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Master thesis

SIRT1 modulation of p53 and DNA damage response in HPV-associated cancer reveals potential therapeutic interventions

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1. ABSTRACT

Human papillomavirus (HPV) is the most common sexually transmitted agent world-wide, responsible for one-third of all the tumors induced by viruses and accounts for 5% of all human cancers. The pathogenesis of these tumors depends on the dysregulated expression of the viral oncoproteins E6 and E7, which target p53 and pRb tumor suppressors, respectively, promoting uncontrolled cellular proliferation. Our group has recently demonstrated a crucial mechanism used by high-risk HPVs to effectively suppress p53 activity, focused around the cellular deacetylase SIRT1. Specifically, we found that inhibition of SIRT1 through its pharmacologically inhibitor, namely EX527, restores a transcriptionally active K382-acetylated p53, leading to cell cycle arrest in G0/G1 phase and reducing cell survival and clonogenicity compared to HPV- cells. We have also demonstrated that treatment with EX527 enhanced the sensitivity of HPV^+ cells to some chemotherapeutic agents. Here, we evaluate the impact of SIRT1 inhibition on the anticancer activity of another standard treatment for these tumours, namely ionizing radiation. Specifically, we show that the treatment with EX527 enhances the sensitivity of HPV^+ cells to ionizing radiation. Indeed, this combinatorial treatment impairs cell viability and clonogenicity in HPV-transformed cells. Lastly, we have also analysed the SIRT1 interactome and we have identified several proteins involved in "DNA Damage Repair" and for this reason we have evaluated the expression of several DDR markers in our model, in order to understand how SIRT1 regulates these pathways in HPV-associated cancers.

Altogether, our findings suggest a crucial role of SIRT1 in HPV-associated cancers and an important target to develop novel therapeutic approaches.

2. INTRODUCTION

2.1 Papillomaviruses

2.1.1 General features and classification

Papillomaviruses (PV) are small, non-enveloped DNA viruses with an icosahedral capsid containing a circular double-stranded DNA genome of about 8 kilobase pairs (kbp) in length encoding for 8 open reading frames (ORFs). They belong to the Papillomaviridae family which contains 450 individual human papillomavirus (HPV) types (Moody, 2022). Papillomaviruses are classified on the basis of the major capsid protein (L1) open reading frame, which is the most conserved gene within the genome, and of their topological position within PV phylogenetic trees. They are characterized by a specific tropism for the squamous stratified epithelia where they can cause cutaneous and mucosal infections that can lead to hyperplastic lesions in a wide range of vertebrates (Morshed R. et al., 2014; Moody, 2022; Burk R.D., 2013; Doorbar et al., 2012). HPV is the most common sexually transmitted agent and is responsible for more than 5% of all cancers worldwide (Estêvão et al., 2019; Egawa N. et. al., 2015). HPVs are divided into five genera based on DNA sequence analysis (*alpha, beta, gamma, mu,* and *nu)* and can be classified as “cutaneous” or “mucosal”, based on their ability to infect skin or mucosal epithelial cells (Figure 1). Among the different genotypes, αHPVs are responsible for both mucosal and cutaneous lesions while the others are able to infect only the skin. In addition, mucosal HPVs are classified into low-risk HPVs (LR-HPVs) and high-risk HPVs (HR-HPVs) based on the propensity for malignant progression. LR-HPVs are highly responsible for benign or low-grade cervical lesions and anogenital warts. On the other side, HR-HPVs are associated with cervical cancer and other mucosal anogenital and head and neck cancers. There are 12 HPVs recognized by the World Health Organization (WHO) as high-risk cancer-causing types: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and, among them, HPV 16 and HPV 18 are responsible for most of the HPV-related cancers (De Sanjosè S. et al., 2018; Muñoz N. et al., 2003; Gheit T. et al., 2019; Cubie H. A. et al., 2013).

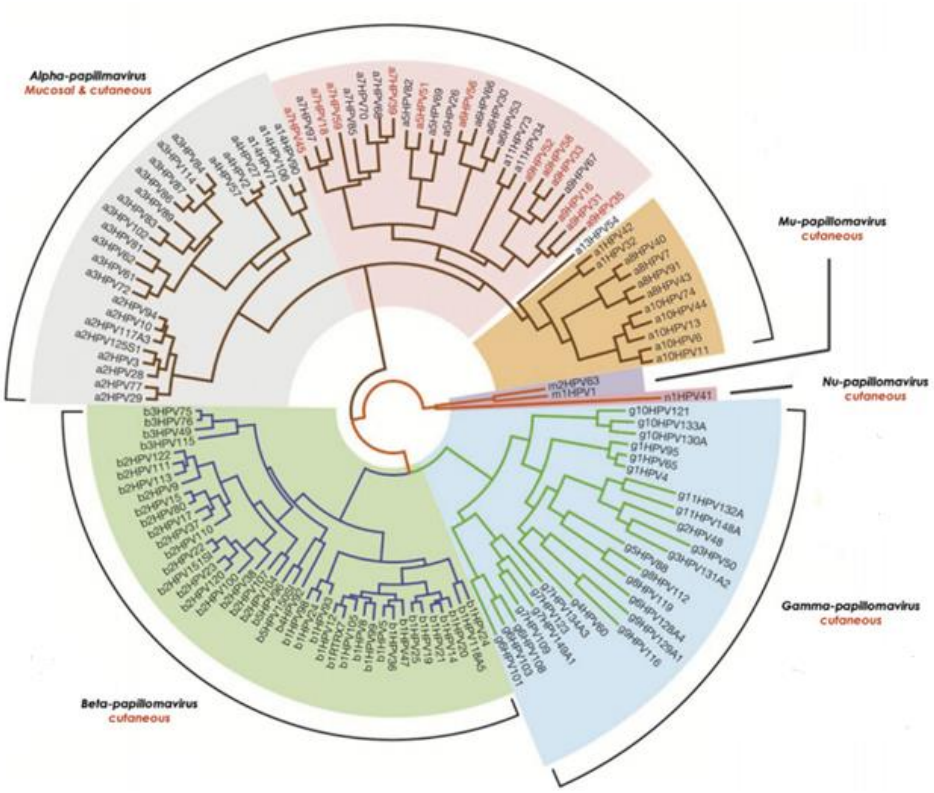


Figure 1. Human Papillomaviruses comprise five evolutionary groups with different epithelial tropisms and disease associations. Alpha-papillomaviruses are responsible for mucosal and cutaneous lesions in human. They are classified as "high-risk" and "low-risk": high-risk types cause pre- and malignant lesions, whereas low-risk types cause benign lesions. Beta, gamma, mu, and nu-papillomaviruses are responsible for cutaneous lesions (Morshed et al, 2014; Doorbar et al., 2012).

2.1.2 HPV structure and genome organization

Human Papillomaviruses are small, non-enveloped, icosahedral DNA viruses of approximately 50-60 nm in diameter. HPV genome is organized into 3 distinct regions: an early region (E), a late region (L), and an upstream regulatory region (URR) that varies among papillomaviruses (Figure 2). The early and late regions encode for non-structural (E1, E2, E4, E5, E6 and E7) and structural genes (L1 and L2) respectively, while the URR region, also known as long control region (LCR), includes regulatory elements (e.g. transcription factor-binding sites, promoter, and enhancer) and the viral origin of replication (ORI) (Doorbar J. et al., 2016; De Sanjosè S. et al., 2018; Yu L. et al., 2022).

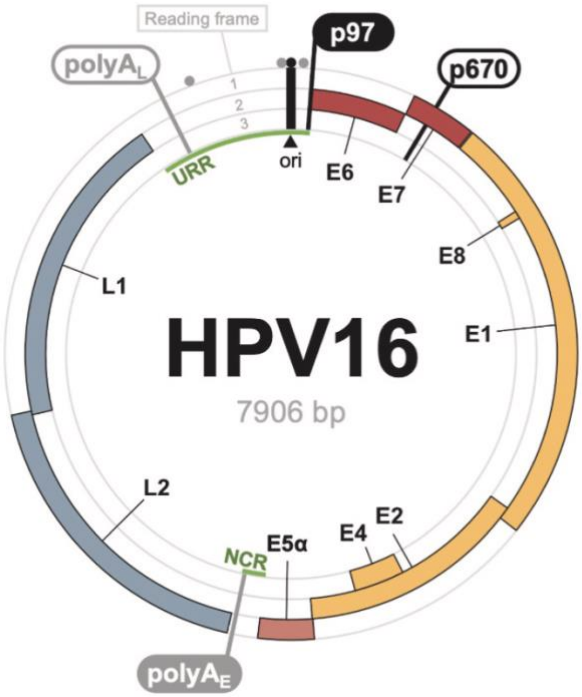


Figure 2. The HPV genome has a circular double-stranded DNA structure. The viral genes are transcribed in a single direction (clockwise). There are genes coding for non-structural proteins (E1, E2, E4, E6, and E7) and structural proteins (L1, L2), and a transcriptional control region (long control region; LCR). LCR contains a DNA replication origin and functions as the regulator for the DNA replication (Kajitani et al., 2012; Nelson et al., 2023).

E1 is an ATP-dependent hexameric DNA helicase necessary for viral DNA replication. Indeed, it is required to increase the copy number of the viral episome during the infection of basal keratinocytes, it is highly conserved among different HPV types and it is the unique HPV viral protein with enzymatic activity (Morshed et al., 2014; Bergvall et al., 2013).

The E2 protein is a coactivator of viral DNA replication, and it is the main transcriptional regulator of the virus; it works primarily by recruiting cellular factors to the viral genome, which activate or repress transcriptional processes. It also acts as a transcriptional repressor of HPV E6 and E7 and occasionally E2 functions can be disrupted due to mutation or integration of the viral genome in the host-cell genome that consequently leads to an overexpression of E6 and E7 proteins (Schwarz et al., 1985; McBride et al., 2013).

The E4 protein is the most abundant expressed viral protein, and it has a role in genome amplification and in viral particles release (Doorbar, 2013).

HPV encodes also for the three oncoproteins E5, E6, and E7 that have a role in the development of HPV-associated carcinomas since they induce cell immortalization and transformation. E5 is an accessory oncoprotein that plays a supportive, but not necessary, role in oncogenesis. This protein is thought to sustain proliferative signalling and to modulate the immune system (Estêvão et al., 2019). However, low-risk HPVs lack E5 or encode different types of the protein with less transforming ability (de Freitas et al., 2017). Instead, E6 and E7 play a pivotal role in driving the cells toward carcinogenesis targeting several oncogenic and tumor suppressor proteins to alter cell cycle. Specifically, the E6 protein from high-risk HPVs is a relative larger protein of about 150 amino acids and is able to inactivate p53 promoting cell proliferation, loss of cell cycle regulation, genomic instability, and evasion of cell death mechanism. In particular, E6 facilitates p53 degradation in HPV-infected cells by promoting its ubiquitination and subsequent proteasome degradation. It forms a heterotrimeric complex E6/E6AP/p53 leading to p53 inactivation and consequent degradation. Consequently, p21, a downstream target of p53, is not transcribed, allowing cells to enter the cell cycle. Indeed, p21 inhibits various cyclin-CDK complexes, preventing cells from progressing to the S phase and inducing G1 phase arrest in response to stimuli (IARC, 2007; Szymonowicz K.A., et al., 2020; McIntyre M.C. et al., 1993; Bhattacharjee R. et al., 2022; Pal. A. et al., 2020). E7, on the other hand, is a small phosphoprotein of about 100 amino acids and targets the retinoblastoma tumor suppressor (pRb) inducing its ubiquitination and leading to the release of the E2F transcription factor. The release of E2F can activate genes required for DNA replication as well as genes involved in regulation the cell cycle, such as cyclin E, cyclin A, and CDK4/6, resulting in uncontrolled cell proliferation and can also stimulate cell cycle progression by promoting the transition of cells from G1 phase to the S phase (Figure 3) (IARC, 2007; Roman A. et al., 2013; Doorbar J, 2015; Estêvão et al., 2019; Nelson CW. Et al., 2023; McBride A.A., 2013).

