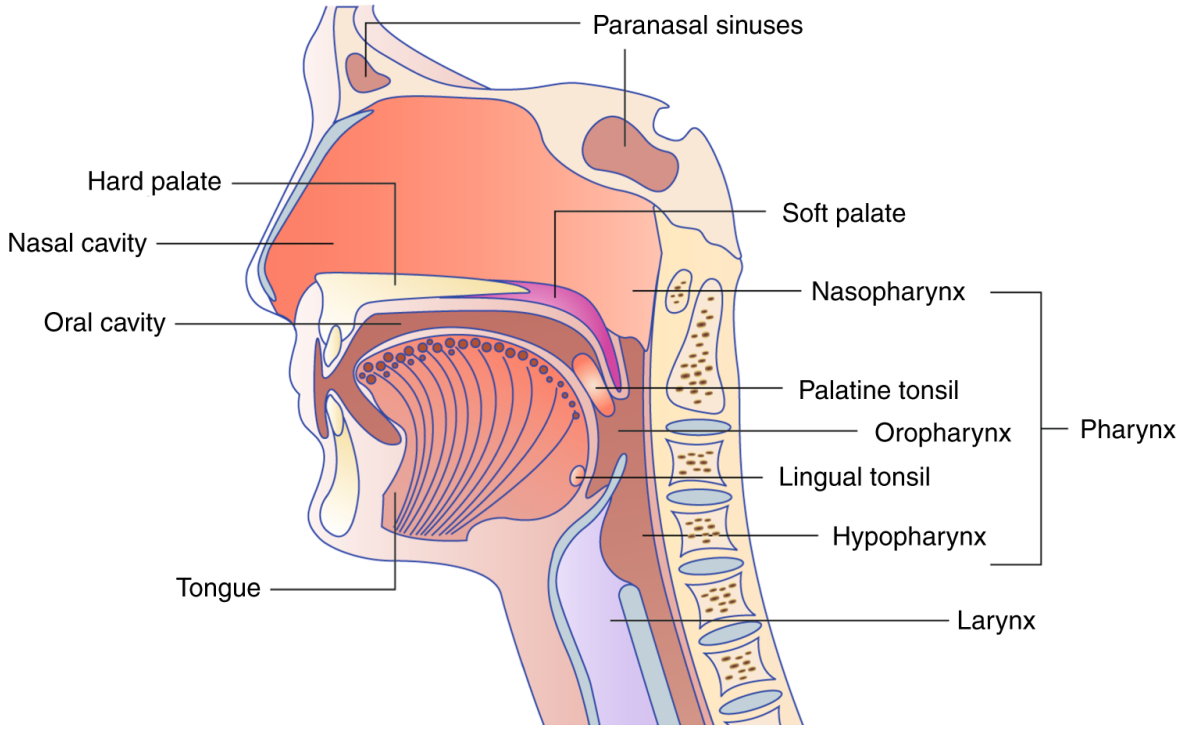


Figure 3 - Schematic representation of the head and neck anatomical region involving HNSCC.

Adapted from Sabatini and Chiocca, BJC 2020.

Every Head and Neck cancer has different risk factors, depending on the various carcinogens each anatomical area is mostly exposed to. As mentioned, excessive tobacco and alcohol consumption represents, aside from HPV, the greatest risk factors for the development of HNSCC.

It is worth noting however that the way these substances are consumed in some cases varies according to geographical area and culture and these differences contribute to different tumor development. In some countries it is common to chew tobacco leaves, mixed with other leaves and nuts (i.e. betel quid and areca nut), known as pan and the groups practicing it have shown high levels of oral SCC. Interestingly, these are also the countries (such as India), which were found to have the highest HNSCC prevalence. Another seemingly deleterious habit practiced in these countries is reverse smoking, where the burning part of the cigarette is kept inside the mouth; this was correlated with the development of SCC in the hard palate. Aside from cigarettes, smoking tobacco using cigars and pipes also increases the risk of developing Head and Neck cancers; specifically, tobacco consumption using a pipe was correlated with a greater incidence in lower lip cancers (Vigneswaran and Williams 2014).

Important risk factors of Head and Neck Cancers are sex and gender. HNSCC is more likely to develop in men than women with a ratio that ranges from 2:1 to 4:1 (Sabatini and Chiocca 2020). This disparity can be attributed to a different gender-based exposure to tobacco and alcohol (Giuliano et al. 2015). However, also HPV-related Head and Neck cancer present a higher incidence in men and many hypotheses have been proposed. One hypothesis is that this difference could be caused by the higher rates of HPV transmission for vaginal–oral rather than penile–oral sex. In accordance, HPV may be transmitted more often from women to men than from men to women, suggesting that transmission rates may differ by sex, although data from literature are still ambiguous regarding male-to-female and female-to-male transmission rates. However, sexual behavior and gender differences in lifestyle cannot fully explain these differences in incidence between the sexes (reviewed in Sabatini and Chiocca 2020).

2.2.3 The role of HPV in Head and Neck cancers

Since Durst et al.’s finding in 1983, it is now widely known and accepted that HPV and in particular HPV16, plays a key role in the development of most cervical cancers (Durst et al. 1983). Its carcinogenic role has been therefore well known, but only recently its importance was also determined in Head and Neck cancers along-

side that of other socioeconomic factors such as tobacco and alcohol abuse (Kobayashi et al. 2018).

A wide collection of studies was analyzed employing the use of polymerase chain reaction (PCR) to detect HPV in HNSCC biopsies and determine the specific strains and their distribution by anatomical area (Kreimer et al. 2005). As mentioned, HPV16 was found to be the most prevalent, especially in oropharyngeal squamous cell carcinomas (OPSCC), closely followed by HPV18 in oral and laryngeal cancers (Kreimer et al. 2005).

More recent studies have further investigated the role of HPV16 in Head and Neck cancers, but also more deeply researched the contribution of other HPV strains (Syrjänen 2010). In particular, a systematic review from 2010 brought together 34 separate studies (33 using PCR, 1 using in situ hybridization), with a total of 5.681 patients, and found that most HPV positive HNSCC concerned oropharyngeal cancers, rather than oral or laryngeal. More specifically, HPV16 was found to be the most common, with a prevalence of 86.69% out of all HPV-positive tumors thus confirming its central role. Again, HPV18 was also found to be present, although much less, together with HPV33 (Dayyani et al. 2010). A few low-risk types such as HPV6 and HPV11, in most cases effectively cleared by the immune system, were also found to contribute in some cases to the carcinogenesis of SCC (Syrjänen 2010).

2.2.4 Primary and secondary prevention for Head and Neck cancer

Unlike many other tumors, successful prevention for HNSCC is possible and has grown considerably in the last decades. The recent anti-smoke campaigns and the laws regulating smoking in public areas, which are more and more common every day, and which have seen a decrease in the number of smokers especially in some developed countries such as North America, represent an effective primary prevention strategy for HPV-negative HNSCC tumors. In the same way, sensibilization on the negative effects of long-term alcohol abuse, also helps towards this purpose (Hashim et al. 2019).

The most successful form of HPV-positive Head and Neck cancer primary prevention, however, is vaccination against HPV. This vaccine in fact represents the first anti-cancer vaccine and was originally developed to protect adolescent girls and

adult women against cervical cancer, which has been established to be caused by several HPV strains (Cutts et al. 2007).

Free mass vaccination of girls and young women not previously vaccinated (11-26 age range) against the known high risk HPV strains, has in the last few years greatly contributed to prevention of cervical cancer and has greatly reduced its burden on healthcare. This has been greatly useful and effective in controlling the rise in cases caused by changing sexual behavior of the young population, especially in Europe and North America, and very recently it was recognized that its use should be extended to young males too. The reason for this is that not only males can contribute to the spread of HPV through sexual intercourse, but they could also develop genital warts and other forms of anogenital cancers (Carlos et al. 2010). Now we know that this protection extends even further to the whole range of HNSCC (D'Souza and Dempsey 2011).

Since HPV vaccines have only been available since 2006, their complete beneficial impact on HPV-positive HNSCC has yet to be fully seen, but the statistics are encouraging with the US Centers for Disease and Prevention reporting between the 2000-2006 (before vaccination) and the 2009-2012 (after vaccination) period, a 64% decrease in HPV infections for adolescents aged 14-19 and a 34% decrease for young women aged 20-24. Similar results were obtained by an Australian study which observed the decreasing incidence of genital warts before and after the start of a vaccination program in 2007, in both boys and girls (Hashim et al. 2019).

Another effective and fundamental behavior to adopt is regular screening. Particularly considering HPV- HNSCC, alcohol and tobacco cause several changes to cells over the course, for example, of years of tobacco smoking, and as these cells acquire mutations favoring their transformation into cancer cells, precancerous lesions might develop. In some cases, these lesions might appear as a red (erythroplakia) or white (leukoplakia) area of the oral mucosa, and therefore be visible enough to be quickly detected by a routine screening or by a patient's dentist (Brouns et al. 2014).

Screening of the head and neck area usually involves visual inspection, followed by biopsies and HPV-testing if abnormal lesions are found; but as HNSCC can involve a wide range of anatomical sites, complete screening is not always possible or completely reliable. However, dysplastic cells are not visible and can only be detected through specific microscopic analysis of a biopsy sample.

To increase the success of these screenings, techniques such as fluorescence imaging and toluidine blue staining were experimented but did not show particularly advantageous outcomes compared to the standard procedure. Another proposed method, which could present a valid and un-invasive solution, are molecular-based test; this however needs a greater knowledge and molecular characterization of HNSCC (Hashim et al. 2019).

This form of secondary prevention, together with the early identification of high-risk individuals, has been proven effective in several other tumors such as colorectal and cervical cancer, allowing an early diagnosis and more chances of full recovery. There are several types of high-risk individuals and several ways they can be monitored. For example, having previously recovered from a tumor is considered as a risk factor for developing another one in the future and for this reason it is approached with regular monitoring. The reason for this is that the development of a past tumor suggests an exposure to the well-known risk factors we previously discussed such as tobacco and alcohol consumption, or a predisposition possibly due to genetic instability and/or mutations (Loomans-Kropp and Umar 2019).

In some particular cases, hereditary genetic diseases such as Li-Fraumeni syndrome and Plummer-Vinson can increase the likelihood of developing one or more malignancies. Fanconi anemia and dyskeratosis congenita (a telomere defect resulting in a bone marrow disorder) for example, were found to be associated with a dramatic increase (about 800-fold) in the chances of developing HNSCC, especially at a young age.

Another factor to be considered is the exposure of oncological patients to chemotherapy or, in the case of transplants following entire organ removal, anti-rejection drugs and the resulting immunodepression (Vigneswaran and Williams 2014).

Despite what is generally thought, the category presenting a higher risk of being diagnosed with a tumor at a late stage, are individuals with no cancer history and no particular predisposition to be taken into account. During the first stages of its development, HNSCC does not present itself with any particularly distinctive or noticeable symptom, making it more difficult for potential new patients to recognize that they should get checked. In this case the most common and frequent risk factors previously mentioned should be considered to identify individuals which

could potentially be at risk of developing a malignancy and to help them undergo periodic site-specific screening.

2.2.5 Treatment strategies for Head and Neck cancer

Despite the growing understanding surrounding these types of tumors, the treatment strategies are still rather standardized and uniform for both HPV-positive and negative HNSCC. As with many other types of tumors, the main strategies used are chemotherapy (mostly for advanced or metastasized tumors requiring a systemic approach), radiotherapy and surgical excision of all or part of the mass. This combination generally gives satisfying results, especially in HPV positive patients which were shown to have a higher overall survival and disease-free survival (80% compared with 50% of HPV negative patients) (Dok and Nuyts 2016).

Still, many factors come into play when treatment success and survival rates are considered, an HPV-positive patient for example might also be a heavy smoker, both factors contributing to the development and molecular landscape of the tumor. For this reason, after the discovery of a possible correlation between years of tobacco smoking and mortality, it was suggested that patients should be classified based not only on the stage of the tumor, the state of their lymph nodes and their HPV status but also on the extent of their tobacco exposure. As mentioned, HPV and tobacco exposure are the main cause of the difference between HNSCC and thus their responsiveness to treatment. The reason for a different response to therapy could be linked to the different path leading to the development of each tumor; as a result, tumor subtypes appearance at the molecular level will also be different and thus the mechanism by which various treatments can act on them (Ang et al. 2010). Still, the reasons for the variable response to treatment of HPV+ and HPV- subsets is not entirely clear and the known side effects of conventional chemoradiation therapies, coupled with the chemoresistance which might inevitably occur, highlights the importance of understanding more about HNSCC to be able to find new treatment strategies.

Additionally, the current therapeutic approach is quite generic, and recurrence or metastases are quite common (in 35-55% of patients). More specific and tailored therapies are needed, and they can be developed only towards better understanding of the unique differences between HPV-positive and negative HNSCC subsets and exploiting them according to each specific tumor.

In the last decade, the use of biologics such as monoclonal antibodies, for the treatment of various forms of cancer has increased and showed a high success rate in many cases. An example of a monoclonal with a potential application in HNC is cetuximab, which specifically targets the epidermal growth factor receptor (EGFR), found to be overexpressed in 90% of HNSCC and correlated with poor prognosis (Xu, Johnson, and Grandis 2017). The use of cetuximab had already been proven rather effective in the treatment of a particular breast cancer subtype, and its recent application for metastatic or recurrent HNSCC in combination with cisplatin (a common chemotherapeutic agent) seems promising (Rivera et al. 2009).

However, the possibility of developing resistance needs to be taken into account for this type of treatment too. Other monoclonal antibodies are being investigated, as well as tyrosine kinase inhibitors both for EGFR and other molecular targets, in order to block downstream signaling of several relevant cellular processes happening in cancer cells (Wise-Draper et al. 2012). It was found that a lower patient age corresponded to a higher success rate in combination treatment where radiotherapy was combined with either cisplatin or cetuximab (Oosting and Haddad 2019). The same cannot be said for older patients, where an improvement was not observed. In this case a better response to treatment was obtained when radiotherapy was coupled with nimorazole, a hypoxia modifier used in certain countries.

2.3 The molecular landscape of Head and Neck cancer

In the last few decades, molecular characterization of precancerous lesions and carcinomas allowed a better understanding of their progression mechanisms. The -omics era allowed a deeper knowledge of genetics events occurring in cancer. In particular, The Cancer Genome Atlas (TCGA) collects sequencing of data of a huge number of patients.

Even if some subgroups of HNSCC based on genetic and epigenetic events underlying tumors have been identified, the true comparable differences in Head and Neck Cancer can be determined by observing the molecular landscape of HPV-positive and HPV-negative subtypes.

As we mentioned before, HPV- incidence in HNSCC is decreasing, while HPV+ is increasing. Age of HPV- patients is higher than in HPV+, socioeconomical status is higher in HPV+ than in HPV- HNSCC. Also risk factors are different, with

tobacco and alcohol consumption stand out. The tumor location is also different, with HPV- tumors that develop in all the head and neck sites, while HPV+ form mainly in the oropharynx. HPV+ tumors have a better prognosis than HPV- (Table 2) (Dok and Nuyts 2016).

Besides the clinical and epidemiological characteristics, also biological and histopathological characteristics vary among the subgroups.

Table 2 - Overview of the main characteristics of HPV-positive and negative HNSCC. *Adapted from Dok and Nuyts, Cancers, 2016.*

	HPV Positive	HPV Negative
Clinical, epidemiological characteristics		
Incidence	Increasing	Decreasing
Age	Younger	Older
Socioeconomic status	Higher	Lower
Risk factors	Sexual behavior, marijuana exposure	Tobacco and alcohol exposure
Location of the tumor	Oropharynx (common in tonsil and BOT)	All head and neck sites (common in floor of mouth, lateral tongue and ventral tongue)
Prognosis	good	poor
Biological and histopathology characteristics		
TP53 pathway	E6 mediated degradation	TP53 mutations
RB pathway	E7 mediated degradation	Inactivating mutations or other alterations in pathway
p16INK4a expression	Commonly overexpressed	Commonly decreased expression (inactivating mutations and hyper methylation)
Histology	Poorly differentiated or basaloid SCC	Modestly to well differentiated, keratinized SCC

Abbreviations: HPV, Human papillomavirus; BOT, Base of tongue; SCC, squamous cell carcinoma.

2.3.1 Molecular features of HPV- HNSCC

Molecular studies found that the number of mutations occurring in these HNSCC cells was directly correlated with the severity of the tumor and that changes in different chromosomes were responsible for different levels of malignancy: the worst outcome relating to HPV- HNSCC was found in 11q, 4q and 8p chromosome arms mutations which were related to a more advanced tumor progression to carcinoma, while mutations in 3p, 9p and 17p seemed to lead to dysplasia (Califano et al. 1996). Interestingly, in 17p resides the p53 gene TP53, whose alteration is responsible for the development of several tumors. Further studies established mutations in TP53 as a tell-tale sign for the development of a tumor. TP53 mutation frequency is 72% in all HNSCCs, almost all HPV-, with typical allelic losses of TP53 locus and not copy number alterations (CNAs), suggesting that it is advantageous for tumors to have a double copy of chromosome 17 or two copies of mutated TP53 (Table 3) (Leemans, Snijders, and Brakenhoff 2018).

Another important event is the alteration in 9p21, with the loss of cyclin dependent kinase inhibitor 2A (CDKN2A) with 22% of mutational frequency and CNA frequency of 32% (Table 3). The protein encoded by CDKN2A, $p16^{INK4A}$, is essential for the tight control and regulation of the cell cycle machine, and its modification disrupts this mechanism leading to abnormal DNA replication (Leemans, Snijders, and Brakenhoff 2018; Xu, Johnson, and Grandis 2017).

Besides the mutation of tumor-suppressor genes into proto-oncogenes, and their inactivation, other genes could undergo activation. For example, the aforementioned EGFR, important in many cellular processes including proliferation, migration, survival and angiogenesis had a low mutation rate, but a high amplification frequency (11%), as well as high expression levels in 90% of cases (Leemans, Snijders, and Brakenhoff 2018). Another interesting change occurring in many cancer cells, is type III EMT (epithelial-mesenchymal transition) which involves modifications in polarity to favor adhesion and migration of tumor cells in order to metastasize (A. Smith, Teknos, and Pan 2013). This is accomplished through the regulation of cell adhesion molecules such as E-cadherin, β-catenin and Vimentin, which can be aberrantly activated through various mechanisms such as the Wingless Int-1 (Wnt) pathway, the EGFR pathway, the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, and many others. These mutations generally also confer radio-resistance to cancer cells, which is in line with the observation that HPV-negative HNSCC usually responds less well to standard treatments (Jiawei Zhou et al. 2020).

Table 3 - Genes frequently mutated in HPV-negative HNSCC. *(Adapted from Leemans et al., 2018).*

Cellular process	Gene	Protein	Type of gene	Mutation frequency (%)	CNA frequency (%)
Cell cycle	*CDKN2A*	$p16^{INK4A}$	Tumour suppressor	22	32
	TP53	p53	Tumour suppressor	72	1.4
	CCND1	G1–S-specific cyclin D1	Oncogene	0.6	25
Growth signals	*EGFR*	EGFR	Oncogene	4	11
Survival	*PIK3CA*	Catalytic p110α subunit of class 1 PI3Ks	Oncogene	18	21
	PTEN	PTEN	Tumour suppressor	3	4
WNT signalling	*FAT1*	Protocadherin FAT1	Tumour suppressor	23	8
	AJUBA	LIM domain-containing protein AJUBA	Tumour suppressor	7*	1
	NOTCH1	NOTCH1	Tumour suppressor	18	4
Epigenetic regulation	*KMT2D*	Histone-lysine *N*-methyltransferase KMT2D	Tumour suppressor	16	0.4
	NSD1	Histone-lysine *N*-methyltransferase NSD1	Tumour suppressor	12*	0.8

Data from Ref. 3. Mutation data were taken from The Cancer Genome Atlas (TCGA) ($n = 504$) using the cBioPortal. CNA, copy number alteration; EGFR, epidermal growth factor receptor.
*Putative passenger mutation that requires further functional studies.

2.3.2 Molecular features of HPV-induced carcinogenesis.

Cells can transduce external stimuli in order to regulate the transcription of specific genes and to control many biological processes. This happens through molecular signaling. HPV is able to affect several distinct signaling pathways implicated in the regulation of important molecular processes that affect cell proliferation and differentiation in order to induce carcinogenesis. Among these, the most extensively studied and described are p53 and pRb. For the importance of p53 in this context, the disruption of the binding between E6 and p53 could be a promising target for HPV-related cancer therapy. In a recent work, Celegato *et al*. studied small molecule inhibitors of p53/E6 interaction, which rescued p53 activity and caused cancer cells growth arrest (Celegato et al. 2020).