L1 and L2 proteins are the major and the minor capsid proteins, respectively. These proteins are required for virion assembly. Indeed, they assemble in capsomers forming icosahedral capsids around the viral genome during the generation of progeny virions. L1 is implicated in the initial attachment of the virion, in the release of viral genome into a new target cell and its sequence is used to define and classify the HPV types, while L2 is involved in the infectious process (McBride A.A., 2022).

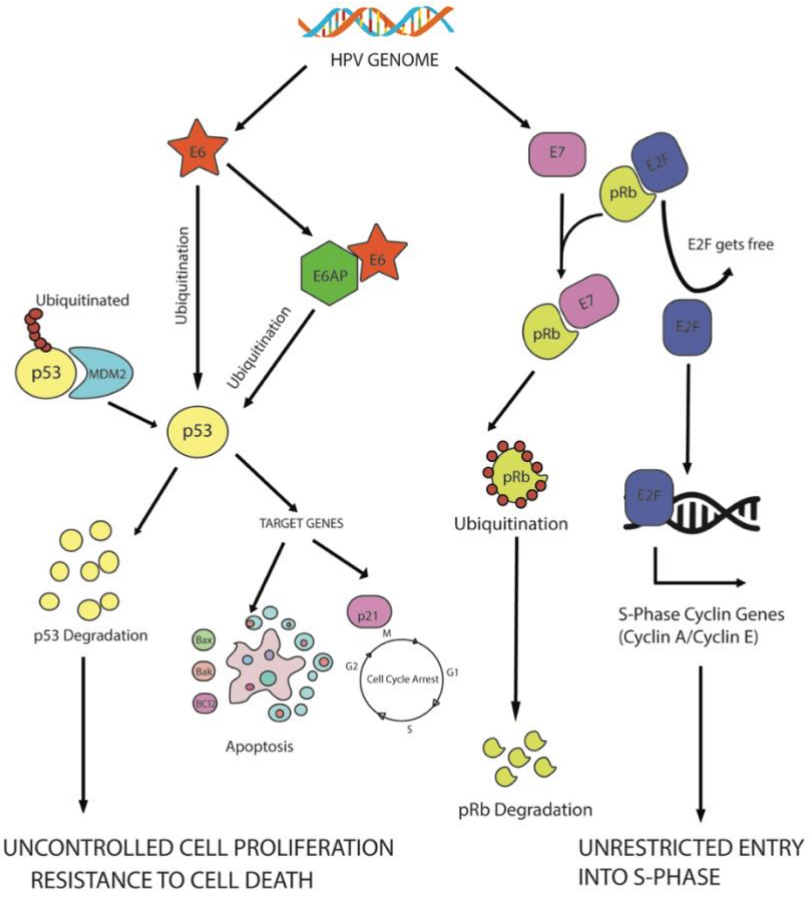


Figure 3. E6-mediated p53 manipulation and E7-mediated inhibition of pRb protein leading to sustained cell proliferation and resistance to apoptotic barrier (Pal A et al., 2020).

2.1.3 HPV life cycle

Human papillomaviruses are strictly epitheliotropic and their life cycle takes place within stratified squamous epithelia. Infection by HPVs is well known to occur through microwounds of the epithelium that expose cells in the basal layer to viral entry. HPVs primarily infect keratinocytes, which form the epidermidis of stratified epithelium. Epidermidis is organized into three layers based on differentiation status: the basal layer containing stem cells, the upper layer, and the uppermost cornified layer consisting of sloughing cells (Longworth et al., 2004; Della Fera A.N. et al., 2021; Doorbar J. et al., 2005). The HPV life cycle takes 2-3 weeks and it is closely associated to the differentiation process of the infected host squamous epithelium. Initially, HPVs infect undifferentiated basal epithelial cells, and then the viral replication occurs in differentiated daughter cells located in the uppermost layers of the epithelium (Harden and Munger, 2016; Graham S.V., 2017).

The infection starts when the HPV L1 capsid protein binds to cellular receptors localized on either the basement membrane or the surface of basal layer cells. Heparan sulfate proteoglycans (HSPGs) appear to be the primary receptors for this initial binding. HSPGs, located in the extracellular matrix and on the surface of most cells, are involved in several biological functions and, because of their localization, they are appropriate molecules for viral infections. The binding to HSPGs induces a conformational change in the viral capsid exposing the N-terminus of the L2 component on the virion surface. The N-terminus of L2 is then cleaved by furin and/or PC5/6 enzymes facilitating the binding to a secondary receptor on the plasma membrane of the target cell (Figure 4). After being internalized, virions undergo endosomal transport, uncoating, and cellular sorting. The L2 protein-DNA complex allows the correct entry of the viral genome into the nucleus, while the L1 protein is retained in the endosome and subjected to lysosomal degradation (Graham et al., 2017; Doorbar et al., 2012; Aksoy P. et al., 2017; Kines R.C. et al., 2022; Gheit T., 2019).

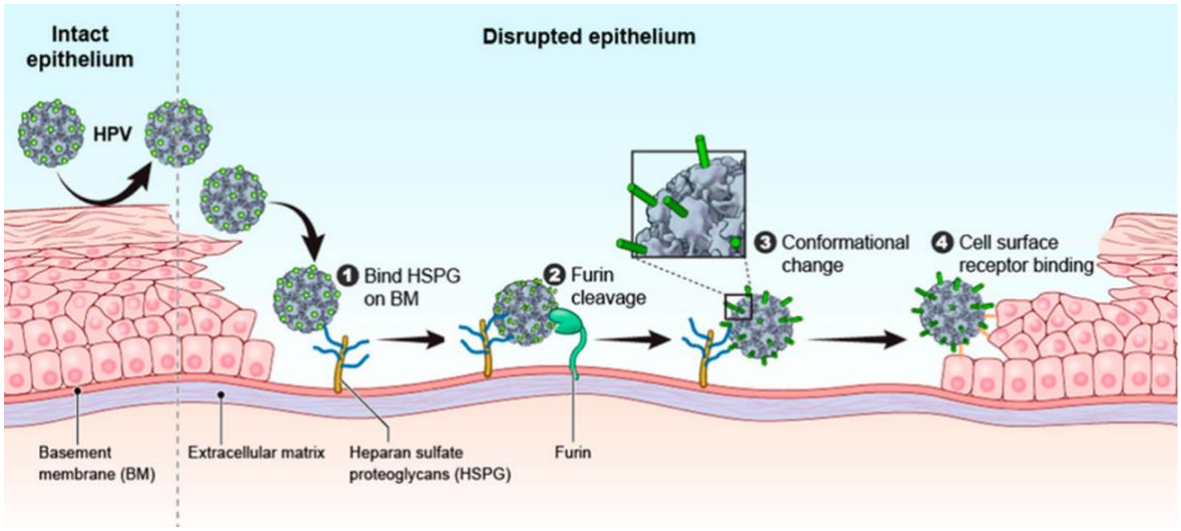


Figure 4. Mechanism of HPV attachment. HPV attaches to HSPG on the exposed basement membrane. The L2 protein is then cleaved by furin and the virion undergoes conformational changes before being able to bind cell surface receptors (Kines R.C. et al., 2022).

Infection and uncoating are followed by an initial phase of genome amplification, and then the virus maintains its genome at low copy number episomes (10-200 copies per cell) in the infected host basal cells of the epithelium, thanks to the expression of E1 and E2 protein, which are also useful to facilitate the correct segregation of episomes during cell division. E2 has one DNA-binding domain and one protein-binding domain. It forms a homodimer that binds to four palindromic sites in the LCR (Long Control Region), three of these sites

are located near the replication origin and are essential for E1-mediated viral replication. E2 binds E1, which then binds the viral replication origin, recruiting the cellular DNA replication machinery. Early viral proteins E6, E7, E1, and E2 are expressed at low levels, presumably to avoid activating the local immune response (Doorbar, 2005; Graham et al., 2017; McKinney C.C. et al., 2015).

In uninfected epithelium, basal cells exit the cell cycle upon migrating into the suprabasal layers and undergo terminal differentiation. During HPV infection, the early viral proteins E6 and E7 are expressed in these cells to abolish the restrain on cell cycle progression and cause a delay in normal terminal differentiation. Additionally, E4 and E5 proteins contribute indirectly to genome amplification by altering the cellular environment, with E5 being involved in koilocytes formation. During the HPV life cycle, the upregulation of E2 levels that results from late promoter activation leads to a decrease in E6 and E7 expression. This reduction allows terminal differentiation of keratinocytes and the expression of the two capsid proteins L1 and L2. High levels of these two capsid proteins results in the self-assembly of capsids that encapsidate viral DNA (Figure 5) (Johansson et al., 2012; Krawczyk et al., 2008; Doorbar, 2005).

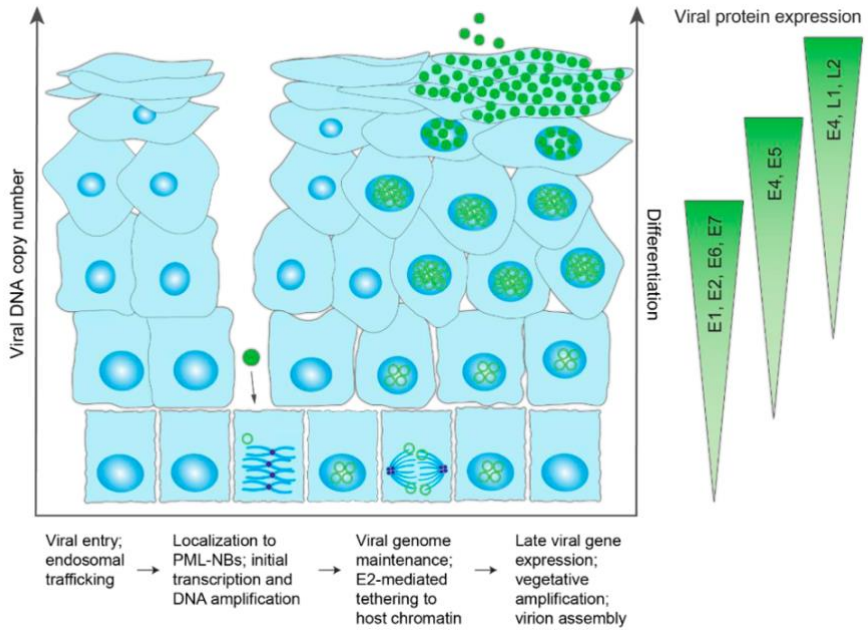


Figure 5. Schematic representation of the differentiated layers of a stratified epithelium infected with HPV. The virus accesses the basal keratinocytes through a microabrasion. Upon cellular entry, the virus is trafficked through the endosome and enters the nucleus following breakdown of the nuclear membrane during mitosis. Within the nucleus, HPV genome localize to promyelocytic leukemia nuclear bodies (PML-

NBs), undergo a limited round of DNA synthesis and become established by binding to host chromatin to maintain the viral genome at a constant copy number in dividing cells. Upon epithelial differentiation, infected cells amplify the viral DNA to high copy numbers, and late viral genes are expressed for virion assembly. Virions detached from the epithelial surface (Della Fera A.N. et al., 2021).