Besides p53 and pRb, many other signaling processes have been tackled by HPV, in order to achieve its own needs, including EGFR, PI3K, Akt, mTOR, MAPKs. In the next sections, an overview of the mechanisms underlying HPV-mediated regulation of these pathways, together with the potential implications in targeted therapy will be analyzed.

2.3.2.1 The EGFR pathway

The tyrosine kinase EGFR is a member of the ErbB/HER (ERBB, from the related avian viral erythroblastosis oncogene; HER, human EGF receptor) family (Roskoski 2014). EGFR contains a transmembrane domain and is activated by the

binding of some ligands, including the epidermal growth factor (EGF) and the transforming growth factor α (TGF-α) (Cha et al. 1996).

EGFR has been extensively studied as proto-oncogene, for its implication in a wide number of cellular processes including proliferation, migration, survival and angiogenesis (Franklin et al. 2002; Hirsch et al. 2003). It is composed by an extracellular part that binds ligands, a transmembrane domain, and an intracellular part with catalytic activity. EGFR is monomeric when inactive, but homodimers form upon activation by the binding of ligands to the receptor. Activation of EGFR induces its autophosphorylation, and activates a signaling cascade to propagate the extracellular mitogenic signals, activating many cellular genes and pathways, such as mitogen-activated protein kinase (MAPK) and Phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt), involved in cell differentiation, mitogenesis, mobility and cell survival (Yarden 2001).

EGFR is important for the proliferation of cancer cells because it is involved in metabolic processes, such as fatty acids and pyrimidines synthesis, glucose catabolism, both directly or indirectly, respectively by phosphorylating specific enzymes, or by activating signaling pathways involved in metabolism, i.e. Akt (Makinoshima et al. 2014; Babic et al. 2013; Makinoshima et al. 2015). High levels of EGFR are associated with poor prognosis in cervical cancer (Pfeiffer et al. 1989).

During HPV infection, E5 is fundamental for hyperproliferation of keratinocytes, because it upregulates EGFR signaling leading to a delayed differentiation. *In vitro* and *in vivo* studies revealed that E5 expression is localized in differentiating suprabasal cells of the stratified epithelium (Ilahi and Bhatti 2020).

The HPV oncoprotein E5 activates and upregulates EGFR pathway dependently on the ligand (Figure 4a) (Ilahi and Bhatti 2020). E5 indirectly upregulates vascular endothelial growth factor (VEGF) and cyclooxygenase 2, by upregulating EGFR. E5-mediated activation of EGFR initiates an intracellular cascade, activating numerous proto-oncogenes. Specifically, it activates MAPKs and the activating protein-1 (AP-1), inducing the expression of the viral oncoproteins E6/E7, as explained in detail in the following sections (Ilahi and Bhatti 2020; S. H. Kim et al. 2006; Su Hyeong Kim et al. 2009).

HPV16 E5 increases EGFR abundance on the surface of cells and EGFR phosphorylation levels, phenomenon that requires EGF binding, by reducing its degradation and internalization rates (Straight et al. 1993; Crusius et al. 1998).

As mentioned above, EGFR is an important target for targeted therapy in HNSCC. Inhibition of EGFR confers higher sensitivity of cancer cells to ionizing radiations in preclinical studies on HNSCC (Dassonville et al. 1993; Buchholz et al. 2005; Sheridan et al. 1997; Balaban et al. 1996). Cetuximab, the monoclonal antibody against EGFR, is currently used for recurrent and metastatic HNSCC (Mehra, Cohen, and Burtness 2008). However, it is not clear whether cetuximab treatment efficacy differs between HPV positive and HPV negative tumors since data from different studies are controversial (Vermorken et al. 2014; Rosenthal et al. 2016).

2.3.2.2 The PI3K/Akt/mTOR axis

The PI3K/Akt/mTOR is involved in promoting cell survival, growth, proliferation, migration, and energy metabolism. Specifically, PI3Ks are activated by phosphorylation when eternal stimuli occur and are regulated by RTKs or G protein-coupled receptors (GPCRs) GTPases. Membrane lipids phosphatidylinositol-3,4,5-trisphosphate (PIP3) and phosphatidylinositol-3,4-bisphosphate (PI3,4P2) are generated by the kinase activity of PI3K with the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PI4,5P2) (Rodon et al. 2013). The presence of PIP3 and PI3,4P2 recruits Akt to the cell membrane, that is activated by phosphorylation by the mammalian target of rapamycin complex 2 (mTORC2).

Phosphatase and tensin homolog (PTEN) inhibits Akt activation by dephosphorylating PIP3. Akt activity regulates many targets involved in cell cycle control, cell proliferation, cell mobilization, angiogenesis, anti-apoptosis and cell survival (Manning and Toker 2017). Tuberous sclerosis complex 2 (TSC2) is inhibited by Akt leading to activation of mammalian target of rapamycin complex 1 (mTORC1). mTORC1 is involved in a wide range of activities, including sensing of energy, oxygen, growth factor, amino acids and stress and is important to ensure adequate resources to activate downstream processes. Among these, mTORC1 activates protein translation, lipid and nucleotide synthesis, and inhibits autophagy (reviewed in Saxton and Sabatini 2017).

For its central role in metabolic control, the PI3K/Akt/mTOR axis is highly deregulated in many cancer types, contributing to malignant growth and resistance to therapy (Lim, Crowe, and Yang 2015). For example, PIK3CA gene, the class I PI3K catalytic subunit, is mutated or amplified in many cancers; in particular, it is

mutated in 17.5% and amplified in 15.7% of HNSCC; moreover, PTEN loss is implicated in carcinogenesis. To note, HPV-positive HNSCC presents higher frequency of PIK3CA and PTEN mutations than HPV-negative HNSCC (Lawrence et al. 2015; Gillison et al. 2019).

The HPV16 E7 oncoprotein induces Akt activation by inactivating pRb. HPV16 E6 can both activate Akt or induce TSC2 degradation, leading to mTORC1 activation (Figure 4b) (Pim et al. 2005; Menges et al. 2006; Contreras-Paredes et al. 2009; Lu et al. 2004). It has been shown that inhibiting mTOR arrests cancer cell growth in a mouse model of HPV (Callejas-Valera et al. 2016). To date, many clinical trials on HNSCC patients are ongoing, in order to test the efficiency of PI3K/AKT/mTOR inhibitors, in particular rapamycin analogs are often used, and show partially promising treatment responses (Z. Wang et al. 2017; T. A. Day et al. 2019; Harsha et al. 2020).

2.3.2.3 The role of MAPKs

MAPK pathways are evolutionarily conserved kinases that connect extracellular signals to important cellular processes such as growth, proliferation, differentiation, migration, and apoptosis. MAPK pathways consist of a three-tier kinase module in which a MAPK is activated upon phosphorylation by a mitogen-activated protein kinase kinase (MAPKK), which in turn is activated when phosphorylated by a MAPKK kinase (MAP3K). In mammals, six groups of MAPKs have been identified, including Jun N-terminal kinase (JNK)1/2/3, extracellular signal-regulated kinase (ERK)1/2, ERK3/4, ERK5, ERK7/8, and the p38 isoforms *α/β/γ*(ERK6)/*δ* (Dhillon et al. 2007).

C-jun N-terminal kinase (JNK) is a subfamily of Ser/Thr kinases from the canonical signal transduction of MAPK, comprising JNK1, JNK2 and JNK, transcribed from three different genes (Zeke et al. 2016; Bode and Dong 2007). JNKs are activated by a cascade signals coming from upstream JNK kinases and JNK kinase kinases, to respond to different external signals, such as infections (both viral and bacterial), cytokines, growth factors, heat shock, UV radiation, and other stresses (Hammouda et al. 2020). JNKs phosphorylate target proteins, such as activating transcription factors (ATF), ETS Like-1 protein (Elk1) and JUN protein family (Kyriakis and Avruch 2001; X. Wang, Destrument, and Tournier 2007; Chadee and Kyriakis 2010).

About HPV role in regulating this pathway, it has been shown that in primary keratinocytes, JNK1/2 phosphorylation is upregulated upon transduction with HPV18 (Chen and Tan 2000). A recent study showed that HPV E6 activates JNK to promote proliferation and oncoviral proteins expression through EGFR in cervical cancer (Morgan et al. 2020).

The ERK pathway regulates a variety of substrates involved in cell proliferation, differentiation, survival and motility (Kohno and Pouyssegur 2006). The cascade starts from the phosphorylation of Ras (from Rat sarcoma) GTPase mediated by membrane receptors, including EGFR, vascular endothelial growth factor receptors (VEGFRs) and platelet-derived growth factor receptor β (PDGFRβ). Active Ras recruits RAF (Rapidly Accelerated Fibrosarcoma), specifically Raf-1, leading to the phosphorylation of MEK1/2 on serines (MAPK/ERK kinase 1 and 2) (Pages et al. 1993; Sun et al. 2015; Lewis, Shapiro, and Ahn 1998). MEK1/2 in turn, phosphorylates tyrosine and threonine residues of ERK1/2, leading to the induction of a downstream pathways (Kohno and Pouyssegur 2006).

Since ERK signaling is involved in the activation of many pathways, it is deregulated in different kinds of cancers (Hoshino et al. 1999; Gioeli et al. 1999). The presence of inhibitors of ERK signaling developed in the last years, attracted the interest in this pathway for targeted cancer therapy (Sebolt-Leopold and Herrera 2004).

ERK pathway is implicated in HPV-related cancers. HPV E6 changes the activity of the Eukaryotic translation initiation factor 4E (eIF4E) protein by acting on ERK and Akt pathways (Morales-Garcia et al. 2020). E6 oncoprotein upregulates HIF-1α (hypoxia inducible factor 1α), VEGF, and interleukin 8 expression by modulating ERK (Liu et al. 2016). Activation of ERK1/2 signaling by benzo[α]pyrene, a molecule derived from burning of cigarettes, upregulates the expression of HPV31 (Bowser, Alam, and Meyers 2011). An interesting therapeutic approach sees the use of the 5-aminolevulinic acid photodynamic therapy to reduce HPV viral load through ERK, Akt, mTOR pathway, with a consequent reduction in cell proliferation (Xie et al. 2019).

An important protein complex downstream of MAPK signaling is AP-1, a dimeric transcription factor that can be composed of proteins coming from 4 different families, including JUN, FOS, ATF/CREB (activating transcription factor, cyclic AMP-responsive element-binding), and Maf (musculoaponeurotic fibrosarcoma)

(reviewed in Eferl and Wagner 2003). AP-1 is involved in the regulation of many pathways, including differentiation, proliferation, and apoptosis. AP-1 regulation is complex, and includes changes in dimer composition, interactions between AP-1 proteins, transcriptional regulations and post-transcriptional mechanisms (Gazon et al. 2018).

JUN and FOS families are the most relevant for AP-1 transcription factors. Jun-Fos heterodimers show the highest affinity to an asymmetric heptameric sequence TGA(C/G)TCA, the so called AP-1 sequence, and a slightly lower affinity to a symmetric octameric sequence TGACGTCA (Nakabeppu, Ryder, and Nathans 1988; Rauscher et al. 1988).

JUN family is composed by c-jun, junB and junD, that can form homodimers (Angel and Karin 1991). As mention before, c-jun activity is regulated by the JNKs and it is phosphorylated at serine 63 and 73 (Hibi et al. 1993).

The FOS family is composed by c-Fos, FosB, Fra-1 and Fra-2, that can only form heterodimers with Jun. c-fos is regulated by the ERK signaling pathway with a dual mechanism: increasing c-fos transcription and increasing its activity by direct phosphorylation (Monje et al. 2005; Monje, Marinissen, and Gutkind 2003; Treisman 1994; Murphy et al. 2002).

AP-1 is essential for the transcription of HPV oncoproteins. The HPV18 promoter 105 (p97 in HPV16) contains an AP-1 consensus sequence, from which AP-1 transcription factors start the transcription of E6/E7 (Figure 4c) (Steger, Rehtanz, and Schnabel 2001). It has been shown that mutations in the AP-1 binding site abolish E6/E7 expression. Moreover, an altered AP-1 is correlated with tumorigenic phenotypes in HeLa cells, while c-fos upregulation induces cervical cancer cells proliferation (Rösl et al. 1997; Soto et al. 1999). AP-1 regulates of E6/E7 expression but also mediates chemoradiation resistance that can be reverted by curcumin (Dhandapani, Mahesh, and Brann 2007). It has been shown that exposure to tobacco upregulates the expression of E6/E7 oncoproteins by increasing AP-1 mediated transcription in cervical cancer cells (Muñoz et al. 2018). On the contrary, inhibiting the AP-1 pathway using the natural alkaloid berberine, induces the suppression of E6/E7 and the restoration of p53 and pRb activity, resulting in growth arrest and apoptosis in cervical cancer cells (Mahata et al. 2011; Warowicka, Nawrot, and Goździcka-Józefiak 2020). Given the importance of this transcription

factor for HPV oncoproteins' expression, AP-1 could be a potential therapeutic target for HPV-induced cancers.

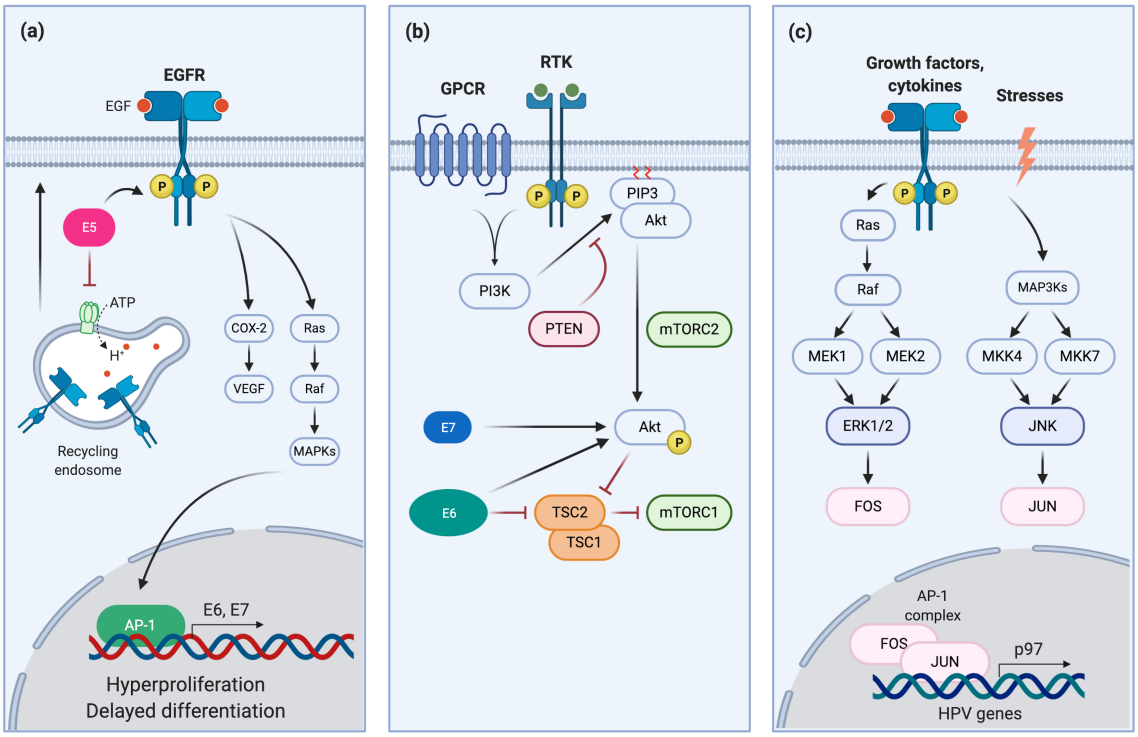


Figure 4 – Signaling pathways involved in HPV-mediated oncogenesis.

(a) E5 mediated regulation of EGFR pathway. (b) HPV oncoproteins regulation of Akt and mTOR. (c) MAPKs effects on AP-1 mediated activation of HPV transcription. Adapted from Medda et al. (submitted).

2.4 Aims of the study

The aim of my thesis is to assess the inhibition of E6/E7 oncoproteins as a potential therapeutic strategy in Head and Neck cancer.

First of all, we tested the effects of E6/E7 downregulation on HPV+ HNSCC proliferation, by using both siRNA and shRNA approaches. We then assessed whether mTOR inhibitors induced a downregulation of E6/E7 expression. Among them, we focused on Sorafenib, a multi-kinase inhibitor already used in clinics. We assessed the potential role of Sorafenib by studying which molecular mechanism it uses to reduce E6/E7 expression. Moreover, we targeted EGFR/ERK/FOS signaling, the specific pathway found to be involved in regulating E6/E7 expression in HPV+ HNSCC cell lines. To conclude, we tested the therapeutic potential of ERK inhibition towards HPV+ HNSCC treatment.

3 Material and Methods

3.1 Cells

Phoenix Ampho and HEK 293T cells were maintained in DMEM with stable glutamine plus 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin to avoid contaminations. A panel of HNSCC cell lines listed in Table 4 was used and cells were maintained in DMEM with stable glutamine plus 10% FBS and supplemented with 1% Non-essential aminoacid solution and kept in 1% Penicillin/Streptomycin to avoid contaminations. All cells were maintained at 37°C with 5% CO^2. Cells were passaged using 0.05% trypsin EDTA solution (Lonza), depending on the confluency. Medium was refreshed 2 or 3 times per week. All cell lines were authenticated by short tandem repeat profiling and tested for mycoplasma contamination every 6 months.

HNSCC cell lines were obtained from different sources. The UM-SCC-4, UM-SCC-6, UM-SCC-10A, UM-SCC-17A, UM-SCC-17B, UM-SCC-18, UM-SCC-19, UM-SCC-23, UM-SCC-28 and UM-SCC-47 cell lines were created by Prof. Thomas E. Carey. The UD-SCC-2 cell line was kindly provided by Prof. Henning Bier: present address LRZ, Munich, Germany. The VU-SCC-147 cell line was kindly provided by Dr. Martin Rooimans, Free University Medical Centre, Amsterdam, the Netherland. The UM-SCC-104 cell line was from Merck spa. The UPCI-SCC-90, UPCI-SCC-152 and UPCI-SCC-154 cell lines were acquired from ATCC®.

Table 4 – List and features of HNSCC cell lines used. *Adapted from* (Citro et al. 2019).

Cell line	Anatomical origin	Sex	HPV status	Reference
UD-SCC-2	Hypopharynx	M	HPV+	(Balló et al. 1999)
UM-SCC-4	Oropharynx	F	HPV-	(Brenner et al. 2010)
UM-SCC-6	Oropharynx	M	HPV-	(Brenner et al. 2010)
UM-SCC-10A	Larynx	M	HPV-	(Brenner et al. 2010)
UM-SCC-17A	Larynx	F	HPV-	(Brenner et al. 2010)
UM-SCC-17B *	Cervical lynph node	F	HPV-	(Brenner et al. 2010)
UM-SCC-18	Oropharynx	M	HPV-	(Brenner et al. 2010)
UM-SCC-19	Oropharynx	M	HPV-	(Brenner et al. 2010)
UM-SCC-23	Larynx	F	HPV-	(Brenner et al. 2010)
UM-SCC-28	Larynx	F	HPV-	(Brenner et al. 2010)
UM-SCC-47	Oropharynx	M	HPV+	(Brenner et al. 2010)
UPCI-SCC-90	Oral cavity	M	HPV+	(White et al. 2007)
UM-SCC-104	Oral cavity	M	HPV+	(Tang et al. 2012)
VU-SCC-147	Oropharynx	M	HPV+	(Steenbergen et al. 1995)
UPCI-SCC-152 **	Hypopharynx	M	HPV+	(White et al. 2007)
UPCI-SCC-154	Oral Cavity (Tongue)	M	HPV+	(White et al. 2007)

- *Metastasis of UM-SCC-17A; ** Recurrence of UPCI-SCC-90.*

3.2 Transductions and Transfections

Phoenix Ampho is a second-generation retrovirus producer cell line for the generation of helper-free amphotropic retroviruses, which expresses amphotropic envelope protein, highly transfectable with either calcium phosphate mediated transfection or lipid-based transfection protocols.

For retroviral transduction, plasmids were transfected into Phoenix Ampho cells by calcium-phosphate method using 10μg of plasmid DNA and 1 mL of the solution was added dropwise to 9mL of medium, for a total 10 mL of volume. After 6 hours medium was changed with 5 mL of fresh medium to concentrate the virus. Cells were transduced with retroviral supernatants in the presence of 8μg/mL of polybrene for 6 hours at 37°C for two consecutive days and selected with the appropriate concentration of Puromycin (Sigma Aldrich) for 3 days or G-418 Sulfate

(Gibco) for 1 week. Cells were then collected for RNA extraction, Western Blot or used for other experiments.