2.1.4 HPV life cycle deregulation and cancer progression

Genital HPV infection is typically contracted through sexual intercourse, with a prevalence among young women. HR-HPVs associated with several types of human cancers are mainly HPV16 and HPV18. HR-HPV infections can lead to cancer progression in only a small percentage of infected women, after a long latency period (zur Hausen H. et al., 1196). However, other secondary events may cooperate with persistent infection to lead to cancer formation. The main risk factors for cervical cancer have been identified as smoking, oral combined contraceptive use, other sexually transmitted infections, particularly Chlamydia, and immune status (immunocompromised or immunosuppressed) (Westrich J.A. et al., 2016; Graham S.V. et al., 2017). During productive HPV infection, low-grade cervical abnormalities (low grade squamous intraepithelial lesions (LSIL) or cervical intraepithelial neoplasia grade 1 (CIN1)) may be clinically detectable in screening, but these lesions are usually transient and resolve without intervention within 1-2 years. Indeed, a high percentage of infected women (90%) clears the infection by immunological mechanisms. However, a minority of HPV infections persist beyond 12 months increasing the risk of carcinogenic progression to cervical pre-cancer (high-grade squamous intraepithelial lesions (HSIL)) or cervical intraepithelial neoplasia grade 2 or 3 (CIN2 or CIN3) and potentially cancer if untreated. The accurate identification of the lesion grade has prognostic significance, as around 20% of CIN1 will progress to CIN2, and around 30% of CIN2 will progress to CIN3 when untreated. CIN3 are generally considered the direct precursors of cervical cancer, and around 40% of CIN3 lesions will progress to cervical cancer in the absence of intervention. More in detail, CIN1 indicate a self-limited sexually transmitted HPV infection while CIN2 and CIN3 are the only true cervical cancer precursors (Doorbar et al., 2012; Gravitt P et al., 2017).

The expression levels of E6 and E7 genes increase as cervical intraepithelial neoplasia progresses from grade 1 to 3 (CIN1 to CIN3), and these changes in gene expression directly underlie the neoplastic phenotype (Figure 6). The majority of cervical cancers contain one

or multiple copies of HPV, which integrate randomly into the host chromosomes and the viral integration frequently occurs within the regulatory E1 or E2 genes. This integration leads to the deregulation of E6/E7 expression by either genetic or epigenetic alterations resulting in their overexpression within the epithelial lesions. E6 suppresses p53 function, preventing apoptosis while E7 inhibits pRb and abrogates cell cycle arrest (Doorbar et al., 2012; Schiffman et al., 2007).

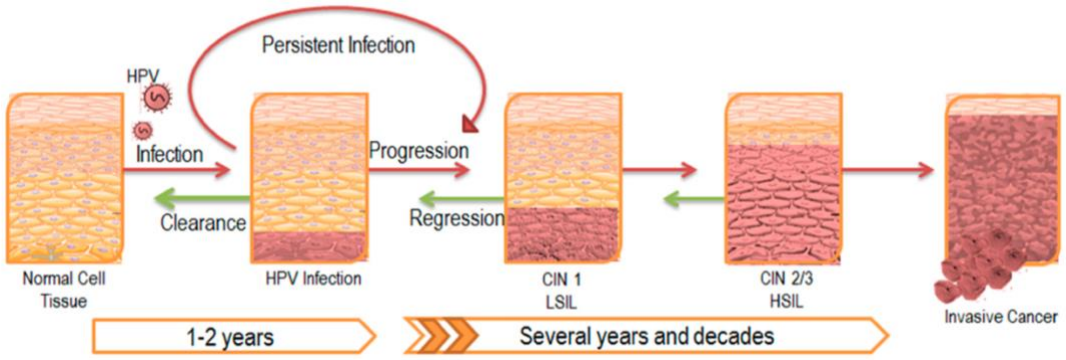


Figure 6. Progression of HPV infection and associated disease. HPV establishes infection in the basal epithelial layer. A majority of these infections are transient and are cleared by the immune system in a couple of years. However, 10-20% of infections persist, leading to disease progression as illustrated by the red arrows. The lesion is known as cervical intraepithelial neoplasia (CIN) and is classified according to its severity. LSIL lesions advance to HSIL lesions, leading to invasive carcinoma. Despite tumor regression in response to initial treatment as illustrated by the green arrows, most cases of latent infection prevent complete clearence of the viral infection, and eventually results in lesion recurrence (Shanmugasundaram and You, 2017).

2.1.5 HPV-derived head and neck squamous carcinomas

Head and neck cancer squamous cell carcinomas (HNSCC) comprise a group of malignancies that affect mucosal linings at different anatomic sites of the upper aerodigestive tract, including the nasopharynx, paranasal sinuses, oral cavity, oropharynx, hypopharynx and larynx (Figure 7) (Sabatini M.E and Chiocca S., 2020). The classical risk factors are tobacco, alcohol, poor oral hygiene and genetic factors. In recent decades, human papillomavirus, and in particular HPV16, have emerged as novel additional risk factor for these cancers, especially for oropharyngeal squamous cell carcinoma (OPSCC), a subtype of HNSCC arising from the crypt epithelium of the palatine and lingual tonsils (Gillson et al., 2000; Snijder et al., 1992). The incidence of HNSCC

depends on the interested anatomical region and the geographical location. Men are more likely to develop HNSCC than women, and the median age of diagnosis is 50-70. Specifically, for non-virally associated HNSCC the diagnosis usually occurs at about 66 years of age, while the median age of diagnosis for HPV-associated oropharyngeal cancer is about 53 years (Marur S., 2010; Johnson D.E et al., 2020; You E.L et al., 2019; Sabatini M.E and Chiocca S., 2020).

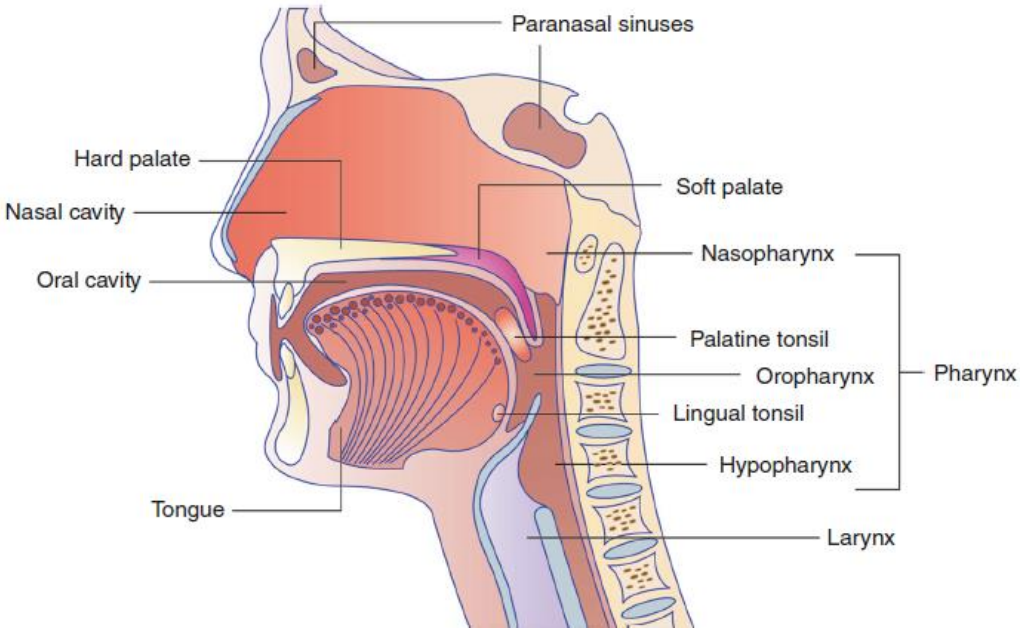


Figure 7. **Anatomical sites of HNSCC development** (Sabatini and Chiocca, 2020).

The most recent edition of the American Joint Committee on Cancer (AJCC) staging system defined HPV-positive and HPV-negative OPSCCs as separate entities, with distinct molecular profiles, tumour characteristics and outcomes (Craig et al., 2019). The biology of HPV^{+} OPSCC is typified by p53 degradation, Rb protein down-regulation and p16 up-regulation. By contrast, HPV- OPSCC is characterized by p53 mutation, p16 down-regulation and Rb protein up-regulation (Seiwert et al., 2015; Gillison et al., 2019; Dogan et al, 2019, Elrefaey et al., 2014). Histologically, progression to invasive HNSCC follows a series of steps that begin with epithelial cell hyperplasia, followed by dysplasia (mild, moderate and severe), carcinoma in situ and, at least, invasive carcinoma. Each stage is characterized by specific genetic events, and in particular, the inactivation of tumor suppressor genes such as TP53 and CDKN2A is involved in HNSCC formation in the early stages, and PTEN in the later stages (Johnson D.E et al., 2020).

In addition, mutations or alterations in genes involved in phosphoinositide 3-kinase (PI3K) pathway are a key feature of HPV^{+} OPSCC (Gillison et al, 2019; Lui et al., 2013; Nichols et al., 2013). Prognosis is distinctly better for HPV-positive OPSCC; indeed, there is a 28% reduced risk of death and a 49% reduced risk of disease recurrence for patients with HPV-positive OPSCC compared to the counterpart (Gillison M.L et al., 2015; Elrefaey S et al., 2014; Seiwert T.Y et al., 2015). Nowadays, patients with an early stage of HNSCC are eligible for surgical intervention, while standard treatment for advanced HSNCC is represented by cisplatin-based chemotherapy in combination with radiotherapy (You E.L et al., 2019).

2.2 HPV prevention and treatments for HPV-related cancer

HPV, the leading global sexually transmitted infection, poses significant health risks, driving urgent vaccination efforts. Persistent high-risk HPV strains can trigger precancerous lesions. Prophylactic vaccines like Gardasil and Cervarix, crucial for prevention, target oncogenic genotypes (Dunne E.F et al., 2013; Cheng L et al., 2020; Rosalik K. et al., 2021; Pils S. et al., 2015). Treatment for HPV-related cancers, including surgery, radiotherapy, and chemotherapy, varies based on cancer type, stage, and patient health (Johnson CA et al., 2019). Combination therapies, such as radiotherapy with cisplatin, improve outcomes, especially for larger cervical lesions.