For lentiviral transduction, 2nd generation lentiviral plasmids were transfected in HEK 293T, by calcium-phosphate method using 10μg of plasmid DNA, 4μg of envelope plasmid (expressing VSV-G) and 5μg of packaging plasmid dR8.91 (expressing Gag, Pol, Rev, Tat) and 1mL of the solution was added dropwise to 9mL of medium, for a total 10mL of volume. After 6 hours medium was changed with 5mL of fresh medium to concentrate the virus. Cells were transduced with retroviral supernatants in the presence of 8μg/mL of polybrene for 6 hours at 37°C for two consecutive days and selected with the appropriate concentration of Puromycin (Sigma Aldrich) for 3 days or G-418 Sulfate (Gibco) for 1 week. Cells were then collected for RNA extraction, Western Blot or used for other experiments.

For overexpression of c-fos using transfection cells were seeded on 60mm plates and transfected with Lipofectamine 2000 (Thermo-Fisher) with pcDNA3-FLAG empty, or pCDNA3-FLAG c-fos following manufacturers' instructions. After 48 hours from transfection cells were harvested for western blot analysis.

For RNAi experiments cells were seeded on 6 well or 60mm plates and transfected with RNAi-max (Thermo-Fisher) with siLuc, sifos or siE6/E7 siRNAs following manufacturers' instruction. After 72 hours, cells were collected and used for RNA or protein extraction.

siRNA sequences are the following: siLuc 5'- CGUACGGGGAAUACUUCGA -3' sense and 5'-UCGAAGUAUUCCCCGUACG-3' antisense, siE6/E7 5'-CUUCGGUUGUGCGUACAAAGC-3' sense, 5'-GCUUUGUACGCACAACCGAAG-3' antisense. Sifos was a commercial mixture of siRNAs (Santacruz Biotechnology).

3.3 Plasmids

pCDNA3-FLAG and pCDNA3-FLAG c-fos were a kind gift by Natoli's group (IEO). pLKO.1 shluc was a gift by Minucci's group (IEO).

pLKO.1 shE6/E7#1 and shE6/E7#1 were generated by inserting specific sense and antisense sequences listed in table 5 into pLKO.1 TRC vector, after removing the stuffer with AgeI and EcoRI. After ligation of plasmid with the insert, Stabl3 cells were transformed with the plasmid and screened for the presence of the insert. After the screening, one bacterial clone of each insert was selected, purified and sequenced.

Table 5 – List of oligos used for cloning of shRNAs.

Insert name	Sense/ Antisense	Sequence
shE6/E7 #1	Sense	CCGGTGTGTGTACTGCAAGCAACTCTCGAGAGTTGCTTGCAG TACACACATTTTTG
	Antisense	AATTCAAAAATGTGTGTACTGCAAGCAACTCTCGAGAGTTGC TTGCAGTACACACA
shE6/E7 #2	Sense	CCGGCTTCGGTTGTGCGTACAAAGCCTCGAGGCTTTGTACGCA CAACCGAAGTTTTTG
	Antisense	AATTCAAAAACTTCGGTTGTGCGTACAAAGCCTCGAGGCTTTG TACGCACAACCGAAG

3.4 RNA Extraction, Reverse Transcription and qRT-PCR

Total RNA was extracted with the Quick-RNA MiniPrep kit (ZYMO RESEARCH). RNA was quantified using Nanodrop 1000 spectrophotometer (Thermofisher). cDNA was generated by reverse transcription with LunaScript™ RT SuperMix Kit (NEB) using 500ng of total RNA. cDNA was diluted 1 to 5 and 10ng per reaction were used. Relative levels of specific mRNAs were determined with the Luna® Universal qPCR Master Mix (NEB). The reactions were performed in 10μL in technical duplicate using specific primers and ran in a Quant Studio 6 Pro real time machine (Thermofisher). The expression of the indicated mRNAs was quantitated by the comparative ΔΔCt method. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) gene primer was used as control for normalization. Primers used are listed in Table 6.

Table 6 – List of primers for qPCR.

Gene	Forward/ Reverse	Sequence
GAPDH	Forward	GCCTCAAGATCATCAGCAATGC
	Reverse	CCACGATACCAAAGTTGTCATGG
HPV16 E6	Forward	ATGTTTCAGGACCCACAGGA
	Reverse	CAGCTGGGTTTCTCTACGTGTT
HPV16 E7	Forward	CAGAGGAGGAGGATGAAATAGATGG
	Reverse	CACAACCGAAGCGTAGAGTCACAC
c-JUN	Forward	CACGTTACAGTGGGTGCCA
	Reverse	CCCCGACGGTCTCTCTTCA
c-FOS	Forward	AAAAGGAGAATCCGAAGGGAAA
	Reverse	GTCTGTCTCCGCTTGGAGTGTAT
FOSL1	Forward	GAACTGACCGACTTCCTG
	Reverse	TGGGCTTCCAGCACCAG

3.5 Immunoblotting and Antibodies

For protein extraction cells were lysed using a sodium dodecyl sulphate (SDS) lysis buffer composed of a 1:3 mixture of buffer I (5% SDS, 0.15M Tris-HCl [pH 6.8] and 30% glycerol) and buffer II (25mM Tris-HCl [pH 8.3], 50mM NaCl, 0.5% NP-40, 0.1% SDS, 1mM EDTA and protease inhibitors) and supplemented with phosphatase inhibitors 0.5mM NaF and 2mM sodium Na_3VO_4.

Protein concentration was assessed using DC protein assay (BD) following manufacturer instructions. Equal amounts of total proteins (40 or 50µg) were resolved by SDS-PAGE under reducing conditions, using a running buffer (25mM Tris, 192mM glycine, 0.1% SDS to separate the proteins on the gel at 100-180 V. Proteins were transferred from the gel to a Polyvinylidene fluoride (PVDF) membrane using a transfer buffer (25mM Tris, 192mM glycine, 10% methanol) at 0.25 mA for 90 minutes. Membranes were blocked in 5% non-fat milk or 5% bovine serum albumin (BSA) in Tris buffered saline (10ml, 1X TBS) + 0.1% Tween-20 (TBS-T).

Immunoblotting was performed with the indicated antibodies: anti-HPV16E7, 1:300 (Santa Cruz Biotechnology); anti-LC3, 1:2000 (Sigma-Aldrich); anti-p53 (DO-

1), 1:1000 (Santa Cruz Biotechnology); anti-pERK, 1:1000 (Cell Signaling); anti-c-jun 1:1000, (Cell Signaling); anti-p-p70S6, 1:1000 (Cell Signaling), anti-p-c-jun 1:1000, (Cell Signaling); anti-c-fos, 1:1000 (Sigma-Aldrich). Anti-GAPDH 1:5000, (Abcam) or anti-Vinculin 1:10000 (Sigma-Aldrich) antibodies were used as loading control.

Membranes were washed three times with TBS-T and incubated with a horseradish peroxidase-conjugated secondary antibody (anti-mouse or anti-rabbit, 1:10000) for 1 hour. Membranes were then washed three times with TBS-T and incubated with Clarity Enhanced chemiluminescent (ECL) reagent (BIORAD). Detection is based on antibodies conjugated to HRP that in contact with the reagent produce chemoluminescence. The membranes were exposed and acquired with Chemidoc (Bio-rad).

3.6 Growth curves

For growth curve upon siE6/E7, cells were seeded on 96-well and in 6-well plates and transfected with RNAi-max (Thermo-Fisher) with siLuc or siE6/E7 siRNAs following manufacturers' instruction. After 2 days from transfection was repeated. After 3 days and 6 days from the first transfection cells from 6-well plates were harvested for protein and RNA extraction. For cells seeded on 96 well plates cell proliferation was assayed after 3 and 6 days using CellTiter-Glo® Luminescent Cell Viability Assay and following the manufacturer's instructions.

To assess the proliferation of HPV+ HNSCC cell lines transduced with shE6/E7 or shluc cells were seeded on 96-well plates at the appropriate density (UD-SCC-2 and UP-SCC-90 at 2000 cells/well; UM-SCC-47, UM-SCC-104, VU-SCC-147, UPCI-SCC-152 and UPCI-SCC-154 at 1666 cells/well) cell proliferation was assayed after 1, 3, 5 and 7 days using CellTiter-Glo® Luminescent Cell Viability Assay and following the manufacturer's instructions.

3.7 Half Maximal Inhibitory Concentration Analysis

To assess the half maximal inhibitory concentration (IC50) of Sorafenib and Ravoxertinib, cell lines were seeded in duplicate at the appropriate density (UD-SCC-2, UM-SCC-10A and UPCI-SCC-90 at 6000 cells/well; UM-SCC-18 and UM-SCC-19 at 4000 cells/well; UM-SCC-4, UM-SCC-6, UM-SCC-17A, UM-SCC-17B, UM-SCC-23, UM-SCC-28, UM-SCC-47, UM-SCC-104, VU-SCC-147, UPCI-SCC-152 and UPCI-SCC-154 at 5000 cells/well) in 96-well plates. Twenty-four hours later cell lines were treated with vehicle or different concentrations of Sorafenib (60, 40, 26.6, 17.7, 11.8, 7.9, 5.26, 3.5 and 2.3μM), or of Ravoxertinib (100, 66.7, 44.4, 29.6, 19.75, 13.2, 8.8, 5.8, 3.9μM) using serial dilutions (1:1,5), for 72h. Cell proliferation was assayed using CellTiter-Glo® Luminescent Cell Viability Assay and following the manufacturer's instructions. The results come from three independent experiments. Data were analyzed using Graphpad Prism software, calculating the IC50 from the non-linear regression fit curve.

3.8 Apoptosis assay and flow cytometry

For apoptosis evaluation, 10^6 cells were plated and treated with 10μM Sorafenib or DMSO for 24h. Cells were harvested keeping the supernatant and fixed with 1% Paraformaldehyde for 20 minutes on ice. Cells were washed and fixed in ethanol. They were incubated with 0.1% triton X-100 for 10 minutes, washed and incubated for 30 minutes with 5% donkey serum. Cells were washed and incubated with anti-cleaved Caspase3 (Cell Signaling) for 1 hour at room temperature, followed by washing and incubation with anti-rabbit FITC for 1h. Then cells were washed, incubated with 2.5μg/ml Propidium Iodide (PI) and RNAse and incubated at 4^{o}C overnight. Samples were acquired using a Celesta flow cytometer (Becton Dickinson), and data were analyzed using FlowjoX software (Becton Dickinson), reporting caspase 3 positive cells. Results were plotted as fold change relative to the control.

3.9 Drugs and Treatments

Cells were treated with 10ng/mL recombinant EGF (Gibco), 5 or 10μM Sorafenib (Selleckchem), 10μM Ravoxertinib (Selleckchem), 10μM Tanzisertib (Selleckchem), 10μM 10μM Rapamycin, 1μM Torin (Sigma Aldrich), 10μM U0126, AG1478 10μM AG1478, 5μM MG132 (Sigma Aldrich) 10nM Bafilomycin A1(Sigma Aldrich), from here on referred to as Bafilomycin, for the time indicated in figure legends. Cells were starved in starvation medium (D-MEM with 0.1% FBS, 1% NEAA) for 16 or 24 hours. Experimental conditions for all treatments were previously determined in preliminary time-course and dose-response experiments.

3.10 Statistical analysis

Statistical significance was evaluated with Graphpad Prism version 9 software. Multiple samples were compared using ordinary one way ANOVA with Dunnett's multiple comparisons test. Paired or unpaired Student's t tests were used to compare two samples normally distributed. Bar represents means ± SEM of the indicated number of biological replicates. Values of $p<0.05$ were considered significant and represented as: * ($p<0.05$); ** ($p< 0.01$); *** ($p< 0.001$); ***($p<0.0001$). ns (not significant; $p>0.05$).

4 Results

4.1 Downregulation of HPV16 E6/E7 reduces cell proliferation in some, but not all HPV+ HNSCC cell lines

To understand the role of E6/E7 on cellular proliferation of HPV+ HNSCC cell lines, we knocked down HPV16 E6/E7 through siRNA and shRNA approaches.

The Human Papillomavirus induces cancer in HPV+ tumors. In particular, the oncoproteins E6/E7 are responsible for uncontrolled proliferation and inhibition of apoptosis. They are the main drivers of oncogenesis and they represent a valid target for therapy. Many studies, both in cervical cancer and HNSCC, showed that inhibition or downregulation mediated by short interfering RNA (siRNA), short hairpin RNA (shRNA) or genome editing performed using clustered regularly interspaced short palindromic repeats/ CRISPR associated protein 9 (CRISPR/Cas9) targeting E6/E7, induced accumulation of p53 and hypophosphorylation of pRb, and resulted in increased cell apoptosis (Jiansong Zhou et al. 2012; D. Hong et al. 2009; Kennedy et al. 2014).

We initially performed a transient knock down of E6/E7 transfecting with siRNA UD-SCC-2, UM-SCC-47, UM-SCC-104, VU-SCC147, UPCI-SCC-152, and UPCI-SCC-154 cell lines and assessed the levels of E7 proteins by western blot after 3 days of knock down (Figure 5). The knock down of E7 was evident in UD-SCC-2, UM-SCC-104 and VU-147-SCC cell lines, while there was only a slightly reduction in UPCI-SCC-152 and UPCI-SCC-154. UM-SCC-47 cell line did not show a reduction of E7, but p53 was increased, indicating a downregulation of E6. Beside the UM-SCC-47 cell line, p53 increased also in UD-SCC-2 and slightly in UPCI-SCC-154, while the other cell line did not show an upregulation of p53. siRNA was not highly efficient in all the cell lines used.

To assess the effects of knock down on E6/E7 on cell proliferation, we checked cell viability in HPV+ HNSCC. Figures 6-11 show the levels of proliferation of siE6/E7 with respect to the control (siluc) after 3 days and 6 days from the knock down. Since the knock down by siRNA is transient, we performed a double knock down, repeating the siRNA transfection 2 days after the first. Only UD-SCC-2 cell line shows a significant reduction on proliferation upon siE6/E7.

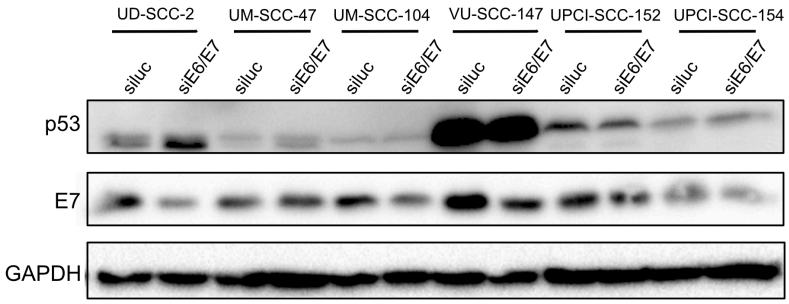


Figure 5 - E7 and p53 protein levels upon siE6/E7.

The HPV+ HNSCC cell lines UD-SCC-2, UM-SCC-47, UM-SCC-104, VU-SCC-147, UPCI-SCC-152 and UPCI-SCC-154 were transfected with siRNA against HPV16 E6/E7 or siluc and harvested after 3 days. Western blot shows HPV16 E7 and p53 protein levels upon knock-down of E6/E7 and GAPDH as the loading control.

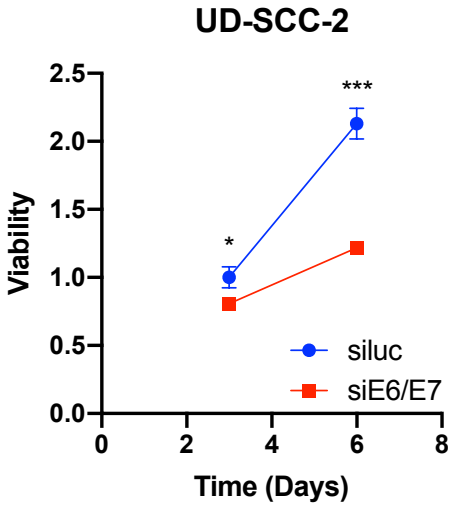


Figure 6 - Effects of siE6/E7 on cell proliferation in UD-SCC-2 HPV+ cell line.

*HPV+ HNSCC UD-SCC-2 cells were transfected with siRNA against HPV16 E6/E7 or luc and 5000 cells were seeded on a 96 well plate in duplicate for each time point. After 48 hours the transfection was repeated in the same conditions. After 3 days or 6 days cell viability was measured using CellTiter-Glo® kit. The graph shows the relative viability with respect to the control. Unpaired student's T test was performed and statistically significant data were reported as * ($p<0.05$), *** ($p<0.001$). Experiment was performed in triplicate.*

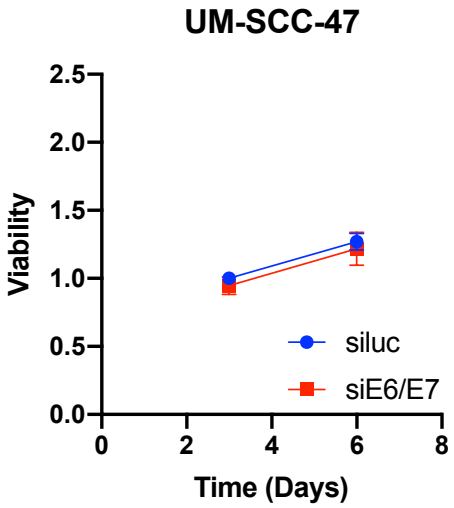


Figure 7 - Effects of siE6/E7 on cell proliferation in UM-SCC-47 cell line.

HPV+ HNSCC UM-SCC-47 cells were transfected with siRNA against HPV16 E6/E7 or luc and 5000 cells were seeded on a 96 well plate in duplicate for each time point. After 48 hours the transfection was repeated in the same conditions. After 3 days or 6 days cell viability was measured using CellTiter-Glo® kit. The graph shows the relative viability with respect to the control. Unpaired student's T test was performed with not significant results. The experiment was performed in triplicate.

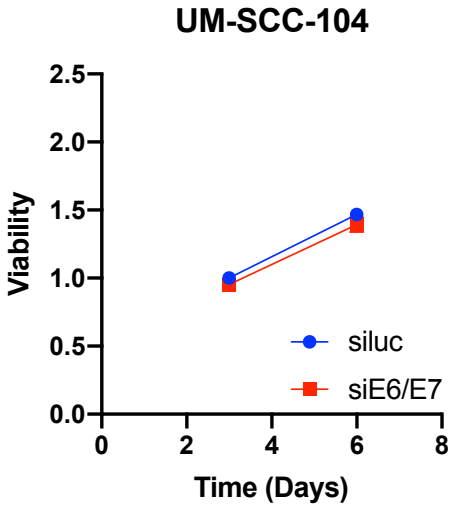


Figure 8 - Effects of siE6/E7 on cell proliferation in UM-SCC-104 cell line.

HPV+ HNSCC UM-SCC-104 cells were transfected with siRNA against HPV16 E6/E7 or luc and 5000 cells were seeded on a 96 well plate in duplicate for each time point. After 48 hours the transfection was repeated in the same conditions. After 3 days or 6 days cell viability was measured using CellTiter-Glo® kit. The graph shows the relative viability with respect to the control. Unpaired student's T test was performed with not significant results. The experiment was performed in triplicate.

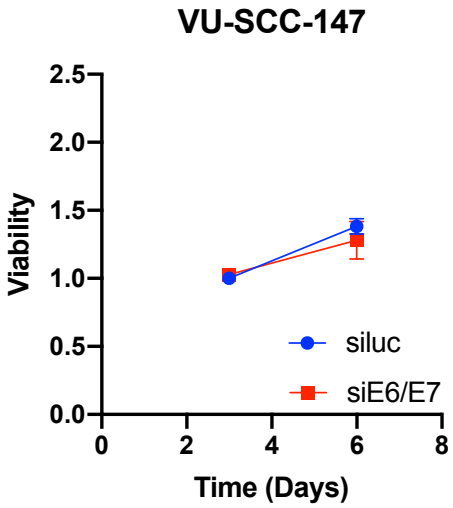


Figure 9 - Effects of siE6/E7 on cell proliferation in VU-SCC-147 cell line.

HPV+ HNSCC VU-SCC-147 cells were transfected with siRNA against HPV16 E6/E7 or luc and 5000 cells were seeded on a 96 well plate in duplicate for each time point. After 48 hours the transfection was repeated in the same conditions. After 3 days or 6 days cell viability was measured using CellTiter-Glo® kit. The graph shows the relative viability with respect to the control. Unpaired student's T test was performed with not significant results. The experiment was performed in triplicate.

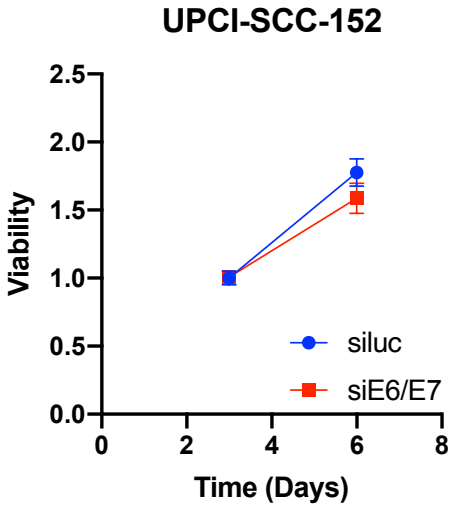


Figure 10 - Effects of siE6/E7 on cell proliferation in UPCI-SCC-152 cell line.