Common surgical methods for early-stage cervical cancer removal include total hysterectomy, radical hysterectomy, LEEP, conization, trachelectomy, and cryosurgery. Radical hysterectomy, often preferred, addresses larger lesions, involving complete uterus and cervix removal. (Burmeister CA et al., 2022; W.-J. Koh et al., 2019; Johnson CA et al., 2019).

Radiotherapy (RT) can be used for the treatment of HPV-associated cancers, as they are considered highly radiosensitive. RT uses high energy x-rays and it is considered the major approach in the management of cervical cancers, since they are generally radiosensitive. However, radiotherapy causes several adverse effects due to the treatment modality and that depend on the duration of treatment and the type location of the cancer. Common negative effects include fatigue, pelvic pain, skin toxicity, abdominal cramps, lymphedema, diarrhea, sexual dysfunction, xerostomia, and stenosis. Another important aspect to avoid side effects is define a precise dose of the

radiation therapy. Specifically, radiotherapy for head and neck cancer is based on weekly doses of 10 Gy, usually divided into fractions of 2 Gy, with a total treatment time of six and a half to seven weeks. At 12 weeks after the end of the treatment, the response to radiotherapy is verified by cervical cytology and biopsies in order to assess the absence or presence of a tumor (Berman TA et al., 2017; Ask A et al., 2005; de Figueiredo FV et al., 2023; Bourhis J et al., 2004; Dijkstra PU., 2004).

A combine treatment based on radiotherapy and chemotherapy Is often used to enhance the efficacy of treatment, especially for larger cervical cancer lesions (more than 4cm in width). The use of radiotherapy in combination with cisplatin has shown a reduction of 30-50% in the risk of death and an improvement in overall survival compared to radiotherapy alone. To treat advanced, recurrent disease or metastasis, chemotherapy is used as the main treatment or as a neoadjuvant therapy to reduce the size of the tumor, cancer cells proliferation and manage the symptoms. Cisplatin is the main used platinum-based chemotherapeutic to treat cervical cancer. Currently, paclitaxel and other non-platinum-based chemotherapeutics, such as 5-fluorouracil and bleomycin, are commonly used in combination with cisplatin (Johnson CA et al., 2019; Berman TA et al., 2017; de Figueiredo FV et al., 2023; Keys HM., 1999).

In advanced-stage oropharyngeal cancer, multimodal therapy combining chemoradiotherapy or chemotherapy with surgery is crucial for optimal outcomes. Several reports indicate that combining Cetuximab, an epidermal growth factor receptor inhibitor, with radiotherapy enhances locoregional control and survival, particularly in HPV-positive patients. Additionally, combining Cetuximab with standard platinum-fluorouracil chemotherapy offers a survival advantage (Berman TA et al., 2017; Wirth LJ., 2016; Vokes EE., 2015).

2.3 SIRT1 and cancer

The sirtuins family is NAD+ dependent class III histone deacetylases (HDACs) involved in several physiological and pathological events, including gene regulation, genome stability maintenance, apoptosis, autophagy, senescence, aging, inflammation, and tumorigenesis. Sirtuins act hydrolyzing NAD+ and simultaneously transferring the lysine-bound acetyl group on the 2’- OH position of ADP ribose, forming nicotinamide and 2’-

O-acetyl-ADP-ribose. (Manjula R et al., 2021; Simmons GE Jr et al., 2015; Alves-Fernandes DK et al., 2019).

Among sirtuins SIRT1 is the best characterized and can deacetylase both histones and non-histones substrates, including p53, FOXO, HIF1α and NF-kB. Different studies have shown ambiguous implications of SIRT1 in cancer, in fact it can acts as both a tumor suppressor and tumor promoter, however this contradictory role seems to be determined by the cell type and by SIRT1 localization. SIRT1 upregulation has already been demonstrated in some cancer cells, such as acute myeloid leukemia and colon, prostate, melanoma and non-melanoma skin cancers, while SIRT1 downregulation was described in breast cancer and hepatic carcinoma (Stünkel at al., 2007; Simmons GE Jr., 2015). Importantly, SIRT1 directly binds and deacetylates p53 on Lys382 residue, one of the observed consequences of this deacetylation is the attenuation of p53 transcription factor activity at the p21 promoter, leading to the inhibition of p53-dependent apoptotic response (Vaziri et al., 2001).

SIRT1 regulates histone modifications, including deacetylation and methylation. SIRT1 recruitment to its target promoter results in deacetylation of histone proteins at H1K26, H4K16, and H3K9. Additionally, SIRT1 promotes the production of H4K20me, H3K9me3, and H3K79me2. Also the mammalian histone methyltransferase SUV39H1 undergoes regulation by SIRT1, and its activity is regulated by acetylation at Lys266 in its catalytic SET domain. SIRT1 interacts with, recruits and deacetylates SUV39H1 contributing to have elevated levels of SUV39H1 that lead to an increase of the levels of the H3K9me3 modifications. (Vaquero A et al., 2007).

The enzymatic function of SIRT1 can be modulated by several proteins, including the tumor suppressor protein deleted in breast cancer 1 (DBC1), which is completely absent in some breast cancer patients. DBC1 acts as a natural inhibitor of SIRT1 by binding to its catalytic domain and leading to a reduction of DBC1 that enhance SIRT1-mediated deacetylation of p53 and inhibits p53-mediated apoptosis (Zhao W et al., 2008).

2.4 SIRT1 and HPV

SIRT1 is an important regulator of high-risk HPV life cycle, including transcription and replication. Specifically, SIRT1 is involved in the E1 and E2 DNA replication complex and is recruited to the HPV viral origin of replication in an E1-E2-dependent manner. In addition,

its expression is significantly upregulated in HPV-positive cells and in cervical intraepithelial neoplasia (CIN), showing a progressive increase from CIN1 to CIN3 (Cohen et al., 2019; Vonsky et al., 2019; Almeida et al., 2019; Das et al., 2017).

The development of invasive squamous cell carcinoma (SCC) of the uterine cervix involves the progression of premalignant lesions and is associated with persistent infection of high-risk human papillomaviruses, such as HPV16/18. Velez-Perez and colleagues evaluated the expression of SIRT1 in 101 tissues specimen from the uterine cervix comprising 29 CIN1, 32 CIN2, 16 CIN3, 22 invasive squamous cell carcinomas (SCC) and 2 micro-invasive SCC. In CIN lesions, a cytoplasmatic overexpression of SIRT1 was found in 13,8% CIN1, in 40,6% CIN2, and in 50% CIN3. Moreover, 68% SCC showed both nuclear and cytoplasmatic expression of SIRT1, 25% of SCC showed cytoplasmatic overexpression, and 4% SCC showed nuclear overexpression. These results show that increased expression of SIRT1 is correlated with disease progression of CINs (Velez-Perez et al., 2016). The biological explanation for the upregulation of cellular SIRT1 in CIN1 and SCC may be related to the viral oncoproteins HPV E7. A study performed by Allison and colleagues demonstrated that HPV16 E7 upregulates SIRT1 levels in human cervical cancer SiHa cell line. In this work, the authors demonstrated that RNAi-mediated silencing of HPV E7 in SiHa cells down-regulates SIRT1 protein levels by 50% 48h post-transfection with E7 siRNA. In addition, they didn't find any effect on SIRT1 mRNA levels, suggesting that the effect of HPV E7 on SIRT1 expression is post-transcriptional (Allison et al., 2009).

Identification of SIRT1 as a downstream target of HPV have both prognostic and therapeutic value, however, further prospective studies are necessary to validate the role of SIRT1 as a biomarker for the malignant transformation of cervical preneoplastic lesions.

Recently, the laboratory of Molecular Virology head by Prof. Gariglio has demonstrated for the first time the role of SIRT1 in the development and maintenance of HPV-associated tumors. In particular, the SIRT1 protein, through its deacetylase activity, participates in the continuous degradation of p53 in HPV^+ cells, and consequently, its pharmacological inhibition allows the restoration of normal p53 activity, which in this context results in a strong reduction in cell proliferation. They found that pharmacological inhibition, using EX527, or genetic silencing of SIRT1 in HPV^+ cell lines leads to the reactivation of a transcriptionally active K382-acetylated p53, leading to cell cycle arrest at G0/G1 phase and reduction of cell survival and clonogenicity compared to HPV- cells (Lo Cigno et al., 2023).

2.5 DNA damage response

The integrity of the eukaryotic genome is maintained through a network collectively referred to as the DNA damage response (DDR) that senses and signals DNA damage arrests the cell cycle and activates repair mechanisms or eliminates the damaged cells through apoptosis. Different types of DNA injury are detected through unique sensors. DNA damage signals are then relayed to effector molecules in a manner similar to signal transduction pathways, including post-translational modifications such as phosphorylation (O'Connor MJ., 2015; Jackson SP., 2019).

Preserving the integrity of the genome is crucial for maintaining its stability, with mechanisms constantly surveilling nucleotide sequence changes to prevent mutations and chromosomal rearrangements. In addition, cells have developed others mechanism wherein signal transducing and effector proteins regulate cell cycle progression, damage repair and apoptosis. Collectively, these mechanisms are known as DNA damage response (DDR). Over 450 proteins participate in DDR, and some of these have been studied as potential targets for therapy and predictive biomarkers (Friedberg EC et al., 2006; Situ Y et al., 2019).

A macromolecular complex specialized in the DNA damage sensing and in the early response to the DSBs is the MRE11- RAD50-NBS1 (MRN) complex. The MRN complex has been reported not only to aid in the sensing and initiation of DNA DSB repair, but also to activate cell cycle checkpoints, as well as to maintain telomeres and manage meiotic recombination (Qiu S et al., 2015; Yuan J et al., 2009). MRE11 facilitates the assembly of the complex by interacting with RAD50 and NBS1, despite the absence of direct interaction between them. The largest subunit of the MNR complex is RAD50, that resides on chromosome 5 and encodes a 1312 amino acids protein. RAD50 molecules possess a hook domain that facilitates their dimerization, allowing them to bind and connect DNA ends together. The core RAD50 is arranged such that the DNA-binding sites on the MRE11 dimer are in proximity to the two RAD50 ATP-ase domains. Located on chromosome 2 and encoding for a protein 744 amino acids in length, NBS1 plays a pivotal role in the rapid re-localization of the complex and mediates numerous interactions with other proteins involved in DNA double-strand break signaling and repair (McCarthy-Leo C., 2022; Qiu S et al., 2015).

The major upstream kinases in the signal transduction pathway that orchestrate the response to DNA damage are members of the phosphatidylinositol 3-kinase-related kinase (PIKKs) family and include Ataxia telangiectasia mutated kinase (ATM) and Ataxia telangiectasia and Rad3-related protein FRAP-related protein 1 (ATR). ATM and ATR appear to regulate the broadest spectrum of downstream factors that contribute to the DDR. In addition, they induce further phosphorylation events through the activation of the Chk1 and Chk2 kinases. ATM is activated in response to double stranded breaks (DSBs), whereas ATR is activated by the presence of single stranded DNA. The downstream events in the DDR signal transduction chain include cell cycle check-points, apoptosis or DNA synthesis to restore the integrity of the DNA molecule. The latter feature of the DDR is exploited by some DNA viruses such as HPV that lacks a DNA polymerase and has evolved to employ the DDR for amplification of the viral genome (Figure 9) (McCarthy-Leo C et al., 2022; Qiu S et al., 2015; Lavin MF et al., 2015; Situ Y et al., 2019; Lafrance-Vanasse J et al., 2015; Jackson SP et al., 2002).

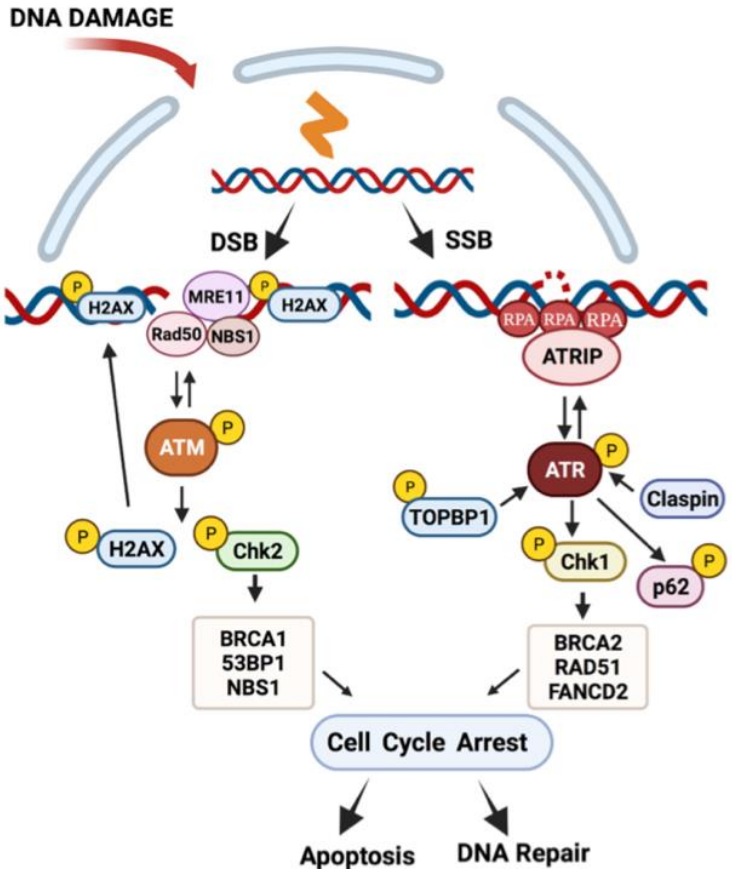


Figure 9. DNA damage repair pathways. The ATM and ATR DNA damage repair pathways are activated in response to dsDNA or ssDNA breaks, respectively. Activated ATM phosphorylates effectors such as CHK2,

leading to the recruitment and phosphorylation of a series of DNA repair factors and cell cycle checkpoint arrest. Similarly, activated ATR phosphorylates effector protein CHK1, leading to activation of additional downstream factors. Both pathways eliminate the damaged cell by apoptosis or senescence (Gusho E et al., 2021).

Human papillomaviruses have developed multiple strategies to successfully complete their viral life cycles, including the use of host cellular machinery to replicate their genome. HPV infection is sufficient to activate DNA damage repair pathway, which can be advantageous for the viral life cycle. In addition to HPV proteins, HPV genome amplification also requires cellular proteins of the ATM and ATR branches of the DDR. ATR is active during all stages of the HPV life cycle, suggesting that this branch of the DDR is necessary for initial-, maintenance- and productive-replication (Nilsson K. et al., 2018; Spriggs CC et al., 2017). Further, TopBP1 that acts upstream of ATR signaling is a required component of the viral replication loci. The HPV E1 and E7 proteins can independently activate ATR and Chk1. Alternatively, this activation is a consequence of the replication stress that arises from replication of the HPV genome, the unspecific DNA helicase activity of E1, the aberrant cell cycle entry created by the viral proteins, or the ssDNA generated during homologous recombination (HR)-mediated productive HPV replication. However, different HPV types seem to have specific effects on the ATR signaling. As the signaling from the ATM and ATR branch overlap, perhaps this reflects a variable ability of HPV proteins to interact with cellular components to elicit the DDR required for genome amplification (Nilsson K et al., 2018; Hong SY., 2017). The HPV infection activates the DDR with the purpose of exploiting the DDR DNA synthesis machinery for HPV genome replication. However, induction of the DDR is accompanied with a risk of inducing p53-mediated apoptosis. To prevent apoptosis, the HPV E6 protein binds and degrades cellular p53. ATM is also active in HPV infected cells and contributes to the productive phase of HPV DNA replication. HR mediated repair creates a large area of ssDNA that invades a sister chromatid to use a homologues sequence as template for synthesis of new DNA. Thus, HPV may specifically activate ATM to recruit HR factors as they offer high fidelity replication in G2-arrested cells upon differentiation. Alternatively, ATM activation is a result of the rolling circle replication used for the productive amplification of the viral genome (Moody CA et al., 2009; Anacker DC et al., 2017). The modified histone γH2AX, a hallmark of DNA damage, is also found on HPV genomes at onset of productive replication. It is aiding in the recruitment of DNA repair

factors to the HPV genome. HPV E7 appears to increase the abundance of these factors, partly through transcriptional activation by E2F. Activation of the DDR by E7 is also mediated by interactions with signal transducer and trans activator 5 protein (STAT5) and the Tip60 acetyltransferase. In conclusion, several cellular DDR factors are required for replication of the HPV DNA genome (Hong S et al., 2013; Gillespie KA et al., 2012).

3. MATERIALS AND METHODS

3.1 Cell culture and treatments

For the realization of the experiments carried out for this thesis work, we used several HPV positive or negative cell lines. HNO150 (HPV-negative laryngeal squamous cell carcinoma-derived cell line) were cultured in DMEM medium (Sigma-Aldrich). CaSki cells, a cervical carcinoma-derived cell line containing integrated HPV16 genome (about 600 copies per cell), were grown in RPMI medium (Thermo Fisher Scientific). Both medium were supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 100 U/mL penicillin, 100 µg/mL of streptomycin and 0.05 mM glutamine. NOKpWPI and NOKs HPV16 E6/E7 (briefly named NOKE6/E7), normal oral keratinocytes stably transduced with both E6 and E7 genes from HPV16 by lentiviral infection that were kindly provided by Frank Rösl (Germany), were cultured in keratinocyte serum-free medium (KSFM) supplemented with 2.6 µg/mL bovine pituitary extract (BPE), 0.16 ng/mL recombinant epidermal growth factor (rEGF), 100 U/mL penicillin and 100 µg/mL of streptomycin. A highly expressing E6 and E7 clone with high proliferation rates was isolated from a pool of NOKE6/E7 cells by limiting dilution. Cells derived from this clone were used for all experiments between passage 10 and 20.

Cells were treated with the SIRT1 pharmacological inhibitor EX527 (6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide, alias Selistat) at a concentration of 20 µM, 40 µM, or 80 µM, depending on experiment settings, for 8h, 16h, 24h, and 48 h or with an equal volume of DMSO (Sigma-Aldrich). In combination with EX527 or DMSO, cells were irradiated with 2Gy or 4Gy.

3.2 MTT assay

Cell growth and viability was measured using the MTT colorimetric assay (Sigma-Aldrich), according to the manufacturer's protocol. This assay is based on the ability of a mitochondrial dehydrogenase from viable cells to cleave the tetrazolium rings of the pale yellow MTT, and form purple formazan crystals, which are impermeable to cell membranes. For this purpose, cells were plated into a 12-well plates (6×10^4 cells/well) and after 24h are treated with EX527 (40 µM) alone or in combination with radiotherapy, based on doses of 4Gy. After 24h of treatment, cells were subjected to a 2-hour incubation at 37°C and 5% CO2 with incomplete DMEM medium added with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma-Aldrich, Milan, Italy) 1:10. At the end of the incubation, the supernatant was removed, and the formed formazan crystals were

solubilized by adding DMSO. The intensity of the dissolved formazan crystals (purple color) was quantified using the Victor3 multiplate reader spectrophotometer (Perkin Elmer) at 570 nm.

3.3 Assessment of cell death (Annexin V / Propidium iodide staining)

To evaluate the percentage of cells undergoing necrosis or apoptosis was performed a PI and Annexin V co-staining (FITC Annexin V Apoptosis Kit with PI, BioLegend). Briefly, cells were plated into 12-well plates (6 x 10^4 cells/well) and after 24h are treated with EX527 (40 µM) alone or in combination with radiotherapy, based on doses of 4Gy. After 24h of treatment, floating and harvested cells were mixed, washed twice with cold BioLegend's Cells Staining Buffer and then resuspended in Annexin V Binding Buffer. FITC-annexin V (5 µl) and Propodium Iodide Solution (10 µl) were added to cell suspension, and then tubes were incubated for 15 min at room temperature in the dark. Subsequently, 400 µl of Annexin V Binding Buffer binding buffer were added to each tube and cells were analyzed by flow cytometry using Attune™ NxT Flow Cytometer (Thermo Fisher Scientific). For each condition 10,000 cells were analyzed. The data generated by flow cytometry were plotted in two-dimensional dot plots in which PI was represented versus Annexin V-FITC.

3.4 Clonogenicity assay (CFA)

The ability of cells to give rise to colonies was performed by clonogenicity assay. A total of 1.5 x 10^3 (CaSki, NOKpWPI and NOKE6/E7) or 3 x 10^3 (HNO150) cells were plated in 6-well plates in triplicate and cultured for 15 days. Following a 24h period post plating, cells are treated with DMSO or EX527 at concentrations of 20 µM or 40 µM and after an additional 24h are irradiated with doses of 2Gy or 4Gy respectively. Media supplemented with EX527 (20 or 40 µM) was changed every 3-4 days. Subsequently, medium was removed, cells were carefully washed with PBS 1X, and fixed with 4% paraformaldehyde (PAF) for 10 minutes. Next, cells were stained with 0.1% crystal violet in 20% ethanol for 10 minutes at room temperature and washed with water. Colonies are scanned and counted with Celigo S Image Cytometer (Nexcelom Bioscience).

3.5 Protein extraction and quantification

To perform the protein extraction, a cell lysate was prepared utilizing a high-salt buffer 1X containing 20mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM DTT. The media was aspirated, and cells was treated by adding fresh media; to harvest cell under non-denaturing conditions, the media was removed, and the cells were washed with ice-cold PBS 1X. Once the PBS was removed, 100 µl of high-salt buffer 1X, with the addition of protease (25 µl/mL Sigma-Aldrich) and phosphatase inhibitors (10 µl/mL, Active Motif), were added to each well of the 6-well plate and the plate was incubated on ice for 5 minutes. The cells were scraped off the plate, transferred into a microcentrifuge tubes, sonicated 4 times for 5 seconds and microcentrifuged at 16000g for 10 minutes at 4°C. The supernatant was collected, transferred in a new tube, and quantified. Protein concentration was determined using Bradford Protein Assay based on an absorbance shift of the dye Coomassie Brilliant Blue G-250. In an acidic environment, the red form of the dye is converted into its blue form, binding to the protein being assayed. The protein-dye complex causes a spectral shift in the absorption maximum of the dye from 465 to 595 nm. The increase of absorbance at 595 nm is proportional to the amount of bound dye, and thus to the amount (concentration) of protein present in the sample. Bovine serum albumin (BSA) was used to calibrate the assay by preparing six serial dilutions of protein diluted with PBS1X to final concentrations of 0, 1, 2, 4, 6, 8 µg/µl. The unknown sample was prepared by adding 2 µl of sample in the final volume of 500 µl of PBS1X. For each test tube 500 µl of Bradford Reagent (Sigma-Aldrich) was added. Absorbance readings were measured at 595 nm using a spectrophotometer and a standard curve was plotted and used to provide a relative measurement of protein concentration of each sample.