HPV+ HNSCC UPCI-SCC-152 cells were transfected with siRNA against HPV16 E6/E7 or luc and 5000 cells were seeded on a 96 well plate in duplicate for each time point. After 48 hours the transfection was repeated in the same conditions. After 3 days or 6 days cell viability was measured using CellTiter-Glo® kit. The graph shows the relative viability with respect to the control. Unpaired student's T test was performed with not significant results. The experiment was performed in triplicate.

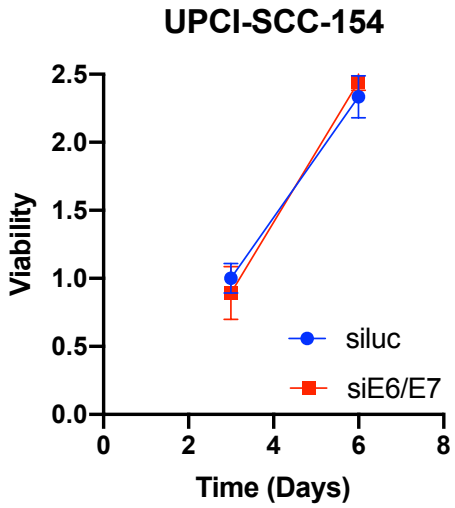


Figure 11 - Effects of siE6/E7 on cell proliferation in UPCI-SCC-152 cell line.
HPV+ HNSCC UPCI-SCC-152 cells were transfected with siRNA against HPV16 E6/E7 or luc and 5000 cells were seeded on a 96 well plate in duplicate for each time point. After 48 hours the transfection was repeated in the same conditions. After 3 days or 6 days cell viability was measured using CellTiter-Glo® kit. The graph shows the relative viability with respect to the control. Unpaired student's T test was performed with not significant results. The experiment was performed in triplicate.

For the difficulties to obtain an efficient knock down using siRNA, we decided to use shRNA on HPV+ HNSCC cell lines. We used two short hairpins to induce the knock down of E6 and E7 (listed in Table 5). All HPV+ HNSCC cell lines in Table 4 were transduced with shE6/E7#1, shE6/E7#2 or the control shluc. To assess the efficiency of knock down we performed western blot analysis (figure 12), showing the reduction in E7 protein expression and the rescue of p53 mediated by E6 downregulation upon expression of shE6/E7. The knock down of E6/E7 did not influence the protein levels of p53 in VU-SCC-147 cell line, which expresses a mutant form of the protein (figure 12) (Steenbergen et al. 1995). Surprisingly, p53 was downregulated by the knock down of E6/E7 in UPCI-SCC-154 cells, harboring wild type p53 (figure 12). Unfortunately, we did not succeed in obtaining an efficient knock down of E6/E7 in UPCI-SCC-152 cell line, and we did not perform a growth curve on this cell line (figure 12).

Again, as for siRNA we performed a growth curve at four different time points on UD-SCC-2, UM-SCC-47, UPCI-SCC-90, UM-SCC-104, VU-SCC147, and UPCI-SCC-154 cell lines transduced with shluc, shE6/E7#1 or shE6/E7#2. The time points were 1 (control), 3, 5 and 7 days from seeding. Figure 13 shows a significant reduction of proliferation rate in UD-SCC-2, between the two shE6/E7 sequences

and the control after 7 days. UM-SCC-104 cells showed a significant reduction of proliferation already after 3 days upon shE6/E7#1, while only after 7 days the reduction in proliferation was visible also upon shE6/E7#2 (figure 16). The other cell lines did not show a significant decrease in proliferation rate, probably suggesting that E6/E7 is not essential for cancer cell proliferation in these cell lines. However, for the UPCI-SCC-90, we saw only a slight increase in cell number after 7 days with respect to control, suggesting that probably these experimental settings are not optimal for this cell line (figure 15).

Together these results show that the knock down of E6/E7 have an effect on proliferation of some, but not all HPV+ HNSCC cell lines tested. This suggests the probably not all HPV+ HNSCC are addicted to E6/E7 oncoprotein expression to survive.

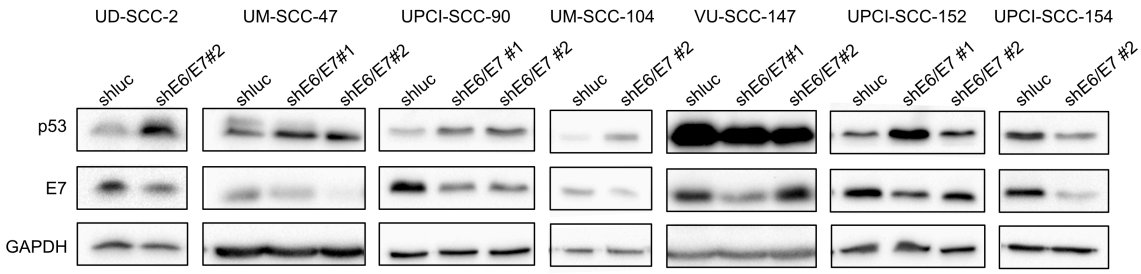


Figure 12 – Knock down of E6/E7 in HPV+ HNSCC cell lines.

Western blot shows HPV16 E7 and p53 protein levels in UD-SCC-2, UM-SCC47, UPCI-SCC-90, UM-SCC-104, VU-SCC-147, UPCI-SCC-152 and UPCI-SCC-154 cells expressing shRNAs against HPV16 E6/E7 (shE6/E7#1 and shE6/E7#2) or luc. GAPDH was used as loading control.

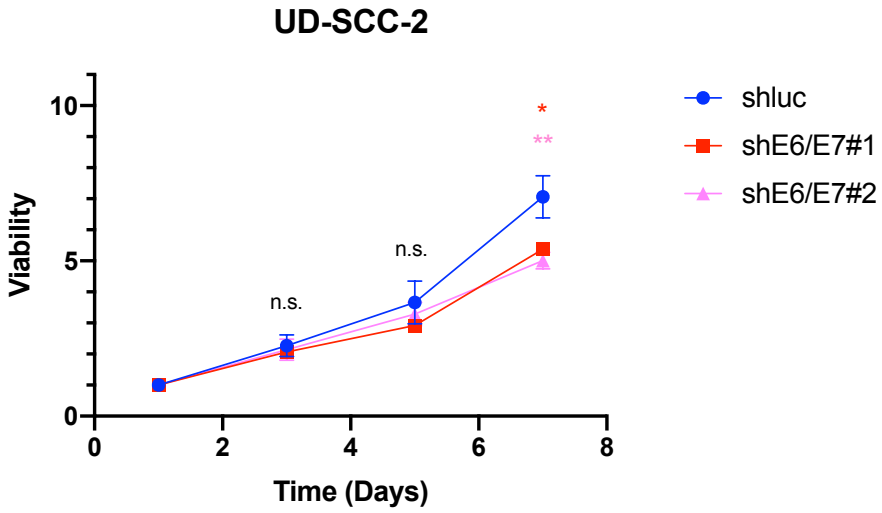


Figure 13 - Effects of shE6/E7 on cell proliferation in UD-SCC-2 cell line.

*HPV+ HNSCC UD-SCC-2 cells, expressing shluc, shE6/E7#1 or shE6/E7#2 were seeded on 96 well plates in duplicate (2500 cells/well) for each time point (1, 3, 5, 7 days). Viability was measured using CellTiter-Glo® kit. The graph shows the relative viability with respect to the control (day1). Ordinary one-way ANOVA was performed between the shE6/E7#1, shE6/E7#2 and shluc for each time point. Statistics is reported in the graph as n.s. ($p>0.05$), *($p<0.05$), **($p<0.01$). Asterisks are of the same color of the sample they represent. This experiment was performed in three different biological replicates.*

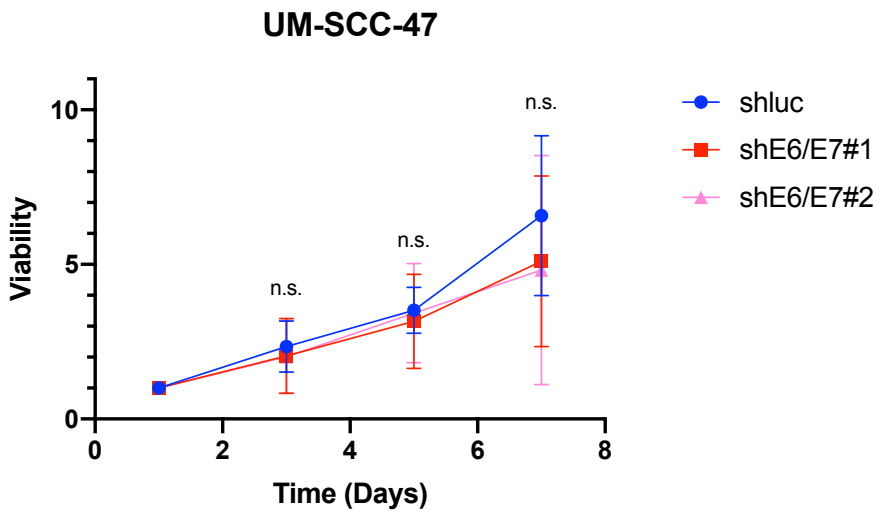


Figure 14 - Effects of shE6/E7 on cell proliferation in UM-SCC-47 cell line.

HPV+ HNSCC UM-SCC-47 cells, expressing shluc, shE6/E7#1 or shE6/E7#2 were seeded on 96 well plates in duplicate (1500 cells/well) for each time point (1, 3, 5, 7 days). Viability was measured using CellTiter-Glo® kit. The graph shows the relative viability with respect to the control (day1). Ordinary one-way ANOVA was performed between the shE6/E7#1, shE6/E7#2 and shluc for each time point. Statistics is reported in the graph as n.s. (p>0.05). This experiment was performed in three different biological replicates.

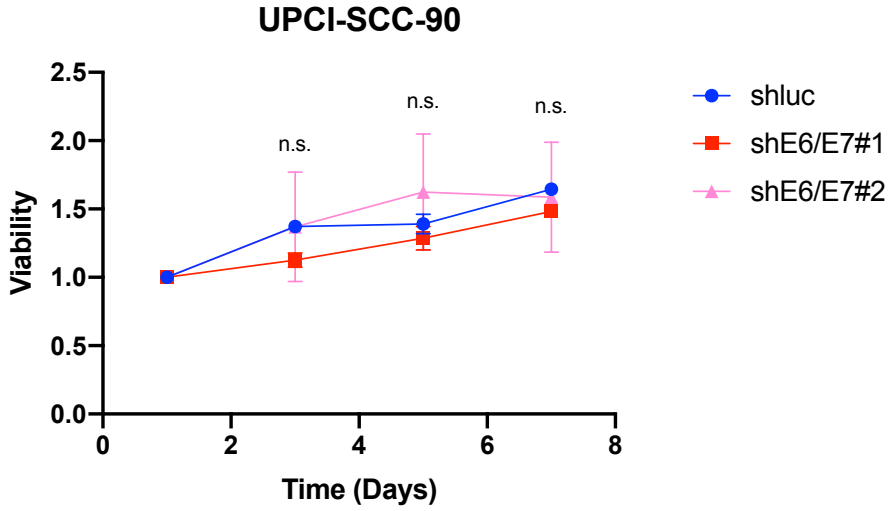


Figure 15 - Effects of shE6/E7 on cell proliferation in UPCI-SCC-90 cell line.

HPV+ HNSCC UPCI-SCC-90 cells, expressing shluc, shE6/E7#1 or shE6/E7#2 were seeded on 96 well plates in duplicate (2500 cells/well) for each time point (1, 3, 5, 7 days). Viability was measured using CellTiter-Glo® kit. The graph shows the relative viability with respect to the control (day1). Ordinary one-way ANOVA was performed between the shE6/E7#1, shE6/E7#2 and shluc for each time point. Statistics is reported in the graph as n.s. (p>0.05). This experiment was performed in three different biological replicates.

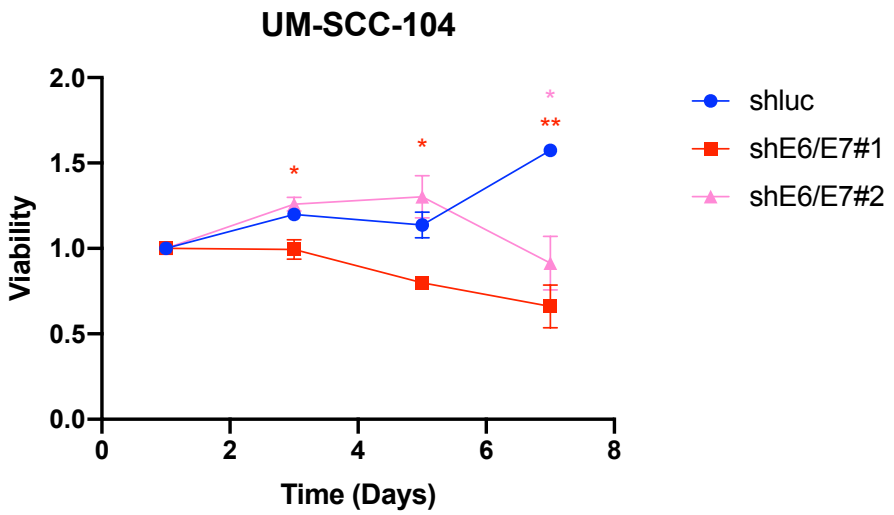


Figure 16 - Effects of shE6/E7 on cell proliferation in UM-SCC-104 cell line.

*HPV+ HNSCC UM-SCC-104 cells, expressing shluc, shE6/E7#1 or shE6/E7#2 were seeded on 96 well plates in duplicate (2000 cells/well) for each time point (1, 3, 5, 7 days). Viability was measured using CellTiter-Glo® kit. The graph the relative viability with respect to the control (day1). Ordinary one-way ANOVA was performed between the shE6/E7#1, shE6/E7#2 and shluc for each time point. Statistics is reported in the graph as *(p<0.05), **(p<0.01). Asterisks are of the same color of the sample they represent. This experiment was performed in three different biological replicates.*

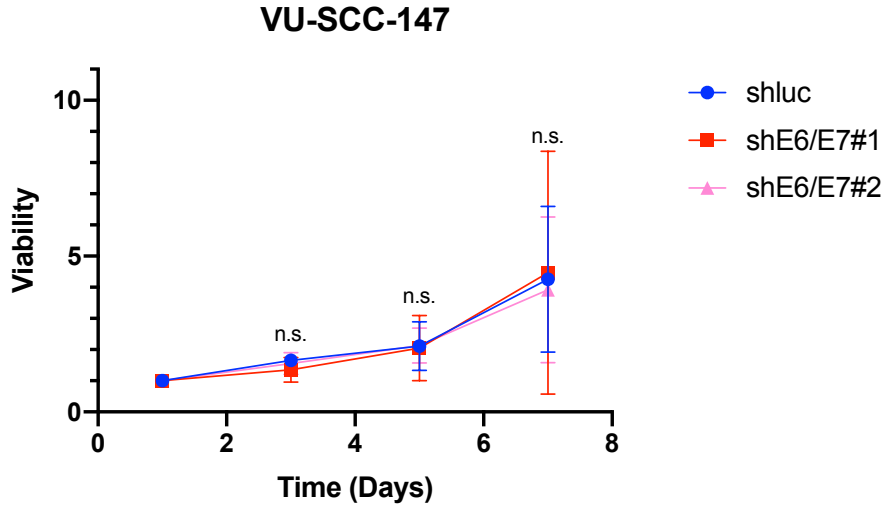


Figure 17 - Effects of shE6/E7 on cell proliferation in VU-SCC-147 cell line.

HPV+ HNSCC VU-SCC-147 cells, expressing shluc, shE6/E7#1 or shE6/E7#2 were seeded on 96 well plates in duplicate 1500 cells/well) for each time point (1, 3, 5, 7 days). Viability was measured using CellTiter-Glo® kit. The graph shows the relative viability with respect to the control (day1). Ordinary one-way ANOVA was performed between the shE6/E7#1, shE6/E7#2 and shluc for each time point. Statistics is reported in the graph as n.s. (p>0.05). This experiment was performed in three different biological replicates.

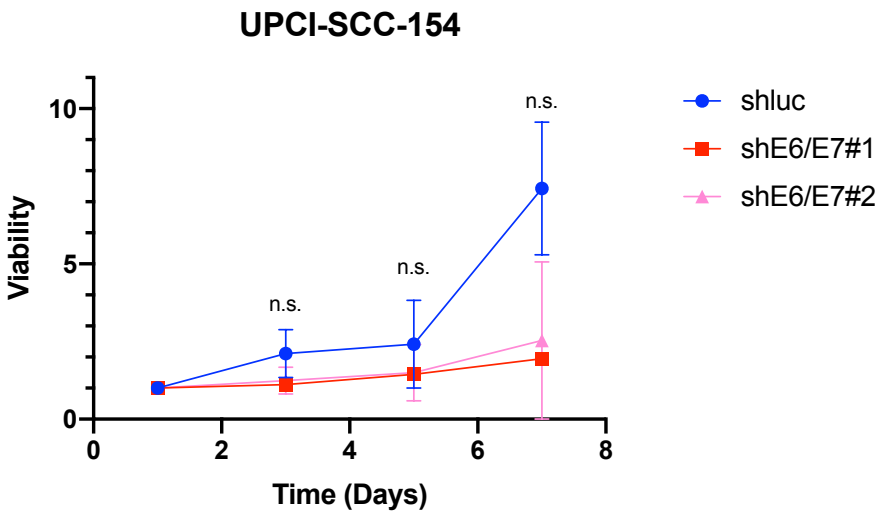


Figure 18 - Effects of shE6/E7 on cell proliferation in UPCI-SCC-154 cell line.
HPV+ HNSCC UPCI-SCC-154 cells, expressing shluc, shE6/E7#1 or shE6/E7#2 were seeded on 96 well plates in duplicate (2000 cells/well) for each time point (1, 3, 5, 7 days). Viability was measured using CellTiter-Glo® kit. The graph shows the the relative viability with respect to the control (day1). Ordinary one-way ANOVA was performed between the shE6/E7#1, shE6/E7#2 and shluc for each time point. Statistics is reported in the graph as n.s. (p>0.05). This experiment was in three different biological replicates.

4.2 Kinase inhibitors decrease HPV16 E6/E7 expression in HNSCC cell lines

The ribosomal protein pS6, a downstream target of mTOR, has been shown to accumulate in multiple HPV-associated cancers, including HNSCC and cervical SCC, as well as anal SCC, suggesting that mTOR activation might contribute to the onset of these cancers (Molinolo et al. 2012). In order to understand whether mTOR pathway is linked to E6/E7 oncoproteins expression in HPV+ HNSCC cell lines, we tested three kinase inhibitors acting on mTOR pathway: Rapamycin, Torin and Sorafenib. We treated HPV+ cell lines for 16 hours with the inhibitors and checked the levels of E7 oncoproteins, p53 and p-p70 S6 kinase, the phosphorylated form of a downstream target of mTOR.

Western blots in figure 19 show that Sorafenib downregulated E7 protein levels in all cell lines, inducing the rescue of p53 in UD-SCC-2, UPCI-SCC-90 and UPCI-SCC-154 cell lines and surprisingly increasing p-p70. Torin strongly downregulated E7 levels only in UPCI-SCC-154, and slightly in UM-SCC-47, UPCI-SCC-90 and UM-SCC104. Rapamycin was able to reduce E7 levels only in UM-SCC-104, and, to a lower extent, in UPCI-SCC-90 and UP-SCC-154. These results suggested that,

since Sorafenib showed a stronger effect and worked on all HPV+ HNSCC cells tested, it could be a valid drug for further investigations.