3.6 Immunoblotting

For protein analysis, 15 µg of protein extracts were dissolved in *Laemmli Sample Buffer* containing 1% bromophenol blue and 1% β-mercaptoethanol and heated at 95 °C for 5 min to denature proteins.

Proteins were separated by their molecular weight under denaturing conditions using ReadyGels (7.5% or 12%; Bio-Rad). Samples, together with a molecular weight ladder, were loaded into appropriate wells, and the gel was run at 300V. Proteins were transferred from the SDS-polyacrylamide gel to nitrocellulose membrane by using *Trans-Blot Turbo Blotting*

System according to manufacturer's instructions (Bio-Rad). In order to confirm the transfer, the membrane was stained with Ponceau stain. To visualize the proteins, the membrane was briefly washed with water and then three times by using TBS-T 1X (10mM Tris-HCl, pH 7.5, 100mM NaCl, 0.1% Tween-20). To minimize any unspecific interaction of the antibody with the membrane, it was blocked in 10% non-fat dry milk dissolved in TBS-T 1X for 1 hour, followed by incubation with primary antibodies at 4°C overnight on a rocker. The following antibodies were used: rabbit monoclonal antibodies anti-ATM (ab32420; Abcam, diluted 1:1000) and anti-ATM (phospho S1981) (ab81292; Abcam, diluted 1:1000), mouse monoclonal antibody anti-Rad50 (GTX70228; GeneTex, diluted 1:500), anti-MCM7 (MA5-14291; Thermo Fischer Scientific, diluted 1:200) anti-p53 (DO-1) (sc-126; Santa Cruz Biotechnology, diluted 1:250), or rabbit polyclonal antibodies anti-NBS1 (GTX103229; GeneTex, diluted 1:3000), anti-p95/NBS1 (phospho S343) (ab47272; Abcam, diluited 1:500), anti-SMC1A (ab9262; Abcam, diluted 1:1000), anti-phospho-SMC1A (Ser957) (a304-147A; Thermo Fisher Scientific, diluted 1:5000. Ab against α-Tubulin (diluted 1:4000) was used as a control for protein loading.

Thereafter, membranes were washed 3 times in TBS-T 1X to eliminate unbound antibody residues and subsequently incubated with the respective species-specific secondary antibody: sheep anti-mouse (NA931; GE Healthcare) or donkey anti-rabbit (A6154; Sigma-Aldrich) immunoglobulin antibodies conjugated to horseradish peroxidase (HRP) and visualized by enhanced chemiluminescence (34580; Super Signal West Pico; Thermo Fisher Scientific) using the instrument ChemiDoc Touch Imaging System (Bio-Rad).

3.7 Statistical Analysis

All statistical tests were performed using Graph-Pad Prism version 9.00 for Windows (GraphPad Software). The data are presented as mean ± standard deviation (SD). For comparisons consisting of two groups, means were compared using two tailed Student's t tests. Differences were considered statistically significant at a P value of < 0.05.

4. OBJECTIVE OF THE THESIS

Currently, the treatment for human papillomavirus (HPV)-associated cancers involves a multidisciplinary approach based on radiotherapy, chemotherapy, and surgery, all associated with devastating effects on the patients. Considering the rising incidence of various HPV-associated cancers within the last years, together with the fact that HPV infection is being established as the principal cause of genital and head&neck (HN) cancers, novel therapeutic options for HPV-associated cancers are stringently necessary.

Starting from this background, the aim of this thesis was to address the feasibility of novel effective therapeutic strategy with the goal of minimizing toxicity and optimizing oncologic outcomes and to understand how SIRT1 is involved in HPV-induced carcinogenesis.

Recently, it was discovered that the cellular deacetylase SIRT1 plays a significant role in HPV-induced transformation in epithelial cells. Specifically, based on our findings and existing literature, it was suggested that the upregulation of SIRT1 by HPV is essential for p53 deacetylation and its destabilization. In particular, the SIRT1 protein, through its deacetylase activity, participates in the continuous degradation of p53 in HPV^+ cells, and consequently, its pharmacological inhibition allows the restoration of normal p53 activity, which in this context results in a strong reduction in cell proliferation. Specifically, pharmacological inhibition, using EX527, or genetic silencing of SIRT1 in HPV^+ cell lines leads to the reactivation of a transcriptionally active K382-acetylated p53, leading to cell cycle arrest at G0/G1 phase and reduction of cell survival and clonogenicity compared to HPV- cells. It has been also found that EX527 treatment enhanced the sensitivity of HPV^+ cells to sublethal doses of standard genotoxic agents (e.g., cisplatin and doxorubicin). Overall, the studies described in this thesis will assess the anticancer efficacy of EX527 and ionizing radiation on the cell growth and survival of HPV-positive cervical carcinoma and HN cancer cell lines upon ionizing radiation. The long-range goal of this thesis work will be the possibility to include this innovative therapeutic strategy in treatments against HPV-driven cancer, with the purpose of optimizing oncologic outcomes while reducing treatment-related toxicity.

5. RESULTS

5.1 SIRT1 inhibition impairs cell viability and enhances the sensitivity of HPV$^+$ cells to ionizing radiation

Recently it has been demonstrated that SIRT1 inhibition increase the sensitivity of HPV$^+$ cells to genotoxic agents, such as cisplatin and doxorubicin, enhancing cell growth inhibition (Lo Cigno et al., 2023). For this reason, now we want to evaluate the SIRT1 inhibition impact on anticancer activity of radiotherapy, another standard treatment for HPV-associated cancers, in order to set up de-escalating strategies aimed to reduce the adverse side effects on patients. To this purpose, cells were treated with EX527 (40 μM) or with vehicle (DMSO), and then we performed an MTT assay to verify whether SIRT1 inhibition impairs HPV$^+$ cell viability. When examining the cell viability of NOKE6/E7 cells treated with the vehicle, we found no significant difference compared to those that were only irradiated. However, HPV$^+$ cells treated with EX527 showed a marked decrease in viability. As shown in Figure 10A, the greatest reduction in cell viability occurred in the precancerous NOKE6/E7 model following co-treatment with EX527 and 4Gy irradiation. Similarly, in CaSki cell lines, there was a gradual decrease in cell viability compared to cells treated with the vehicle alone. In contrast, no significant changes in growth inhibition were observed in the HPV-negative HNO150 cell line upon combined treatment compared to each single treatment.

Next, we evaluated the extent of early and late apoptosis following treatment with EX527 (40 μM) alone or in combination with radiotherapy (4Gy). Flow cytometry analysis using annexin V/propidium iodide (PI) staining was employed for this assessment. As shown in Figure 10B, an increase in apoptotic cells was observed in HPV-positive cell lines, while no significant changes were detected in HPV-negative ones. The percentage of apoptotic cells increased significantly after the combined treatment based on EX527 (40 μM) and IR (4Gy) compared to each treatment alone. These set of experiments consistently support the hypothesis that SIRT1 inhibition reduces cell viability and increases the sensitivity of HPV$^+$ cells to ionizing radiation.

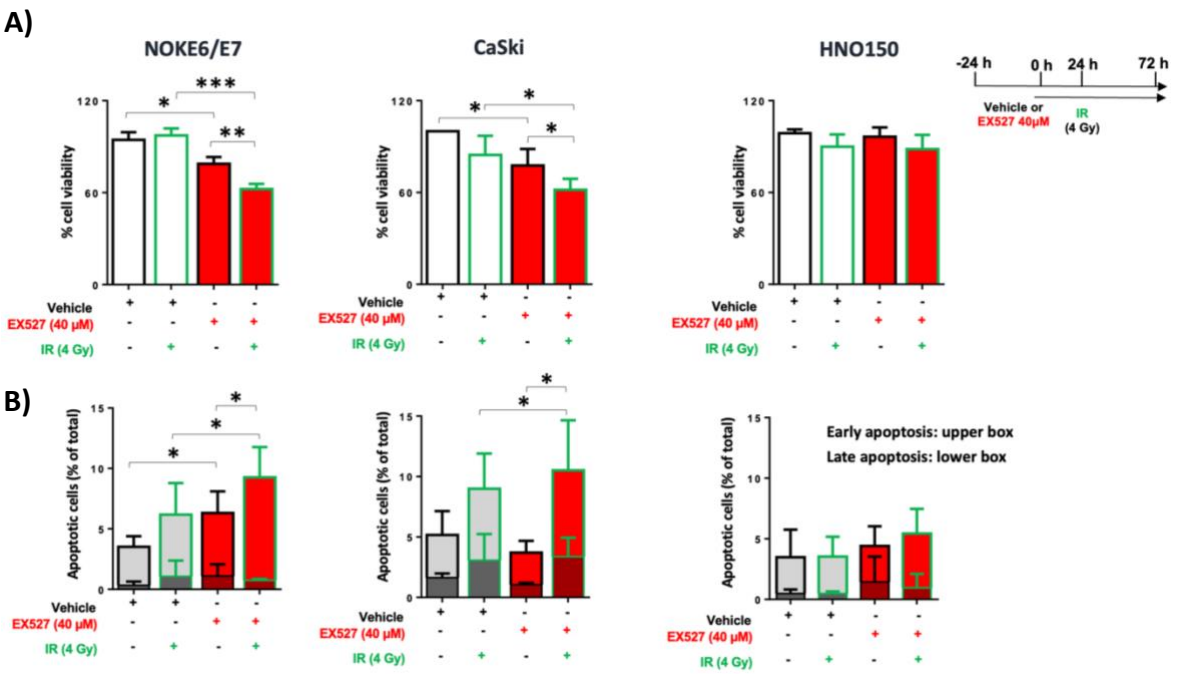


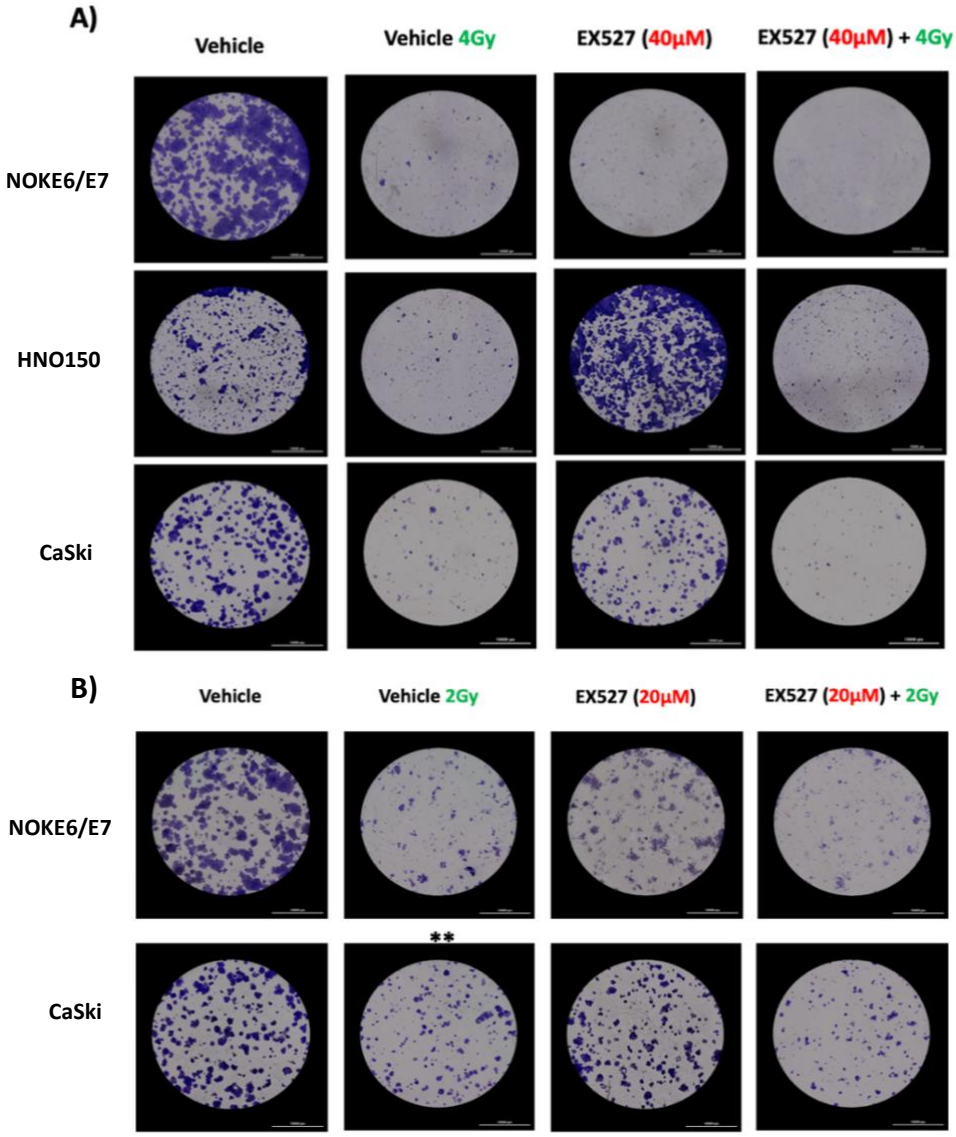
Figure 10. SIRT1 inhibition impairs cell viability and enhances the sensitivity of HPV⁺ cells to ionizing radiation. (A) Cells were treated with EX527 (40 μM) or with vehicle (DMSO), with or without ionizing radiation (4GY) and then cell viability was determined by MTT assay at the indicated time points. The data shown are representative of three independent experiments. Error bars indicate SD. * P < 0.05, ** P < 0.01, *** P < 0.001, unpaired t-test. **(B)** Cell lines after treatment with EX527 (40 μM) or with vehicle (DMSO) for 72h, with or without ionizing radiation (4GY), were incubated with Annexin V-FITC in a buffer containing propidium iodide (PI) and analyzed by flow cytometry. Graphs show the percentage of cells in early apoptosis (annexin V-positive and PI-negative) and late apoptosis/necrosis (annexin V-positive and PI-positive). Data shown are representative of three independent experiments. Error bars indicate SD. * P < 0.05, ** P < 0.01, *** P < 0.001, unpaired t-test.

5.2 SIRT1 inhibition enhances the anticancer activity of ionizing radiation impairing clonogenicity of HPV⁺ cells

To assess whether SIRT1 inhibition in combination with radiotherapy was also associated with a reduction in long-term proliferation, we performed a colony forming assay (CFA) on HPV-positive cells treated with EX527and ionizing radiation. Initially, we used doses of 40 μM EX527 and 4Gy radiation. As shown in Figure 11A, these high doses resulted in excessive cytotoxicity and inhibited cell growth. To address this, we adjusted the treatment regimen to lower doses: 20 μM EX527 and 2Gy radiation. Cells were treated with either the vehicle or EX527 for 24 hours before irradiation. The CFA results demonstrated a significant reduction in the colony area of HPV-positive cells treated with the combination of EX527

and ionizing radiation compared to those only irradiated. Specifically, upon co-treatment the colony area decreased by 6,8% and by 1,4% in NOKE6/E7 and in CaSki cell lines respectively, as illustrated in Figure 11B.

In both NOKE6/E7 and CaSki cells, radiotherapy alone results in a noticeable decrease in colony numbers (Figure 11A and B). This reduction in clonogenic survival demonstrates the effectiveness of ionizing radiation in damaging HPV-positive cancer cells and impairing their ability to proliferate. However, using the combined treatment based on EX527 and ionizing radiation, the decrease in colony formation becomes even more pronounced. This combined therapy results in a significantly greater reduction in the number of colonies compared to radiotherapy or EX527 alone (Figure 11B). These results indicate that the inhibition of SIRT1, along with ionizing radiation, impairs the clonogenic potential of these cancer cells offering a promising approach to improving clinical outcomes for patients with HPV-positive malignancies.



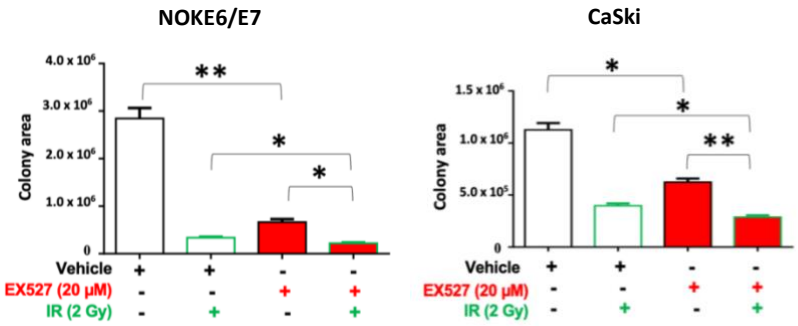


Figure 11. SIRT1 inhibition enhances the antineoplastic activity of ionizing radiation impairing clonogenicity of HPV⁺ cells. (A) Representative images of colony-forming assay on cells treated with EX527 (40 µM) or vehicle (DMSO), and with or without ionizing radiation (4Gy). **(B)** Representative images of colony-forming assay on cells treated with EX527 (20 µM) or vehicle (DMSO), and with or without ionizing radiation (2Gy). The colony area is indicated as mean values of three biological triplicates. Error bars indicate SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, unpaired t-test.

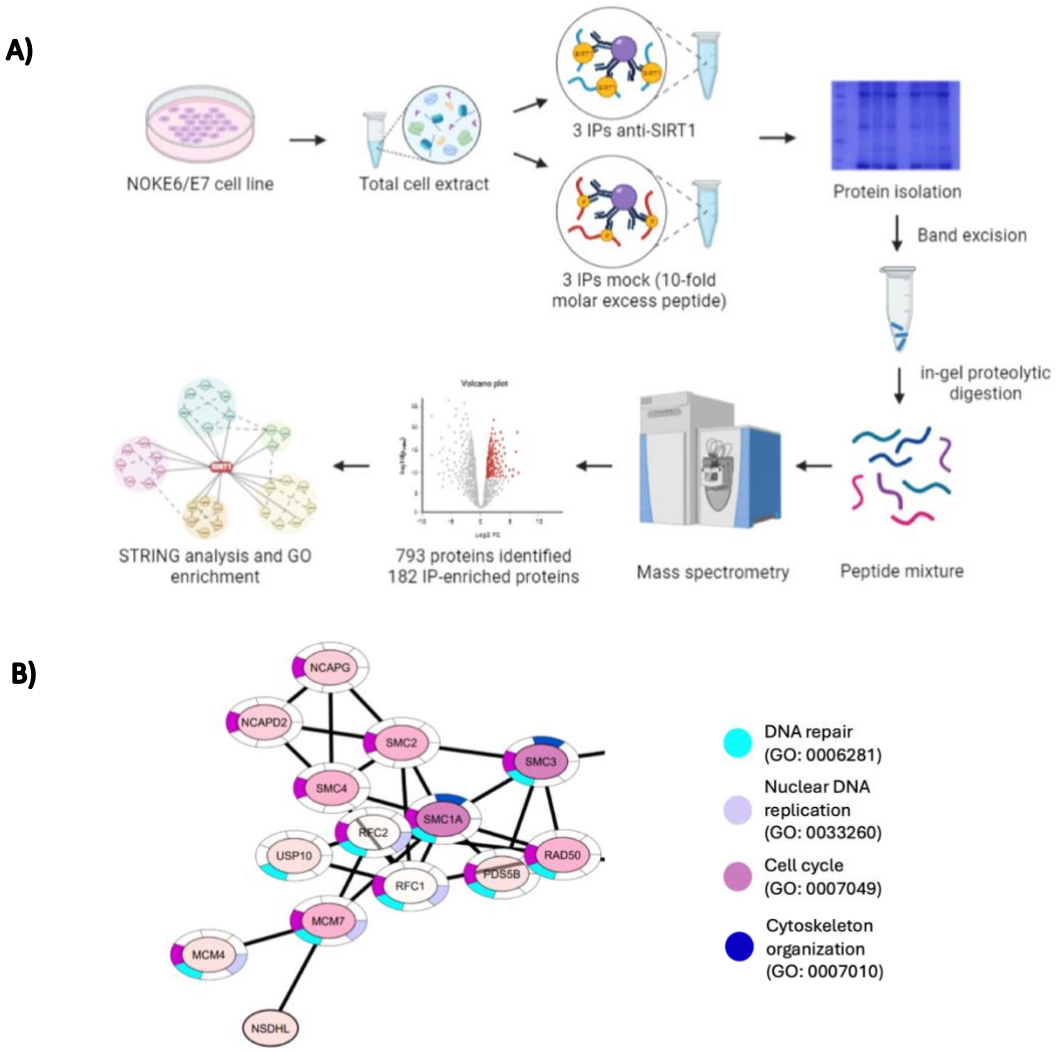
5.3 SIRT1 inhibition enhances the sensitivity of HPV⁺ cells to ionizing radiation through DNA Damage Response (DDR) pathway

Since we have demonstrated that SIRT1 inhibition combined with ionizing radiation leads to a decrease of cell viability and an increase of apoptotic cells, we decided to focus on molecular mechanism that cause these effects. To gain more insight the molecular mechanism underlying SIRT1 action in HPV-driven cancer we have performed an interactome analysis, in order to evaluate the SIRT1 interacting proteins in NOKE6/E7 cells in collaboration with Dr. Tiziana Bonaldi's group (Laboratory of Quantitative Proteomics and Gene Expression Regulation (IEO, Milan). We have found 182 high-confidence interacting proteins specifically associated with SIRT1. Then, a STRING and GO term analysis identified a network of interacting proteins, among which we have observed several factors involved in the DNA Damage Response (DDR) (Figure 12A and B).