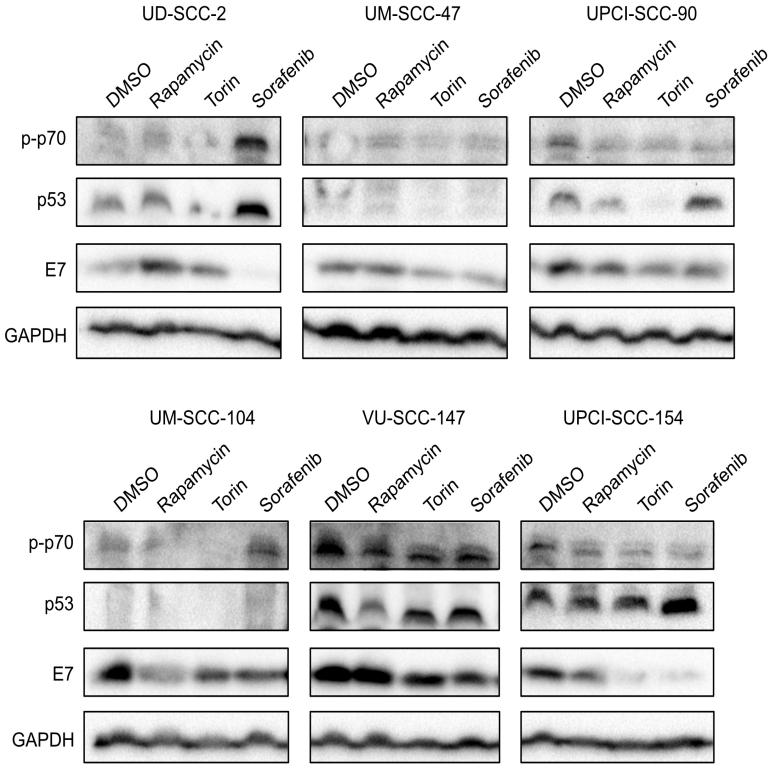


Figure 19 – Effects of mTOR inhibitors on E7 protein expression in HPV+ HNSCC. HPV+ HNSCC cell lines.

Western blots show HPV16, p-p70, E7 and p53 protein levels in UD-SCC-2, UM-SCC47, UPCI-SCC-90, UM-SCC-104, VU-SCC-147, UPCI-SCC-152 and UPCI-SCC-154 treated with 10 μM Rapamycin, 1μM Torin, 10μM Sorafenib, or DMSO for 24 hours. GAPDH was used as loading control.

4.3 Sorafenib induces downregulation of E7 in all HPV+ HNSCC cell lines

Among the inhibitors used, Sorafenib showed a stronger effect in decreasing E6/E7 expression. Next, we thus assessed the concentration of Sorafenib needed to reduce E6/E7 protein levels. Sorafenib is a drug approved for the treatment of advanced renal cell carcinoma, hepatocellular carcinoma, acute myeloid leukemia and radioactive iodine resistant advanced thyroid carcinoma (reviewed in Keating and Santoro 2009). It is a kinase inhibitor acting on multiple kinases, including RAF, PDGFR and VEGFR; it is known to induce autophagy and to inhibit mTOR (Wilhelm et al. 2008; Prieto-Domínguez et al. 2016). We tested Sorafenib on HPV+ HNSCC

cell lines at different concentrations for 24 hours. Sorafenib was able to reduce E7 levels already at 5μM, but the effect was stronger at 10μM, as shown in figure 21. Notably, p53 expression was not rescued in all cell lines upon treatment with Sorafenib: only in UD-SCC-2 and UPCI-SCC-154 cell lines there was a clear increase of p53 levels; which is present in UPCI-SCC-90 only at 5μM, in UM-SCC-47 cells only a slight increase of p53 could be observed; UM-SCC-104 and UPCI-SCC-152 cell lines did not show an increase of p53.

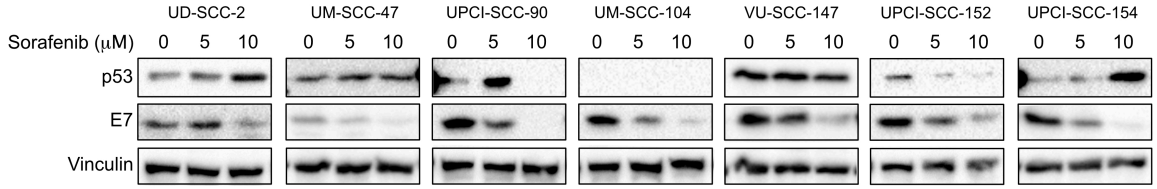


Figure 20 – Effects of Sorafenib on p53 and E7 protein expression in HPV+ HNSCC cell lines. *Western blots show HPV16 E7 and p53 protein levels in UD-SCC-2, UM-SCC47, UPCI-SCC-90, UM-SCC-104, VU-SCC-147, UPCI-SCC-152 and UPCI-SCC-154 treated with 5μM or 10μM, or DMSO for 24 hours. Vinculin was used as loading control.*

4.4 Sorafenib downregulates E6/E7 also in the presence of autophagic and proteasomal inhibition

To understand the mechanism by which Sorafenib downregulates E6/E7 we investigated if it relied on post translational regulations. We treated UPCI-SCC-152 cells with Sorafenib, the proteasomal inhibitor MG132 and the autophagic inhibitor Bafilomycin, alone or in combination for 8 hours. As showed in figure 21, Sorafenib induced the downregulation of E7 even in the presence of both the inhibitors, suggesting that probably Sorafenib does not reduce E6/E7 expression inducing its degradation by the proteasomal or autophagic degradation. The increase of p53 upon MG132 treatment indicated that the treatment worked. Light chain 3 (LC3) protein is an autophagic marker, whose increase in the low molecular weight form indicates the inhibition of autophagic degradation and it was used as a control for Bafilomycin treatment.

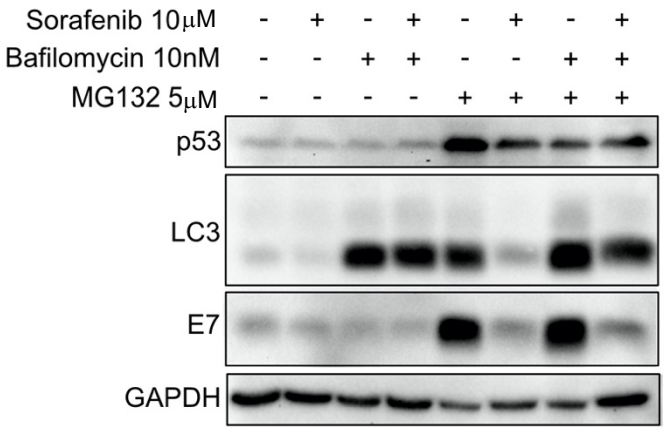


Figure 21 – Combination of Bafilomycin, MG132 and Sorafenib treatment in HPV+ HNSCC cells.

Western blot shows HPV16 E7, LC3 and p53 UPCI-SCC-152 cells treated with 10μM Sorafenib, 10nM Bafilomycin, 5μM MG132, or DMSO, alone or in combination, for 8 hours. GAPDH was used as loading control.

4.5 Sorafenib downregulates E6/E7 mRNA levels

To test the hypothesis that Sorafenib impinges E6/E7 mRNA levels, we performed RT-qPCR on HPV+ HNSCC cell lines upon treatment with Sorafenib for 24 hours. We measured the relative amount of E6/E7 mRNA, as shown in figure 22. Unpaired Student's T test confirmed that there was a significant reduction of E6/E7 mRNA upon Sorafenib treatment in the cell lines tested (figures 22-23). These results suggest that Sorafenib induces the downregulation of E6/E7 transcription.

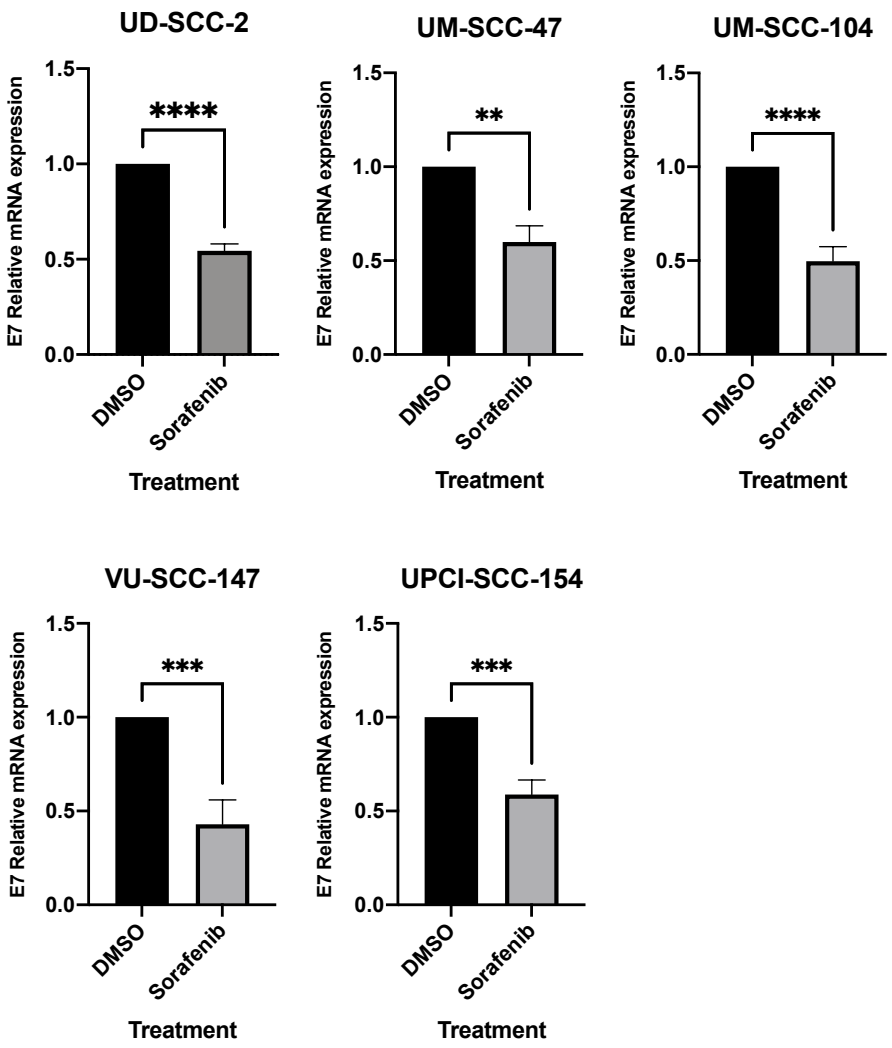


Figure 22 - Sorafenib effects on HPV16 E7 mRNA expression in HPV+ HNSCC cell lines.

*Total RNAs from UD-SCC-2, UM-SCC47, UM-SCC-104, VU-SCC-147 and UPCI-SCC-154 cells treated with Sorafenib (10μM) or DMSO for 24 hours were isolated for RT-qPCR. HPV16 E7 expression was normalized to GAPDH and expressed as means ± SD of at least three independent experiments. Unpaired Student's T test was calculated, and the p value was expressed as ** (p<0.01), *** (p<0.001), or **** (p<0.0001).*

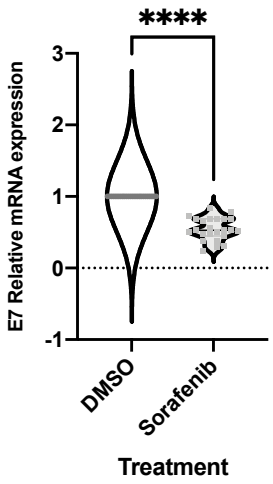


Figure 23 - Global effect of Sorafenib on HPV16 E7 mRNA expression in HPV+ HNSCC cell lines.

*Results shown in Figure 22 were put together and analyzed. Unpaired Student's T test was calculated, and the p value was expressed as **** ($p<0.0001$).*

4.6 Sorafenib induces apoptosis in some HPV+ and HPV- HNSCC cell lines

To test the potential therapeutic effect of Sorafenib in Head and Neck cancer, we evaluated whether it was able to induce apoptosis in both HPV+ and HPV- HNSCC cell lines. We treated UD-SCC-2, UM-SCC-47, UPCI-SCC-90, UM-SCC-104, UPCI-SCC-152, UPCI-SCC-154 (HPV+), and UM-SCC-4, UM-SCC-6, UM-SCC-17A, UM-SCC-17B, UM-SCC-18, UM-SCC-23 (HPV-) with 10μM Sorafenib for 24 hours. Fixed cells were stained with an anti-cleaved caspase 3 antibody and observed with flow cytometry. Caspase 3 cleavage occurs during apoptosis and the presence of this marker indicates that cells are undergoing apoptosis. In figure 24 the fold change of cleaved caspase 3 positive cells treated with Sorafenib with respect to the control is reported. Among HPV+ cell lines, only UD-SCC-2, UPCI-SCC-90 and UPCI-SCC-152 showed an increase in apoptotic cells upon treatment with Sorafenib, while, UM-SCC-47, UM-SCC-104, UPCI-SCC-154 showed a decrease in cleaved caspase 3 positive cells. An analogous situation could be observed in HPV- cell lines, with an increase in apoptotic cells upon treatment with Sorafenib in UM-SCC-4, UM-SCC-17B, UM-SCC-23, and a decrease in UM-SCC-6, UM-SCC-17A, UM-SCC-18.

Figure 25 compared the results obtained in HPV+ with HPV-. The graph showed that there was not a significant difference in the capability of Sorafenib to induce apoptosis between HPV+ and HPV- cells.

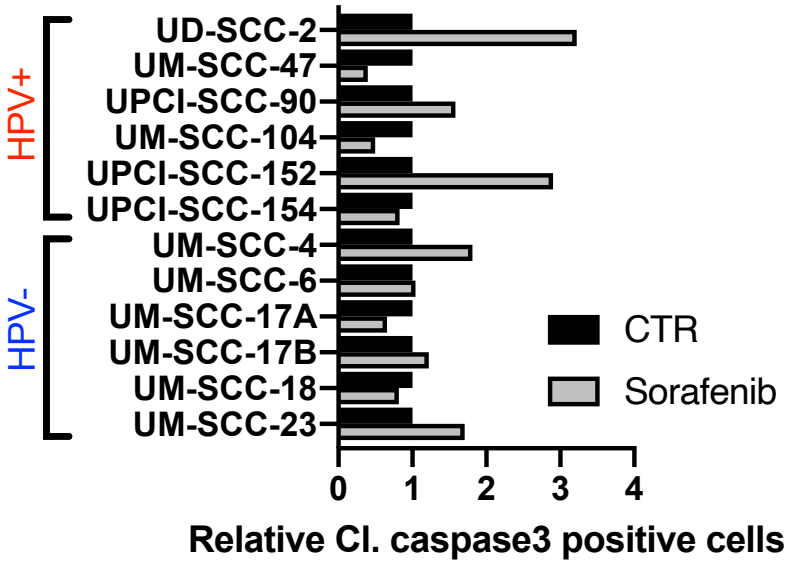


Figure 24 Induction of apoptosis induced by Sorafenib in HNSCC cell lines.

The HPV+ UD-SCC-2, UM-SCC-47, UPCI-SCC-90, UM-SCC-104, UPCI-SCC-152, UPCI-SCC-154 and the HPV- UM-SCC-4, UM-SCC-6, UM-SCC-17A, UM-SCC-17B, UM-SCC-18, UM-SCC-23 HNSCC cell lines were treated with 10μM Sorafenib or DMSO for 24 hours. Cells were harvested, fixed, stained with cleaved Caspase3 antibody, and analyzed by FACS. The graph shows the fold change of cleaved Caspase3 positive (Cl. Caspase 3 positive) cells treated with Sorafenib compared to control treated with DMSO (CTR).

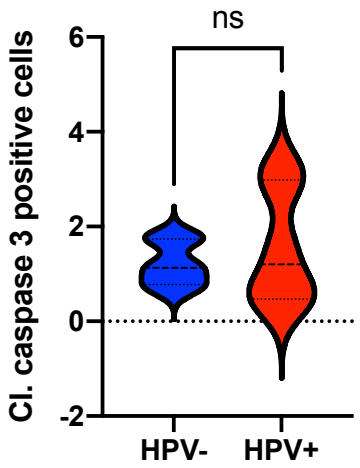


Figure 25 – Sorafenib induces apoptosis in both HPV+ and HPV- HNSCC cells.

Results from each cell line in figure 24 were put together and analyzed. Student's T test analysis was performed with $p > 0.05$ (ns).

4.7 Sorafenib sensitivity varies among HNSCC cell lines but not between HPV+ and HPV- cells

Since Sorafenib induced the downregulation of E6/E7, we wanted to understand whether HPV+ cells were more sensitive than HPV- HNSCC cell lines to this drug. We treated all the cells in Table 4 with 9 different concentrations of Sorafenib and the control for 72 hours and then measured cell viability. From these results we calculated the IC50 of Sorafenib in HPV- (in blue) and in HPV+ (in red) cell lines as showed in figure 26. Figure 27 summarized the results and showed that there were no significant differences in IC50 between HPV+ and HPV- HNSCC cell lines.

These results suggest that Sorafenib cannot be considered a specific molecule for HPV+ tumors. This is probably due to the fact that Sorafenib inhibits many kinases and can act on different pathways, not being a specific molecule. For this reason, it is important to understand the exact pathway by which it downregulates E6/E7 expression and specifically target that pathway.

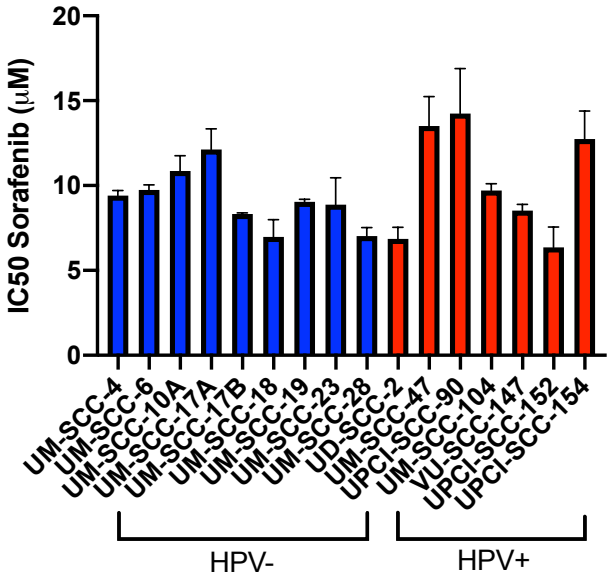


Figure 26 – IC50 of Sorafenib in HNSCC cell lines.

All the cell lines in Table 4 were seeded in 96 well plates and treated with different concentration of Sorafenib. After 72 hours cell quantification was performed using CellTiter-Glo® kit. Nonlinear regression curve was calculated and IC50 concentration was extracted. The graph shows the IC50 of Sorafenib in HPV- (in blue) and HPV+ (in red) HNSCC cell lines expressed as means ± SD of three independent experiments.

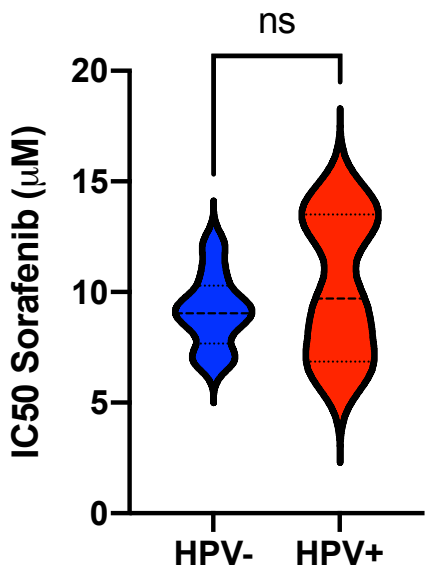


Figure 27 – Sorafenib sensitivity in HPV- and HPV+ cell lines.

The graph shows the IC50 of Sorafenib in HPV- HNSCC cell lines (in blue) with respect to HPV+ HNSCC cell lines from the experiment in figure 26. Student's T test was calculated, with p>0.05 (ns).

4.8 EGFR/MEK/ERK/JNK pathways regulate E7 expression

One of the known enzymes inhibited by Sorafenib is RAF1, upstream of MEK in the ERK signaling pathway, and regulated by EGFR cascade. To test whether E6/E7 downregulation is mediated by the inhibition of this pathway, we used upstream, downstream inhibitors and also a JNK inhibitor, and checked the expression levels of E7 by western blot. We treated UPCI-SCC-90, UCI-SCC-152 and UPCI-SCC-154 cell lines with AG1478 to inhibit the catalytic activity of EGFR, U0126 to inhibit MEK, Ravoxertinib to inhibit ERK and Tanzisertib to inhibit JNK (figure 28). Upon treatment, UPCI-SCC-90 cell line showed only a slightly reduction of E7 expression; in UPCI-SCC-152 cell line Ravoxertinib treatment resulted in a high reduction of E7 expression, while Tanzisertib induced very little variation; in UPCI-SCC-154 cell lines only AG1478 was able to reduce E7 expression. Moreover, c-fos expression was down regulated by all the inhibitors in UPCI-SCC-154 cell line, while the protein was not detected in UPCI-SCC-90 cells; in UPCI-SCC-152 cell line results were not easy to be interpreted. p-ERK protein levels should indicate the activation of the pathway.

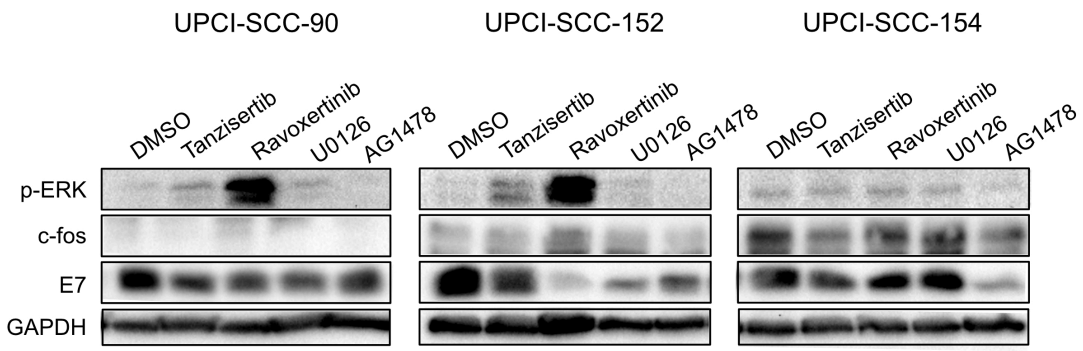


Figure 28 – Effects of EGFR and ERK signaling pathway inhibitors on E7 protein expression in HPV+HNSCC.

UPCI-SCC-90, UPCI-SCC-152 and UPCI-SCC-152 cells were treated with, 10μM Tanzisertib, 10μM Ravoxertinib, 10μM U0126, 10μM AG1478 or DMSO for 24 hours. Western blots show p-ERK, c-fos, HPV16 E7 protein levels or GAPDH as loading control.

4.9 Effects of Ravoxertinib and Tanzisertib on E6/E7 expression upon EGF stimulation

EGF is an important growth factor that regulates cell growth and differentiation through its binding to EGFR. Since we observed that inhibition of EGFR activity by AG1478 induced the downregulation of E7, we wanted to assess whether the use of EGF could have an effect on HPV oncoprotein expression.

We starved UPCI-SCC-90 and VU-SCC-147 cells for 16 hours removing serum in order to turn-off EGFR signaling. We then treated cells with its ligand EGF for 2 hours. Western blots showed that EGF treatment upregulated ERK phosphorylation and c-fos levels, and consequently E7 expression in UPCI-SCC-90 and VU-SCC-147 (figure 29). To check if EGF affects E7 expression specifically through EGFR activation, we inhibited UM-SCC-104 cells with AG1478 before treating them with EGF for 2 hours. Western blot showed that E7 upregulation induced by EGF was reverted in the presence of AG1478, confirming that EGF reduces E7 expression upon EGFR activation (figure 30).

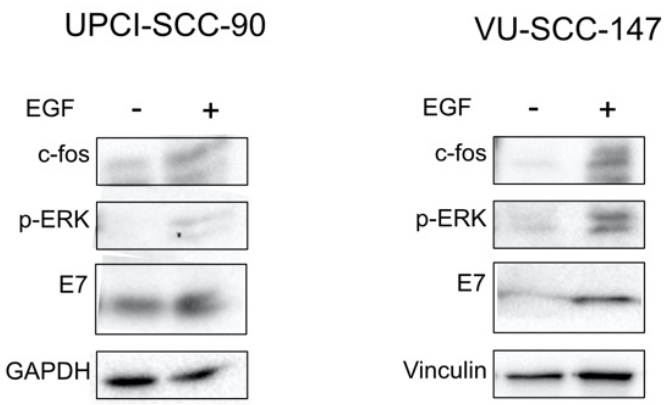


Figure 29 - Effects of EGFR activation on E7 protein expression in HPV+ HNSCC.
UPCI-SCC-90 and VU-SCC-147 cells were starved for 16 hours and then treated with 10ng of EGF or water for 2 hours. Western blots show p-ERK, c-fos, HPV16 E7 protein levels and Vinculin or GAPDH as loading control.

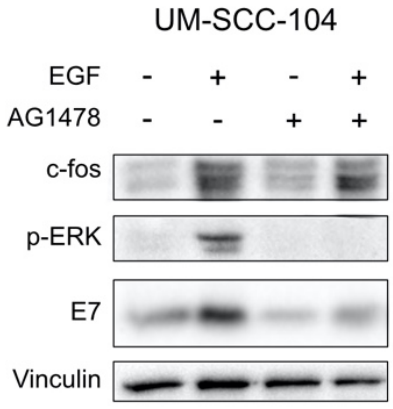


Figure 30 - Effects of EGFR activation on E7 protein expression in UM-SCC-104.
UM-SCC-104 cells were starved for 16 hours and then treated with AG1478 10μM or DMSO for 30 minutes and then with 10ng of EGF or water for 2 hours. Western blot shows p-ERK, c-fos, HPV16 E7 protein levels, or GAPDH as loading control.

As mentioned before, AP-1 transcription factor is fundamental for E6/E7 mRNA expression.

Ravoxertinib and Tanzisertib inhibit ERK and JNK respectively, upstream signals of c-jun and c-fos, members of the AP-1 complex. Since these two inhibitors showed a negative effect on E7 expression, we decided to better investigate these two inhibitors on HPV+ HNSCC cell lines upon EGF treatment. HPV+ HNSCC cell lines were starved for 24 hours and then treated with EGF, Ravoxertinib, Tanzisertib alone or in combination for 6 or 24 hours. Figure 31 shows that in UPCI-SCC-152

cells Ravoxertinib downregulated E7 protein levels alone or in combination with Tanzisertib, already after 6 hours, but with a stronger effect after 24 hours of treatment. P-c-jun and c-fos are respectively downregulated with Tanzisertib and Ravoxertinib. Moreover, the same effects can be observed also at mRNA levels (figure 32); the efficacy of the inhibitors can be observed by the reduction of the downstream JUN and FOS mRNA expression (figure 33).

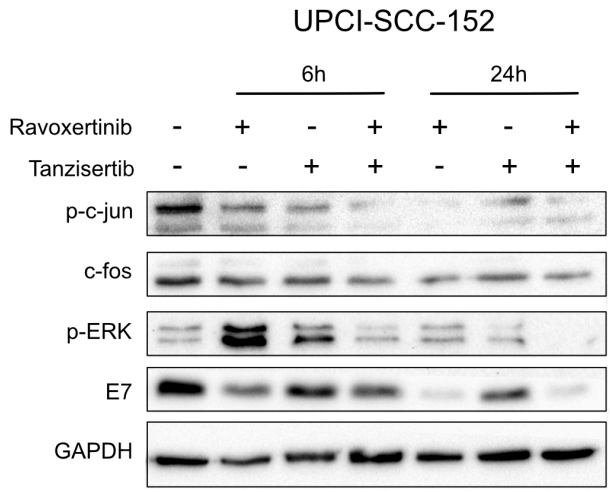


Figure 31 - Effects of Tanzisertib and Ravoxertinib inhibition on E7 protein expression in HPV + HNSCC cells.

UPCI-SCC-152 cells were starved for 24 hours and then treated with 10ng of EGF, and after 2 hours cells were treated with Ravoxertinib 10μM, Tanzisertib 10μM or DMSO for 6 or 24 hours. Western blot shows p-c-jun, p-ERK, c-fos, HPV16 E7, and GAPDH as loading control.

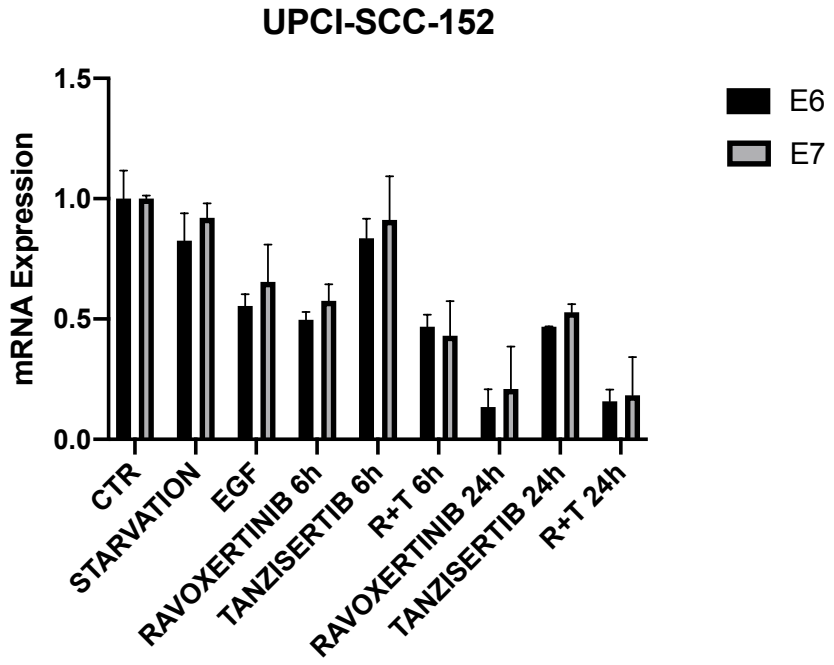


Figure 32 - Effects of Tanzisertib and Ravoxertinib inhibition on E6 and E7 mRNA expression in UPCI-SCC-152.

UPCI-SCC-152 cells were starved for 24 hours and then treated with 10ng of EGF or water, and after 2 hours cells were treated with Ravoxertinib 10μM, Tanzisertib 10μM or DMSO for 6 or 24 hours. Total RNAs were isolated for RT-qPCR. HPV16 E6 and E7 expression was normalized to GAPDH expressed as means ± SD of two independent experiments.

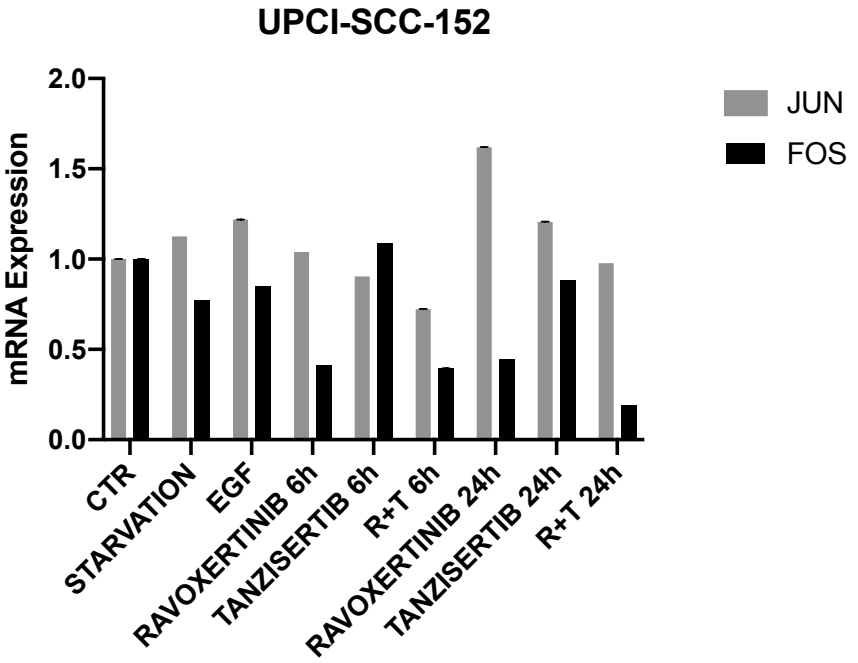


Figure 33 - Effects of Tanzisertib and Ravoxertinib inhibition on JUN and FOS mRNA expression in UPCI-SCC-152.

JUN and FOS expression of experiment in figure 63 was normalized to GAPDH and expressed as means ± SD of two independent experiments.

4.10 Gain and loss of function of c-fos affect HPV16 E6/E7 expression

Since the inhibition of ERK negatively regulated E6/E7 expression, we investigated the effects of c-fos overexpression on E7 protein levels. Transient overexpression of c-fos did not affect E7 expression in all cell lines, inducing E7 upregulation only in UM-SCC-104, VU-SCC-147 and UPCI-SCC-152 cell lines (figure 34).

We then tested the effect of knock-down of c-fos on E7 expression in UD-SCC-2, UPCI-SCC-90 and UM-SCC-104. We transfected cells with sifos or siluc for 72

hours and then evaluated mRNA levels of E7, FOS and JUN. RT-qPCR showed a downregulation of E7 upon knock down of FOS in all 3 cell lines, and also a downregulation of JUN in UD-SCC-2 and UM-SCC-104 (figure 35). These results taken together suggest that c-fos could be a potential target for the treatment of HPV+ HNSCC.

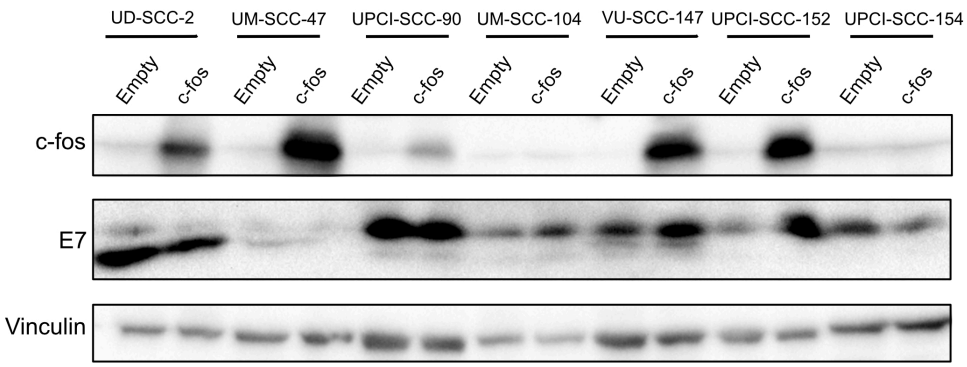


Figure 34 – Effects of fos overexpression on E7 protein levels.

All the HPV+ HNSCC cell lines UD-SCC-2, UM-SCC-47, UPCI-90, UM-SCC-104, VU-SCC-147, UPCI-SCC-152 and UPCI-SCC-154 were transfected with a plasmid overexpressing c-fos or the empty vector and harvested after 48 hours. Western blot shows HPV16 E7 and c-fos protein levels and Vinculin as the loading control.

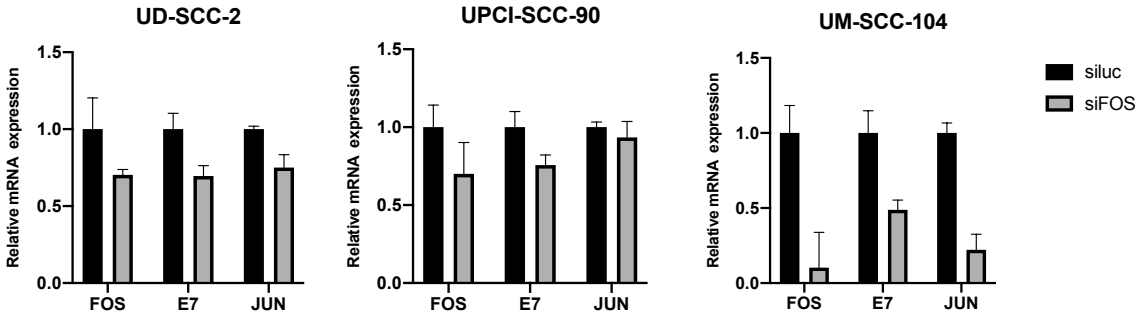


Figure 35 - Effects of siFOS on E7 and c-jun mRNA levels in HPV+ HNSCC cell lines.

UD-SCC-2, UPCI-SCC-90 and UM-SCC-104 cells were transfected with a mix of siRNA against fos or luc and harvested after 72 hours. Total RNAs were isolated for RT-qPCR. HPV16 E7, FOS, and JUN expression was normalized to GAPDH. expressed as means ± SD of two independent experiments.

4.11 HPV16 E6/E7 mRNA expression correlates with JUN and FOS

Since c-jun and c-fos appear to regulate E6/E7 expression, we wanted to check whether c-jun and c-fos expression correlated with E6 and E7 mRNA expression. We performed RT-qPCR on mRNA extracted from UD-SCC-2, UM-SCC-47, UPCI-SCC-90, UM-SCC-104, VU-SCC-147, UPCI-SCC-152, UPCI-SCC-154 cells and monitored E6, E7, c-jun (JUN) and c-fos (FOS) gene expression and calculated the Pearson r correlation coefficient. In figure 36 and 37 E6 expression was compared to JUN and FOS respectively, finding a good correlation (r = 0.6527 and r = 0.6631) even if not significant. An analogous situation could be observed in E7; E7 and JUN expression were high correlated (r = 0.8662), as well as E7 and FOS (r =0.8197) (figures 38, 39).

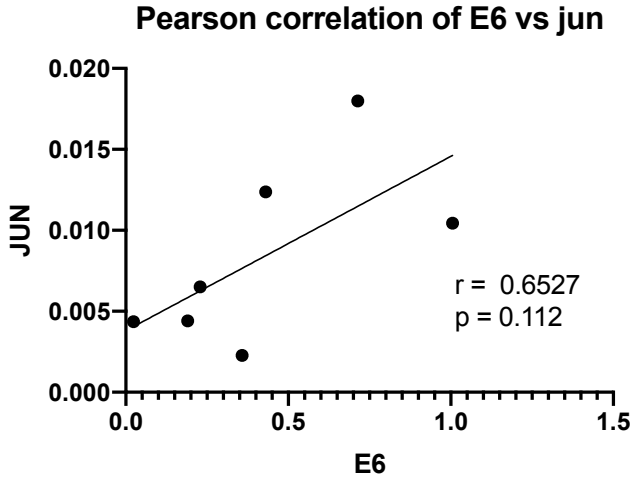


Figure 36 – Correlation of HPV16 E6 to JUN mRNA expression.

HPV16 E6 mRNA levels from the 7 HPV+ HNSCC cell lines were correlated to JUN mRNA levels. This graph shows the results of three independent experiments. Pearson correlation coefficient r and p value are showed in the graph.

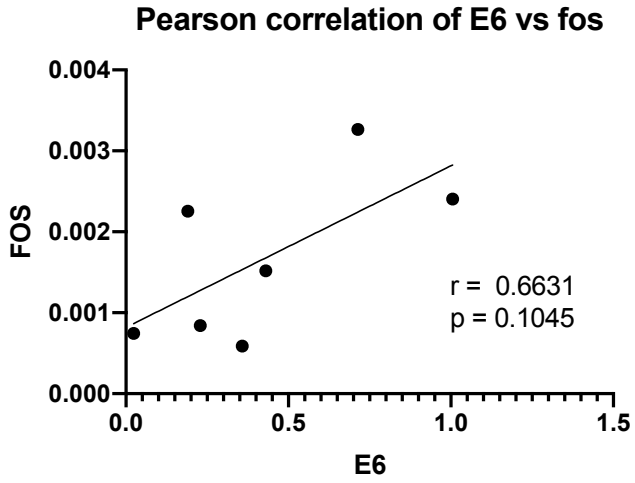


Figure 37 – Correlation of HPV16 E6 to FOS mRNA expression.

HPV16 E6 mRNA levels from the 7 HPV+ HNSCC cell lines were correlated to FOS mRNA levels. This graph shows the results of three independent experiments. Pearson correlation coefficient r and p value are showed in the graph.

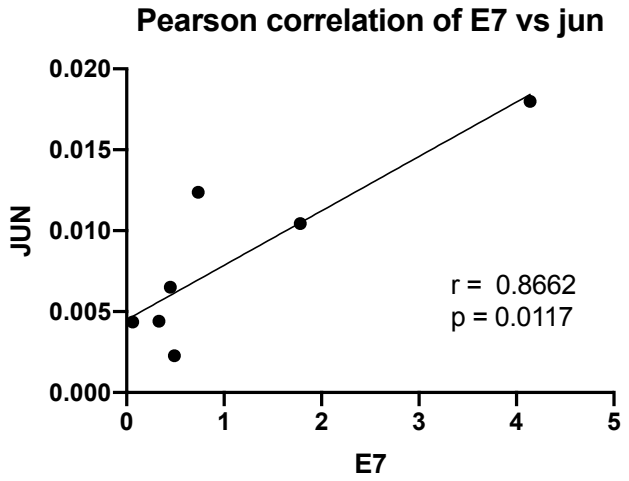


Figure 38 – Correlation of HPV16 E7 to JUN mRNA expression.

HPV16 E6 mRNA levels from the 7 HPV+ HNSCC cell lines were correlated to JUN mRNA levels. This graph shows the results of three independent experiments. Pearson correlation coefficient r and p value are showed in the graph.

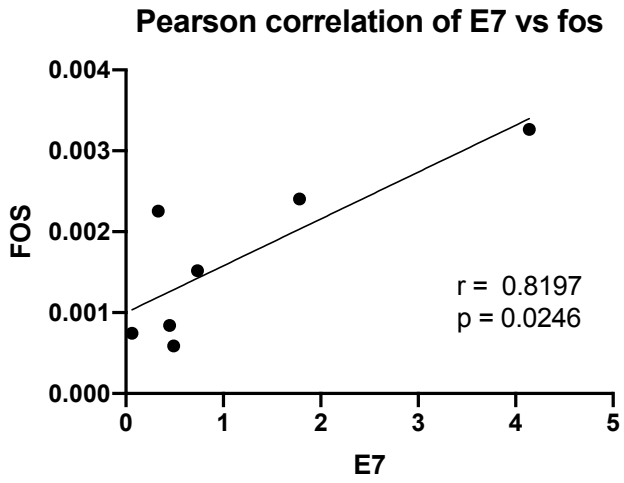


Figure 39 – Correlation of HPV16 E7 to FOS mRNA expression.