Several studies show that SIRT1 plays an important role in the regulation of the DDR pathways and it is well known that some pathways involved in DDR are constitutively active in HPV transformed cells (Langsfeld et al., 2015; Yuan et al., 2007). Indeed, In HPV⁺ cells, the DDR pathways facilitate the viral replication since HPVs are exploit the host cell's DDR machinery, particularly the ATM pathway, to create an environment advantageous to viral genome maintenance and replication. Moreover, ionizing radiation induces double-strand breaks (DSBs) in DNA, which in turn activate several critical pathways of the DDR. Among

these pathways, the ataxia-telangiectasia mutated (ATM) pathway plays a central role. The ATM protein kinase is quickly recruited and activated at the sites of DSBs, initiating a cascade of signaling events that coordinate DNA repair to maintain genomic integrity.

To assess the involvement of the DNA repair system, we focused on the expression of DNA damage response (DDR) markers associated with the ATM pathway. These markers are critical indicators of DDR activation and efficacy in response to DNA damage. As illustrated in Figure 12C, we performed an immunoblotting assay on two cell lines: NOKpWPI, a non-transformed human keratinocyte line, and NOKE6/E7, a transformed cell line expressing HPV E6 and E7 oncoproteins. The immunoblotting assay allowed us to compare DDR marker levels between these cell lines, revealing a significant higher expression of some DDR markers such as pNBS1 (Ser343), Rad50, and pSMC1 (Ser957) in NOKE6/E7 cells compared to NOKpWPI cells. These results indicate an enhanced activation of DDR markers in NOKE6/E7 cells, providing insight into how HPV exploits the DNA repair machinery and highlighting potential targets for therapeutic intervention.



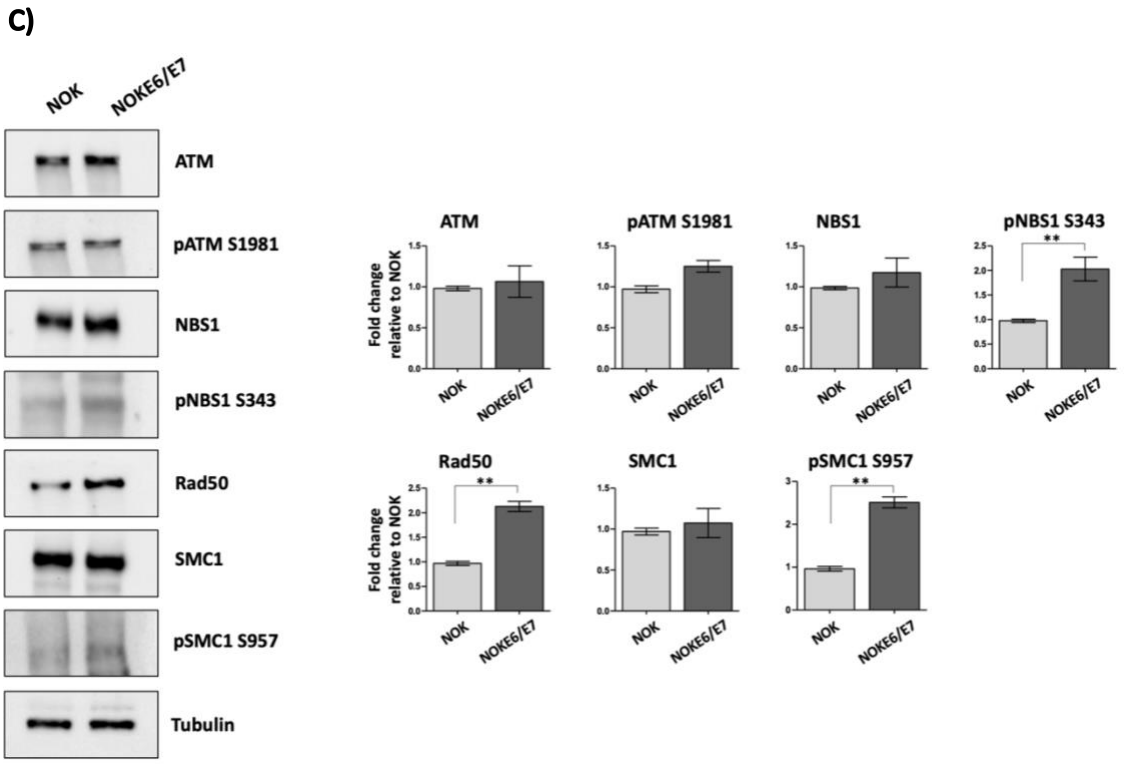


Figure 12. SIRT1 inhibition enhances the sensitivity of HPV^{+} cells to ionizing radiation through DNA Damage Response (DDR) pathway. (A) Experimental workflow of a MS analysis on NOKE6/E7 total cellular extracts immunoprecipitated with anti-SIRT1 antibody. **(B)** Highlight of the STRING network of the SIRT1 interactome involved in DNA repair, DNA replication, and cell cycle. The STRING network was visualized using Cytoscape and a gene ontology (GO) analysis. **(C)** Immunoblot analysis for the indicated proteins of total extraction from NOKpWPI and NOKE6/E7 using antibodies against ATM, phospho-ATM, NBS1, phospho-NBS1, Rad50, SMC1A, phospho-SMC1 and Tubulin as a loading control.

6. DISCUSSION

Papillomaviruses are small, non-enveloped, epitheliotropic, double-stranded DNA viruses that infect cutaneous and mucosal epithelia in a wide variety of higher vertebrates in a species-specific manner, leading to cellular proliferation. Human Papillomaviruses (HPV) are divided into five genera *(alpha, beta, gamma, mu and nu)* and can be classified as cutaneous or mucosal, based on their ability to infect skin or mucosal epithelial cells. Mucosal HPVs are classified as “high-risk” and “low-risk” based on the propensity for malignant progression. Among high-risk HPVs, HPV16 and HPV18 (α-HPVs) are the most prevalent in HPV-positive cancers and are associated with cervical cancer and other mucosal anogenital and head and neck cancers, with an incidence of 5% of the cancer occurrences worldwide (Doorbar, 2006; Morshed et al, 2014; Pal and Kundu, 2020). Currently, the treatment for HPV-related cancers involves radiotherapy, chemotherapy, or surgery, all with devastating effects on the targeted anatomical sites. Consequently, there is an urgent clinical need for alternative approaches that can de-escalate current treatments, optimizing outcomes and reducing acute and long-term toxicities (Mody M.D. et al., 2021; Harari, 2019; Orlandi and Licitra, 2018).

The progression and maintenance of HPV-associated cancers depend on the expression of two oncoproteins, E6 and E7. These proteins use various strategies to bypass the host immune surveillance, allowing viral persistence and deregulating cell cycle and apoptosis control, facilitating the accumulation of DNA damage. Specifically, E6 and E7 target and inhibits the p53 and pRB tumor suppressor proteins, respectively, which are crucial for cell cycle regulation and maintaining genomic stability, thereby driving HPV-induced carcinogenesis (McLaughlin-Drubin and Münger, 2009; Münger et al., 2004; Roman and Munger, 2013; Spriggs and Laimins, 2017; Bester et al., 2011).

Furthermore, different studies demonstrate that exogenous expression of HPV E7 in primary human keratinocytes induces abnormally high levels of the SIRT1 protein, similar to those observed in human cervical cancer cells, and that HPV E7 is required to maintain the abnormally high levels of SIRT1 protein expressed in cervical cancer cells. SIRT1, the mainly NAD+-dependent deacetylase in mammalian cells, functions by deacetylating various substrates, including histones and non-histone targets such as p53. The acetylation of p53 at the K382 lysine residue is crucial for both its stabilization and transcriptional activity (Allison et al., 2009; Lo Cigno et al., 2023; Lee et al., 2013). Consistent with its role in promoting p53 degradation in HPV^{+} cell lines, our findings

indicate that SIRT1 inactivation, through its specific pharmacological inhibitor EX527, significantly impacts HPV-induced carcinogenesis. Indeed, we have demonstrated that pharmacologically inhibition of SIRT1 impairs cell viability and proliferation rates only in HPV^+ cells, confirming that p53 is functional in HPV^+ cells but not in HPV^- cells. In addition, our group have also demonstrated that HPV^+ cells treated with EX527 show more sensitivity to traditional genotoxic agents commonly used to treat HPV-associated cancers, such as doxorubicin and cisplatin (Lo Cigno et al., 2023).

For this reason, in this thesis we want to evaluate the impacts of SIRT1 inhibition on the antitumor activity of another standard treatment for HPV associated cancers, namely radiotherapy, with the aim of setting up de-escalation strategies to reduce the negative side effects. We observed the maximum reduction in cell viability after co-treatment with EX527 and IR in the precancerous NOKE6/E7 model (Figure 10A). We have similar results in CaSki cell line, while no significant changes in growth inhibition were observed in the HPV-negative HNO150 cell lines during combinatorial treatment. In addition, we assessed the impact of the co-treatment based on EX527 and ionizing radiation also evaluating the percentage of early and late apoptosis and we show an increase of apoptotic cells after combination treatment compared with each treatment alone (Figure 10B).

In order to evaluate the effects of the combined treatment on anticancer activity in a long-term proliferation assay, we performed a colony formation assay. We observed a significant reduction in colony area of HPV^+ cells after treatment with EX527 and especially after co-treatment (Figure 11B). This reduction in clonogenic survival demonstrates the effectiveness of ionizing radiation in damaging HPV-positive tumor cells and impairing their ability to proliferate and offers us a promising approach to improve clinical outcomes for patients with HPV-positive malignancies.

To gain more insight the molecular mechanism underlying SIRT1 action in the contest of HPV-driven cancer, we performed a mass spectrometry analysis to identify SIRT1 interacting partners in NOKE6/E7 cells, and we found 182 high-confidence interacting proteins. STRING and GO term analyses identified a network of SIRT1 interacting proteins involved in “DNA repair” and “nuclear DNA replication”. Since several studies show that some pathways involved in DDR, particularly the ATM pathway, are constitutively active in HPV-transformed cells, we evaluated the involvement of the DNA repair system by

focusing specifically on the expression of DNA damage response (DDR) markers associated with the ATM pathway. For this reason, we conducted an immunoblotting assay that allowed us to compare the levels of DDR markers between two distinct cell lines (NOKpWPI and NOKE6/E7), and our results demonstrated markedly higher expression of these markers in NOKE6/E7 cells compared with NOKpWPI cells. These results showed higher expression of some factors involved in DDR, providing further insight into how HPV exploits the DNA repair mechanism and highlighting potential targets for therapeutic intervention.

In conclusion, all our findings confirm the crucial role of SIRT1 in HPV-driven carcinogenesis and it could be considered an important target for novel therapeutic approaches. Indeed, our results show an important increase e of the antitumor effects of traditional treatment, namely radiotherapy, after the combined treatment based on EX527 and ionizing radiation, suggesting an important strategy to set up a de-escalating combinatorial treatment in order to decrease the negative side effects on patients of current radio-chemotherapy-based therapies, improving patient outcomes. Ongoing preclinical investigations aim to translate this approach into clinical practice.

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