HPV16 E6 mRNA levels from the 7 HPV+ HNSCC cell lines were correlated to JUN mRNA levels. This graph shows the results of three independent experiments. Pearson correlation coefficient r and p value are showed in the graph.

4.12 HPV+ HNSCC cell lines are more sensitive to Ravoxertinib than HPV- HNSCC cell lines

We wanted to understand if Ravoxertinib and Tanzisertib could be potential strategies for HPV+ HNSCC therapy. Thus, we treated all the panel of HNSCC cell lines with 9 different concentration of Ravoxertinib and Tanzisertib and the control as described for Sorafenib. We calculated the IC50 of the two compounds in all cell lines. Unfortunately, Tanzisertib was able to kill HNSCC cells only at very high concentrations, and it was not possible to calculate the IC50 (data not shown).

Instead, we were able to calculate the IC50 of Ravoxertinib in 13 out of 16 HNSCC cell lines (figure 40). In figure 41 HPV+ cell lines were compared to HPV- cell lines. HPV+ cell lines were more sensitive to Ravoxertinib compared to HPV-, suggesting a potential application for HPV+ HNSCC.

Unfortunately, it was not possible to calculate the IC50 to Ravoxertinib of 3 HPV+ HNSCC cell lines since they were resistant to this drug. These cell lines were UM-47-SCC, VU-SCC-147, UPCI-SCC-154, the same cell lines that were not affected by the knock-down of E6/E7 in terms of proliferation.

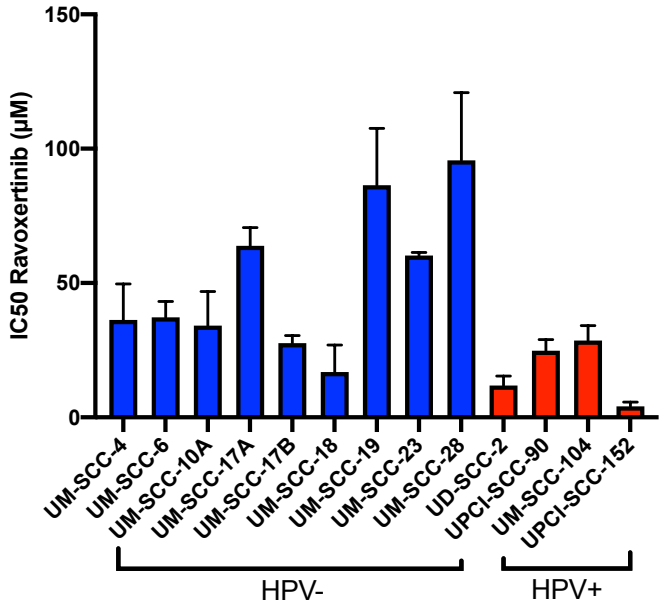


Figure 40 –HNSCC cell lines sensitivity to Ravoxertinib.

All the cell lines in table 4 were seeded in 96 well plates and treated with increasing concentrations of Ravoxertinib. After 72 hours cell quantification was performed using cell CellTiter-Glo® kit. Nonlinear regression curve was calculated and IC50 concentration was extracted. The graph shows the IC50 of Ravoxertinib in HPV- (in blue) and of four HPV+ (in red) HNSCC cell lines expressed as means ± SD of three independent experiments. For UM-SCC-47, VU-SCC-147 and UPCI-SCC-154 was not possible to calculate IC50 and these cell lines were excluded from the graph.

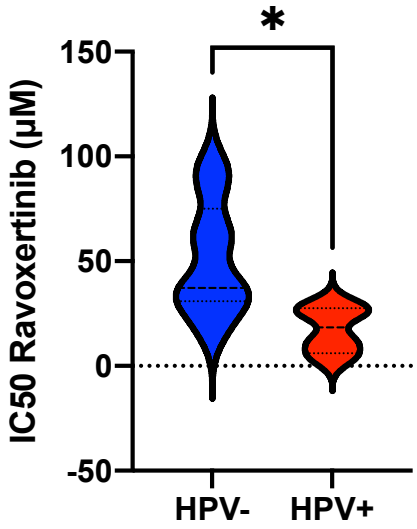


Figure 41 – Ravoxertinib sensitivity in HPV- and HPV+ cell lines.

The graph shows the IC50 of Ravoxertinib in HPV- HNSCC cell lines (in blue) with respect to HPV+ HNSCC cell lines from the experiment in figure 40. Student's T test was calculated, with $p<0.05$ (*).

5 Discussion

HPV oncoproteins E6/E7 are the main driver of carcinogenesis in many types of HPV-related cancers, such as HNSCC, cervical cancer, non-melanoma skin cancer and other anogenital malignancies.

During the last decades, many approaches were attempted to find targeted therapies for HPV-driven malignancies. Among them, considering the central role of E6 and E7 oncoproteins in carcinogenesis, many studies focused on E6/E7 inhibition, especially in cervical cancer.

In particular, many findings showed that downregulation of the HPV oncogenes expression, mostly using RNAi approaches, caused cell growth inhibition, and induction of apoptosis (Yeo-Teh, Ito, and Jha 2018). Unfortunately, this transient approach, with only temporary modulation, can be used only for mechanistic studies, but not as a therapeutic approach *in vivo*.

Thus, mechanisms that utilize permanent genome editing capabilities seem to be more promising in the treatment of cervical cancer patients in the clinic. For example, an ongoing Phase I clinical trial by Hu and colleagues, described the use of transcription activator-like effector nucleases (TALENs), which are restriction enzymes engineered to cut specific sequences of DNA. In the same trial, they have also studied the most revolutionary technique in genome editing of the last decades, CRISPR/Cas9, in which, by delivering the Cas9 nuclease together with a synthetic guide RNA (gRNA) into a cell, they removed HPV16 E6/E7 or HPV18 E6/E7 to treat cervical cancer patients (Clinical Trial identifier: NCT03057912) (Z. Hu et al. 2014, 2015).

In a recent work, Bortnik and colleagues used CRISPR/Cas9 system in two HPV16+ HNSCC cell lines (UD-SCC-2 and UM-SCC-104), to see the effects of the knock-out of E6 and E7 in specific pathways. They showed that only the knock-out of both E6 and E7 was able to induce apoptosis in the 50% of the cells (Bortnik et al. 2020).

In this work, we wanted to assess the effects of the knock down of HPV oncoproteins E6/E7 extending it to all the available HPV16 positive HNSCC cell lines. We used RNAi approaches, both in a transient (using siRNA) and a stable manner (using shRNA), and we evaluated the effects on proliferation. The levels of knock down were variable, in particular using the transient approach. In siRNA

experiments, only UD-SCC-2 cell line showed an effect on proliferation upon knock down of E6/E7. This could be due by the fact the siRNA was delivered by transfection, a process that is not 100% efficient in all the cell lines. For this reason, we established cell lines that stably express a shRNA against E6/E7 upon transduction with lentiviral vectors. In this case the knock down efficiency was high in all cell lines, excluding the UP-SCC-152 which showed only a slight reduction of HPV oncoproteins and thus was not used for growth curve analysis. Among the 6 cell lines evaluated, UD-SCC-2 and UM-SCC-104 cell lines were the only cell lines showing a significant reduction in cell proliferation in the presence of shE6/E7, replicating the results obtained by Bortnik et al. To note, the UP-SCC-90 cells did not show a high increase in proliferation for 7 days, suggesting that probably the settings used for this cell line were not optimal and to be better set in the future.

Even in the presence of good levels of knock down, not all the cell lines seemed to suffer the absence of E6/E7. It is true that RNAi is only able to reduce E6/E7 levels, and probably the CRISPR/Cas9 approach could be more reliable. For this reason, and since it has been performed only in 2 HPV16 positive HNSCC cell lines, we are currently setting the use of this technology to knock-out E6/E7 oncoproteins, in order to have wider and reliable overview of E6/E7 importance in HPV+ HNSCC cell lines.

While in cervical cancer HPV is the most prevalent cause of tumorigenesis, in HNSCC, not only HPV, but also smoke and alcohol, are very important for the pathogenesis of head and neck tumors. It is relevant to highlight the fact that HPV+ positive patients are often also alcohol and smoke consumers. In these cases, it is still not clear what is the effect of the combination of these risk factors in the development of tumors, but also in the molecular phenomena that occurs in the infected region. We still do not know if all HPV+ HNSCC tumors are addicted to E6 and E7 oncoproteins, and this could be due by the presence of other risk factors that contributed to carcinogenesis, that somehow interfere or stand above HPV in the contest of carcinogenesis. Moreover, E6/E7 expression is not sufficient to induce carcinogenesis, and beside integration itself (that is present in all HPV+ HNSCC cell lines but UM-SCC-104), many mutations, amplifications and epigenetic events need to occur for the onset of tumorigenesis, inducing the deregulation of many pathways. Even upon removing E6/E7 these alterations are still present, and it is possible that these mutational events alter the molecular landscape of that

specific tumor with respect to HPV, in a way for which E6/E7 are not essential for tumor growth and maintenance.

It is important to highlight that all the HPV16+ HNSCC cell lines available come from smoker patients. We still do not know what is the impact of smoke on HPV-induced molecular events, but some studies in cervical cancer and in lung cancer suggest that smoke molecules are involved in the activation of HPV gene expression through EGFR, mTOR, Akt pathway (Muñoz et al. 2018; Peña et al. 2015).

Additionally, the VU-SCC-147 cell line harbors a mutation in p53 (L257R), that could inhibit E6-dependent degradation of p53, suggested by the fact that upon knock-down of E6/E7 in our data we do not see an increase of p53 levels (figure 6,13).

Besides RNAi and genomic editing approaches, some works showed the development of molecules which block the activity of either E6, E7, or E6AP, through the use of peptides, organic compounds, RNA molecules, nucleotide analogs, small molecule inhibitors, zinc-ejecting inhibitors, heparin-like molecules or naturally-derived biopolymers (reviewed in Jung et al. 2015). During the last years, also natural compounds derived from plants were studied, which have shown the ability to reduce the viral infection in patients with HPV-positive cervical cancer. In particular, a polyherbal formulation, administration of *Praneem* to patients positive for high-risk HPV16 for a period of 30 days led to non-detection of the virus via PCR-based methods (Shukla et al. 2009). Another example is the natural alkaloid berberine, that downregulated E6/E7 levels by inhibition of AP-1 activity in cervical cancer (Mahata et al. 2011).

It has been shown that mTOR activation might contribute to HPV+ Head and Neck cancer carcinogenesis (Molinolo et al. 2012). Thus, we used some mTOR inhibitors (Rapamycin, Torin and Sorafenib) to evaluate whether the mTOR pathway is important for E6/E7 oncoproteins expression in HPV+ HNSCC cell lines. We showed that Rapamycin and Torin could induce a downregulation of E7, but the effect is cell lines dependent. For example, Rapamycin was effective on downregulation of E7 in UPCI-SCC-90, UM-SCC-104 and UPCI-SCC-154, while Torin induced E7 downregulation in UM-SCC-47, UPCI-SCC-90, UM-SCC-104 and UPCI-SCC-154.

Sorafenib suppressed E7 expression in all HPV+ cell lines. For this reason, we focused our attention on this molecule, a kinase inhibitor already used in clinics for

the treatment of renal cancer, hepatocellular carcinoma and some forms of acute myeloid leukemia (AML). Sorafenib reduced E7 protein level and rescued p53 levels, as a secondary effect of downregulation of E6. This effect was not mediated by a post translational mechanism since inhibition of proteasomal and autophagic degradation were not able to block Sorafenib effects.

Moreover, quantitative PCR analysis revealed reduction of E7 mRNA levels upon Sorafenib treatment, suggesting that this drug acts on E7 transcription.

We also investigated whether Sorafenib was able to kill HPV+ HNSCC cells more efficiently than HPV- HNSCC by staining cleaved caspase 3 cells, a marker of apoptosis upon treatment with Sorafenib. No significant difference in apoptosis induction between HPV+ and HPV- HNSCC cell lines was observed upon Sorafenib treatment. We also checked the sensitivity of HNSCC cell lines to Sorafenib, calculating the IC50 in all the cell lines, but we could not find significant differences between HPV+ and HPV- HNSCC cell lines. This could be due to the fact that Sorafenib is a multi-kinase inhibitor that inhibits 5 different kinases and the lack of specificity confers susceptibility to all HNSCC cell lines.

To understand the molecular mechanism of E7 downregulation, we focused our attention on Raf/MEK/ERK inhibition, one of the kinase pathways inhibited by Sorafenib, and we investigated this pathway with different inhibitors and activators.

We showed that inhibition of ERK or EGFR, with a consequent downregulation of c-fos, resulted in reduced E6/E7 expression, while the activation of the pathway by EGF increased E7 levels in HPV+ HNSCC cell lines, suggesting that EGFR and ERK are involved in E6/E7 gene expression in HNSCC cell lines. However, the inhibition of MEK or ERK not always induced the downregulation of c-fos protein levels, and this will be further investigated. Moreover, treatment with Ravoxertinib increased phosphorylation of ERK, which is also reported for other ERK inhibitors (Bhagwat et al. 2020). The increase in pERK is likely caused by the loss of negative feedback activation through ERK-mediated inhibition of CRAF phosphorylation (Dougherty et al. 2005).

We then combined JNK and ERK inhibitors (Tanzisertib and Ravoxertinib) with EGF treatment, showing that Ravoxertinib downregulated E7 expression both at protein and mRNA levels, while Tanzisertib had only a very slight effect. Even if c-jun and c-fos are both members of the AP-1 complex, that binds on a consensus sequence in the HPV p97 promoter resulting in E6/E7 transcription, it seems that

only the downregulation of c-fos and not of c-jun is important for E6/E7 downregulation.

We showed that overexpression of c-fos was able to upregulate E7 expression only in UM-SCC-104, VU-SCC-147 and UPCI-SCC-152 cell lines, while the downregulation of c-fos reduced E6/E7 mRNA levels. These results are in line with the fact that E6/E7 expression is regulated by the AP-1 complex. According to this, we found that the expression of E7 positively correlated with JUN and FOS mRNA expression. Also E6 expression showed a good correlation with JUN and FOS, even if not significant. This result suggests that JUN and FOS genes and their expression are directly involved in E6/E7 expression also in HPV+ HNSCC cell lines.

The specific proteins belonging to the JUN and FOS family, and composing the AP-1 dimer are important for target discrimination and for the activity of the transcription factor. We have not studied in deep this aspect yet, but we will perform immunoprecipitation experiments to understand which are the monomers that compose AP-1 in HPV+ HNSCC cell lines. Moreover, we will perform Chromatin-Immunoprecipitation, followed by qPCR (ChIP-qPCR) to understand which proteins of the JUN and FOS families that form the AP-1 complex in HPV+ HNSCC cell lines bind the p97 promoter of HPV, in presence or absence of ERK and JNK inhibition, to clarify which proteins are essential for E6/E7 transcription.

We will also try to understand what is the effect of ERK and JNK inhibition on the activation of the promoter of HPV p97 in HNSCC cell lines. To do so, we will use luminescent reporters to check the activation or inhibition of the p97 promoter in a wild-type form or mutant forms lacking the AP-1 DNA binding site.

In this work we showed that the ERK inhibitor Ravoxertinib induced E6/E7 downregulation, while the JNK inhibitor Tanzisertib only partially induced this effect. These inhibitors are very specific for ERK and JNK inhibition respectively and are currently used in clinical trials. Tanzisertib showed an effect on cell viability in HNSCC cell lines only at very high concentrations (100μM), whereas Ravoxertinib showed a significant different sensitivity between HPV+ and HPV- cell lines. Thus, HPV+ HNSCC seemed to be more sensitive to ERK inhibition. It is important to note that we excluded 3 HPV+ HNSCC cell lines (UM-SCC-47, VU-SCC-147, and UPCI-SCC-154) because they were resistant to Ravoxertinib. This could be explained by the fact that the same cell lines were also not sensitive to E6/E7 downregulation.

Until now, we only evaluated the IC50 of Ravoxertinib in HNSCC cell lines. We will focus our attention also on phenotypical effects of this inhibition in HPV+ with respect to HPV- HNSCC cell lines. Specifically, we will perform cleaved Caspase 3 staining to study the induction of apoptosis upon treatment with Ravoxertinib, but also migration using trans-well and wound healing assays.

Even if these results are interesting, we would like also to investigate if E6/E7 downregulation mediated by the described kinase inhibitors induce some vulnerabilities in HPV+ HNSCC cells. Specifically, we will combine these inhibitors to Cisplatin to understand whether the combination of Ravoxertinib to chemotherapy is more effective compared to chemotherapy alone and allows a de-escalation of Cisplatin doses in HPV+ with respect to HPV- HNSCC cell lines.

5.1 Conclusions and outlook

To summarize, we showed that targeting EGFR and ERK signaling, and in particular downregulating c-fos expression results in the downregulation of E6/E7 oncoproteins expression, leading to HPV+ cell death. In our model showed in figure 42, the inhibition of ERK signaling results in the downregulation of the AP-1 complex members c-jun and c-fos, with consequently inhibition of HPV E6/E7 transcription from the p97 promoter. E6 and E7 downregulation could probably result in the restoration of p53 and pRb activity, with a consequent induction of apoptosis and inhibition of cell cycle progression in HPV+ HNSCC cell lines.

Looking at the therapeutic application of Ravoxertinib, it could be interesting to have a preclinical evaluation of this inhibitor for HPV+ HNSCC therapy. *In vivo* models using Patient Derived Xenografts (PDXs) of HPV+ HNSCCs could be useful preclinical approaches in this context (Facompre et al. 2017).

Another preclinical approach is the establishment of HNSCC organoids that showed to be efficient in predicting *in vivo* drug sensitivity. Some studies showed the use of organoids coming from individual patient's tumors with promising results for the use of this application in the research field (Tanaka et al. 2018; Driehuis et al. 2019). The constant contact between the Chiocca's Lab and IEO's clinicians will help to develop organoids derived from IEO patients to be used to preclinically evaluate the efficacy of novel therapeutic approaches in the lab, including the use of Ravoxertinib for HPV+ tumors.

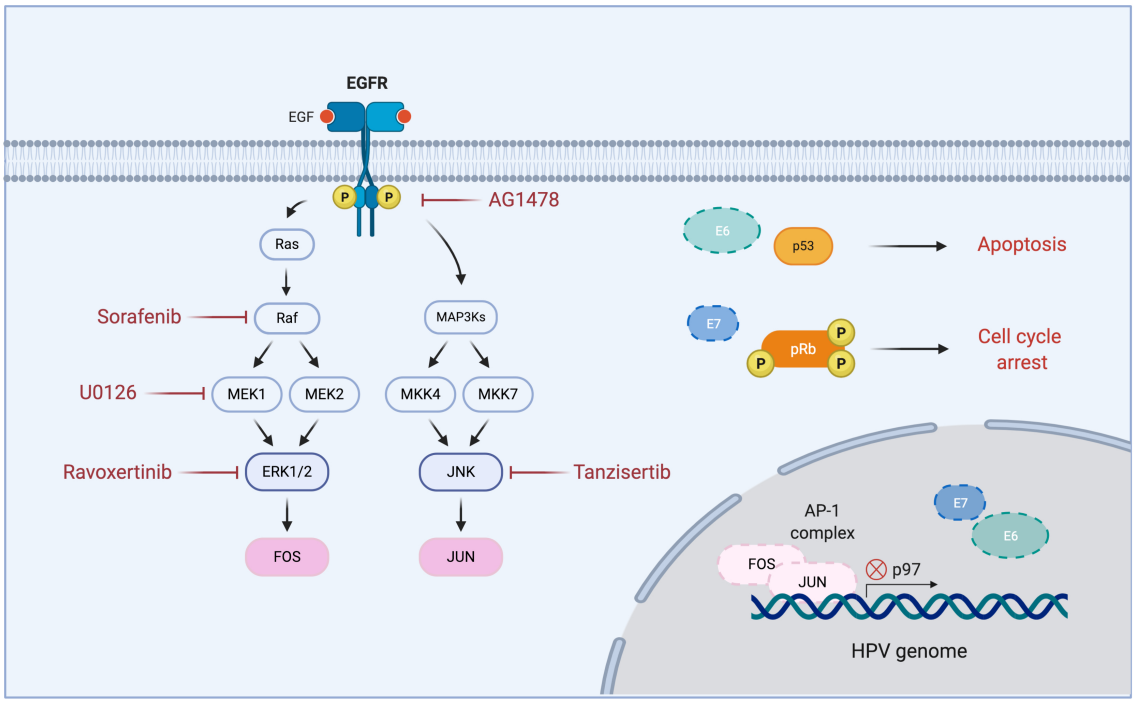


Figure 42 – Model of the molecular mechanisms of EGFR/ERK inhibition in HPV+ HNSCC cell lines.

EGFR, Raf, MEK, ERK and JNK inhibitors downregulate JUN and FOS levels, inhibiting E6 and E7 transcription from the p97 promoter, leading to p53 and pRb rescue and induction of apoptosis and cell cycle arrest.

6 Appendix A - HPV and autophagy in Head and Neck Cancer

6.1 Introduction

Autophagy is a self-eating process used by cells to retain homeostasis. During autophagy cellular proteins are degraded to recycle cellular components and to respond to nutrient stress. Autophagy is implicated in long-lived and misfolded proteins degradation, recycling of damaged mitochondria, and in the elimination of intracellular pathogens (Gross and Graef 2020). Autophagy is mysregulated in different types of cancers (Levy, Towers, and Thorburn 2017).

Three types of autophagy exist: microautophagy, chaperon-mediated autophagy and macroautophagy (hereafter referred as autophagy). In the autophagic process autophagosomes, double-membraned organelles containing cargoes from different origins, fuse with lysosomes, degradative organelles with an acidic environment, to form autolysosomes in which the cargo is degraded (AM, SW, and B 2013). The formation of the small cup-shaped membrane precursor called phagophore starts with the initiation phase, with the unc-51 like autophagy activating kinase 1 and 2 (ULK1/2) kinase complex (Hamasaki et al. 2013). Autophagy factors are recruited to the phagophore and form a double-membrane layer with a curved shape that detaches from the membrane it originates from. During elongation process the phagophore expands thanks to the class III PI3K complex I composed by the vacuolar protein sorting 34 (VPS34), PI3K, autophagy related gene 14L (ATG14L), VPS15, and Beclin1. At this point, the microtubule-associated protein 1 light chain 3 (LC3) is recruited to the autophagosome membrane by conjugation to phosphatidylethanolamine, a process performed by two ubiquitin-like conjugation systems (Stanley, Ragusa, and Hurley 2014). The lipidated form of LC3 (LC3-II), localized on the autophagosome, is a widely used indicator of autophagic flux, and it regulates membrane elongation and autophagosome maturation (Mizushima, Yoshimori, and Levine 2010). p62 is another marker of autophagy that interacts with LC3 and localizes in the autophagosomes. Class III PI3K complex II, composed by VPS34, VPS15, Beclin 1, and UV radiation resistance-associated gene protein (UVRAG), which activates Ras-associated protein-7 (Rab7), is responsible for the

maturation of autophagosomes, by mediating the fusion with lysosomes and formation of autolysosomes (Liang et al. 2008).

The Human Papillomavirus can impinge the autophagy pathway. HPV16 E5 downregulates the mRNA expression of autophagic genes, including autophagy related gene 4a (ATG4a), autophagy related gene 5 (ATG5), autophagy related gene 7 (ATG7), LC3, ULK1, ULK2, and Beclin 1, with a consequent inhibition of phagophore assembly (reviewed in Mattoscio, Medda, and Chiocca 2018). Also HPV16 E6/E7 affect autophagy, specifically by inhibiting autophagosome-lysosome fusion. Oncoproteins' overexpression in primary human keratinocytes (HK) upregulate both the lipidated LC3 and p62, indicating autophagosome accumulation (increase in LC3-II) (Mattoscio et al. 2017). Moreover, HPV oncoprotein E7 induces the degradation of Ambra1 protein, inhibiting autophagy and sensitizing HNSCC cells to cisplatin-induced apoptosis (Antonioli et al. 2020).

The centrality of autophagy for HPV opens the possibility to target autophagy in HPV+ HNSCC. mTOR pathway components, also involved in repressing autophagy, are highly active in HNSCC (Gao et al. 2012), supporting the notion that autophagy is inhibited in this tumor type.

The impact of HPV on HNSCC cells has not been well characterized yet. In this section we focused our attention on the effects of HPV on the autophagy pathway and in particular in HNSCC cell lines.

6.2 Material and methods

Only material and methods different or from the ones in chapter 3 will be described.

6.2.1 Cells

Skin biopsies were collected from healthy skin of donors from IEO hospital. To obtain adult human epidermal keratinocytes, skin biopsies from donors were digested with Dispase (10U/mL; Gibco) for 4 h at 37°C to remove the epidermis, followed by a trypsinization step (Trypsin 500mg/L) for 30 min at 37°C to obtain isolated cells. Primary cultures of the isolated cells were then maintained in Keratinocyte Serum-Free Medium (KSFM; Gibco) containing bovine pituitary extract (BPE, 30µg/mL; Gibco) and epidermal growth factor (EGF, 0.2ng/mL; Gibco). Cells

from passages 2–5 were used for retroviral transduction. All cells were cultured at 37°C in a 5% CO2 buffered incubator.

6.2.2 Transductions, transfections, and plasmids

For retroviral transduction, plasmids were transfected into Phoenix Ampho cells by calcium-phosphate method. The following day, primary keratinocytes were transduced with retroviral supernatants for 6 h at 37°C for two days and selected with G-418 Sulfate (Gibco) for 1 week and finally collected for RNA extraction, WB or treated with drugs. pLXSN-16 E6/E7 was a kind gift by Massimo Tommasino, International Agency for Research on Cancer, Lyon, France.

6.2.3 Library preparation, RNA sequencing and GSEA

500ng of total RNA from 4 biological replicates of HK was used to prepare library for RNA sequencing using Truseq RNA Sample Prep Kit V2set B(Illumina). Sequencing was performed using NovaSeq 6000 system (Illumina). Bioinformatic analysis was performed using HTS-flow framework described in Bianchi V, Ronchi A, and Morelli n.d. ,with which Differential Gene expression (DEGs) and RPKMs (Read Per Kilobase Million) were extracted. Gene Set Enrichment Analysis (GSEA) (Barbie et al. 2009) was run using default parameters on the Broad Institute public genePattern server (http://genepattern.broadinstitute.org/gp/pages/index.jsf) on the C5 GO gene sets.

6.2.4 Electron microscopy

Cells were fixed with 1% glutaraldehyde for 1 hour and postfixed (1% OsO4, 1.5% potassium ferrocyanide in 0.1M cacodylate buffer pH 7.4), *enbloc* stained with 1% uranyl acetate over/night at 4°C, dehydrated with ethanol, embedded in EPON 812 and cured in an oven at 60°C for 48 hours. Ultrathin sections (70-90nm) were cut on an ultramicrotome (Leica FC7, Leica microsystem). Grids were stained with uranyl acetate and Sato's lead solutions and observed in a Leo 912AB Zeiss Transmission Electron Microscope (Carl Zeiss). Digital micrographs were taken with a 2Kx2K bottom mounted slow-scan Proscan camera (ProScan, Lagerlechfeld, Germany) controlled by the EsivisionPro 3.2 software (Soft Imaging System).

6.2.5 Ethics Statement

HKs were isolated from skin biopsies collected via standardized operative procedures approved by European Institute of Oncology Ethical Board. Informed consent was obtained from all patients (donors).

6.3 Results

6.3.1 Autophagic compartments are deregulated by HPV16 in Head and Neck Cancer cells

HPV oncoproteins E6/E7 are able to impinge autophagy in HK and in cervical cancer cells (Mattoscio, Medda, and Chiocca 2018a; Mattoscio et al. 2017), but what happens in HNSCC cells is still unknown. To evaluate whether HPV oncoproteins have an effect on the autophagic machinery in HNSCC cells, we evaluated autophagy-related structures in three HPV-negative and three HPV-positive HNC cell lines by electron microscopy (figure 43). The HPV-positive cell line UD-SCC-2 presented a lower number of autophagic vesicles (AV) compared to the HPV-negative UM-SCC-19 cell line (figure 44). HPV-positive cell lines presented a significant lower number of AVs per cell compared to HPV-negative, except for UM-SCC-104. These results suggest that HPV deregulates autophagy machinery in HNC cells.

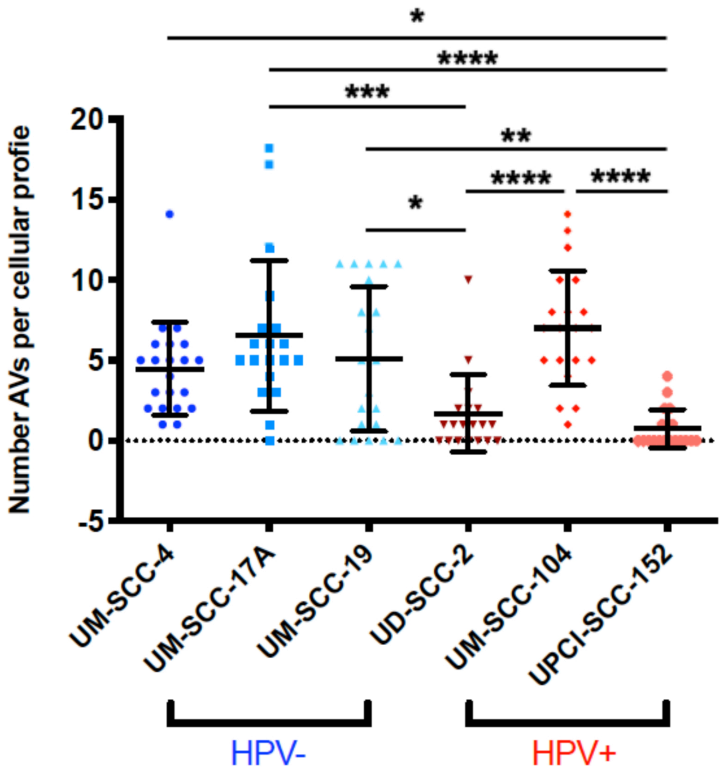


Figure 43 - Differences in autophagic compartment between HPV- and HPV+ HNSCC cell lines. *The graphs represent the quantification of autophagic vesicles of 3 HPV- cell lines (UM-4-SCC, UM-SCC-17A, UM-SCC-19) and 3 HPV+ cell lines (UD-SCC-2, UM-SCC-104, UPCI-SCC-152) in terms of number of AVs per cell detected by Electron microscopy. ANOVA analysis was performed on these samples and the p value was expressed as * (p<0.05), ** (p<0.01), *** (p<0.001), or **** (p<0.0001).*

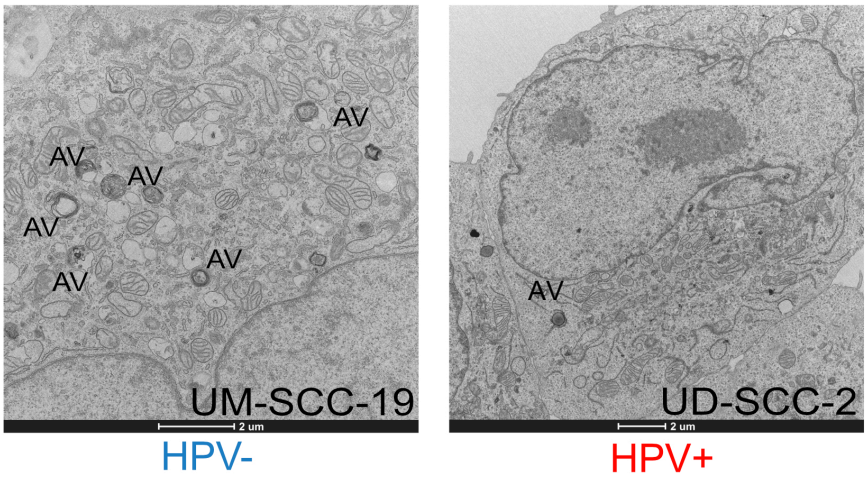
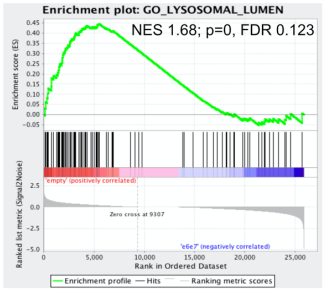
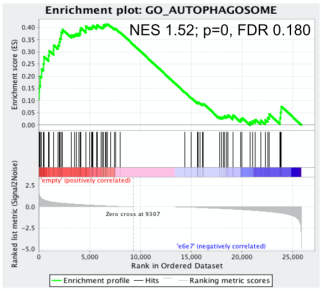


Figure 44 Comparison of HPV- and HPV+ HNSCC cells in terms of AVs.
Representative electron microscopy pictures of HNC cells, indicating the autophagic vesicles present (AV). UM-SCC-19 cell line and UD-SCC-2 cell line were used.

6.3.2 HPV16 E6/E7 oncoproteins downregulate autophagy genes expression

To understand whether the impairment in the number of AVs in HPV-positive cells is due to a transcriptional regulation of autophagic machinery, we performed RNA sequencing on primary keratinocytes transduced with E6/E7 or the empty vector. We launched a Gene Set Enrichment Analysis (GSEA) on RPKMs (Read Per Kilobase Million) from the RNA sequencing and found an enrichment in "GO_LYSOSOMAL_LUMEN" and "GO_AUTOPHAGOSOME" gene sets, observing that HPV oncoproteins downregulate autophagy gene expression (figure 45). As shown in figure 48, 15 genes involved in the autophagic process, were found to be differentially expressed, 14 of them downregulated by HPV16 E6/E7 expression.





Gene ID	log2FC	pvalue	padj
TP53INP1	-2.40	3.09E-75	3.04E-71
GABARAP	-1.79	6.67E-04	1.34E-02
TP53INP2	-1.44	7.32E-08	7.52E-06
ATP6V0A1	-0.87	2.14E-03	3.23E-02
ATG4A	-0.78	1.74E-04	4.76E-03
RAB4A	-0.63	2.52E-03	3.66E-02
WIPI2	-0.58	3.30E-03	4.39E-02
CTSA	-0.56	1.14E-03	2.03E-02
CTSB	-0.54	5.62E-04	1.18E-02
RAB8B	-0.52	3.21E-03	4.31E-02
TSC1	-0.47	1.06E-03	1.94E-02
RAB5B	-0.46	1.07E-03	1.94E-02
ATP6V0E1	-0.44	9.25E-04	1.73E-02
GABARAPL2	-0.43	2.45E-03	3.60E-02
ATP6V1E2	0.73	1.47E-04	4.25E-03

Figure 45 – Effects of E6/E7 on the expression of autophagy genes in HK.
RNA from primary keratinocytes transduced with HPV16 E6/E7 or the empty vector from 4 donors was used to perform RNA sequencing. Differentially expressed genes are listed in the table. GSEA was performed the enrichment plots "GO_LYSOSOMAL_LUMEN" and "GO_AUTOPHAGOSOME" are showed.

Then, we assessed whether there was a difference in the expression of autophagic machinery genes between HPV-positive and HPV-negative HNSCC tumors. To tackle this point, we checked public databases, and in particular the Cancer Genome Atlas (TCGA), with a panel of 523 cases of HNSCC (487 of them with known HPV status). We found that six genes were significantly downregulated in HPV-positive compared to HPV-negative patients: ATP6V0A1, CTSB, LAMP1, MAP1LC3B, RAB7A, TFE3 (figure 46). Specifically, four of them are involved in late stages of autophagy, in line with Mattoscio et al. 2017, that showed the inhibition of late stages of autophagy by HPV16 E6/E7 in primary keratinocytes.

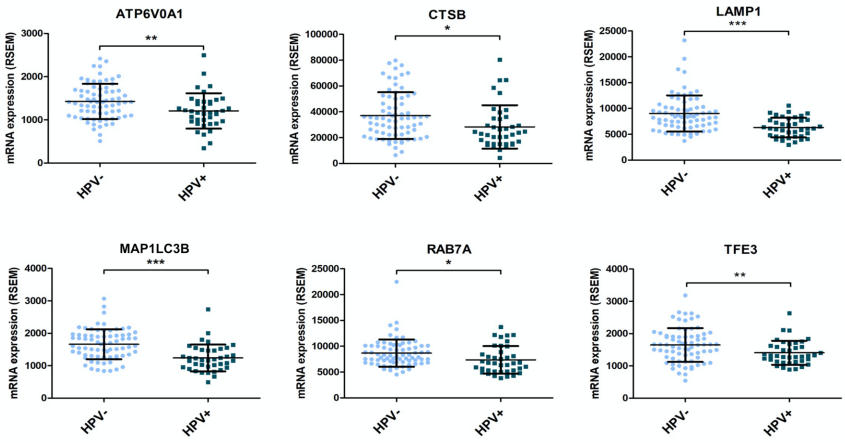


Figure 46 – Difference in autophagy gene expression between HPV+ and HPV- HNSCC patients from TCGA data.

*The graphs represent gene expression of some autophagy genes in HNSCC samples obtained from The Genome Cancer Atlas (TCGA). Each dot represent a patient and they are divided based on HPV presence or not. Student's T test was used to assess the statistical difference between the two groups. P value was represented as * (p<0.05), ** (p<0.01), or *** (p<0.001).*

6.3.3 TP53INP1 is regulated by E6/E7 expression

Taking into consideration the differentially expressed genes in primary keratinocytes, we observed that TP53INP1 (tumor protein p53-induced nuclear protein 1), a cofactor of p53 important in autophagic cell death, was the most significantly downregulated upon E6/E7 transduction. Quantitative PCR analysis on mRNA extracted from HK transduced with E6/E7 confirmed that E6/E7 regulated TP53INP1 gene expression (figure 47).

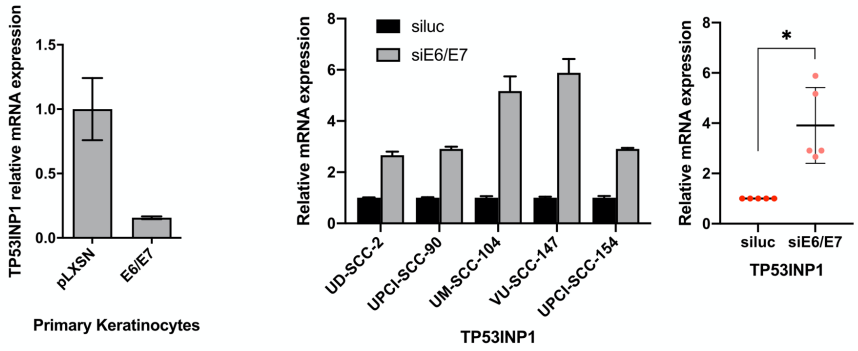


Figure 47 - Effects of E6/E7 oncoproteins on TP53INP1.

*The graphs represent from the left the relative mRNA expression of TP53INP1 gene: in HK transduced with E6/E7 or the empty vector, in 5 different HPV+ HNSCC cell lines transfected with RNAi silencing E6/E7 or luc for 72 hours, and the analysis obtained putting together all the 5 cell lines. Student's T test was performed on the 5 different cell lines and p value expressed as * (p<0.05).*

To check whether this effect was conserved in HNSCC, we silenced E6/E7 using siRNA in 5 HPV-positive HNSCC cell lines and assessed TP53INP1 relative expression (figure 47). E6/E7 downregulation induced a significant increase in TP53INP1 mRNA level in HNSCC cell lines, suggesting a potential implication of this gene in HPV-induced carcinogenesis.

6.4 Discussion

It has been shown in many studies that HPV oncoproteins impinge autophagy for their own needs (reviewed in Mattoscio, Medda, and Chiocca 2018). In this section, we showed that autophagy compartments are less abundant in HPV+ compared to HPV- HNSCC cells, suggesting an impairment in the autophagic processes mediated by E6/E7. This was confirmed by analysis of TCGA data in which some autophagy related genes were found to be less expressed in HPV+ with respect to HPV- HNSCC tumors. We also performed RNA sequencing on primary keratinocytes transduced with HPV16 E6/E7 to check the effects of E6/E7 expression on autophagy genes expression. We showed that some autophagy genes are downregulated upon transduction with E6/E7, confirming the observation in TCGA. Specifically, TP53INP1 was the most significantly downregulated gene involved in autophagy pathway upon transduction with E6/E7. We also demonstrated that the silencing of E6/E7 in HNSCC cell lines restored TP53INP1 gene expression, suggesting that downregulation of TP53INP1 is conserved in HPV-induced carcinogenesis. TP53INP1 silencing has been shown to inhibit p53-mediated apoptosis and its expression is regulated by p53 (Okamura et al. 2001; G. Zhou, Liu, and Myers 2016). TP53INP1 is a dual regulator of autophagy and transcription, interacting with LC3 and other autophagic proteins and it is involved in autophagic cell death (Seillier et al. 2012; Sancho et al. 2012). For its fundamental functions in the autophagy process, further investigations could uncover the role of TP53INP1 in autophagic impairment mediated by E6/E7.

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