Study of the Carcinogenic Potential of the E6 Protein of Human Papillomavirus type 16

by

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Abstract

While most Human papillomavirus (HPV) infections are asymptomatic and self-resolved, high-risk types, such as HPV-16 and HPV-18 are responsible for 99% of cervical cancers worldwide, whereas low-risk types, such as HPV-6 and HPV-11, are responsible for 90% of genital warts. While the different types of HPV and their varying oncogenicities have been studied extensively, it is still not clear what features of a HPV type make it more oncogenic than another. Two aspects which could affect the oncogenicity of HPV were studied: HPV variants and the E6 protein's interaction with membrane associated guanylate kinase (MAGUK) proteins.

Previous studies have shown that some HPV-16 variants may be more oncogenic than others. The first goal of this work was to characterise the HPV-16 variants in a Manitoba cervical cancer sample population to possibly identify mutations which could be associated with an increased risk of developing cervical cancer. Seventy-five samples from different individuals were sequenced in three distinct regions: the long control region and the E6 and E7 open reading frames. The DNA sequences obtained from these genomic regions were then compared between HPV-16 cervical cancer samples and Manitoba HPV-16 non cancer samples to identify any mutations that were exclusive to the cervical cancer samples. No specific mutations in any of the regions could be associated with cervical cancer.

It is also proposed that HPV16 E6 protein's interaction with MAGUK proteins contributes to its oncogenicity since low-risk E6 proteins lack this ability. The second goal

of this work was to investigate which regions of high-risk HPV E6 proteins are needed in order to achieve MAGUK protein degradation, more specifically MAGI-1 degradation. Wild-type high-risk HPV16E6, low-risk HPV6E6, as well as mutants, were synthesized and cloned into vectors. *In vitro* translated proteins were used in MAGI-1 degradation assays. The ability of both wild-type HPV6 and HPV16 E6 proteins to degrade MAGI-1 was confirmed. Based on the performance of the different mutants in these degradation assays, it was determined that the PDZ-binding domain is necessary but not sufficient to induce E6-induced MAGI-1 degradation. In conclusion, it was determined that the entire HPV16 E6 protein is needed for the induction of MAGI-1 degradation.

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°C	Degrees Celsius
A2t	Annexin A2 heterotetramer
a.a	Amino acid
AA	Asian American
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CDK	Cyclin-dependent kinase
cDNA	Copy deoxyribonucleic acid
CKI	Cyclin-dependent kinase inhibitor
C-terminal	Carboxy terminal end
DNA	Deoxyribonucleic acid
dNTP	Mixture of deoxynucleotides
ds	Double stranded
E1	E1 gene/protein
E2	E2 gene/protein
E4	E4 gene/protein
E5	E5 gene/protein
E6	E6 gene/protein
E6-AP	E6 associated protein
E7	E7 gene/protein
EV	Epidermodysplasia verruciformis
g	Grams
HCl	Hydrogen chloride
hDlg	Human discs large protein
hSCRIB	Human scribble protein
hTERT	Human telomerase reverse transcriptase
HPV	Human papillomavirus
HSPG	Heparan sulfate proteoglycans
IARC	International Agency for Research on Cancer
IRF	Interferon regulatory factor
kDa	Kilodalton
L1	L1 gene/protein
L2	L2 gene/protein
LB	Luria-Bertani
LCR	Long control region
MAGI	MAGUK with inverted domain protein
MAGUK	Membrane associated guanylate kinase
mA	milliamps
$MgCl_2$	Magnesium chloride
ml	Milliliter
mM	Millimolar

mRNA	Messenger ribonucleic acid
MW	Molecular weight
NCBI	National Centre for Biotechnology Information
N-terminal	Amino terminal end
ORF	Open reading frame
p53	p53 protein
PAE	Early polyadenylation
PAGE	Polyacrylamide gel electrophoresis
PAL	Late polyadenylation
PCR	Polymerase Chain reaction
PDZ	PSD95/Dlg/ZO-1
PHFK	Primary human foreskin keratinocytes
PV	Papillomavirus
PVDF	polyvinylidene difluoride
Rb	Retinoblastoma protein
rcf	Relative centrifugal force
rpm	Revolutions per minute
RNA	Ribonucleic acid
RRP	Recurrent respiratory papillomatosis
SDS	Sodium dodecyl sulphate
TBS	Tris-Buffered Saline
TBST-T	Tris-Buffered Saline with Tween 20
µG	Micrograms
µl	Microliter
µM	Micromolar
U	Units
URR	Upstream Regulatory Region
V	Volts
VLP	Virus-like particle
WHO	World Health Organization

1.0. GENERAL INTRODUCTION

1.1 Human Papillomavirus

Mucosal human papillomaviruses (HPV) cause sexually transmitted infections which affect both men and women worldwide. Although most HPV infections are asymptomatic and self-limiting, lesions (benign or malignant) may also result from infection[12]. HPV infections have been linked to the development of various cancers, but most notably, cervical cancer [8]. According to the World Health Organization (WHO), every year approximately 500,000 women develop cervical cancer and 274,000 die from the disease worldwide[128]. Cervical cancer is the second most common cancer among women worldwide, and in developing countries it is the most common cancer causing death in women[128]. HPV is found in 99% of cervical cancers[125], 70% of which are due to HPV type 16 (HPV-16) or HPV type 18 (HPV-18)[57]. It was in the late 1970`s that Harald zur Hausen first proposed that HPVs had a role in the development of cervical cancer[138, 139]. Subsequently, in 1983 he was able to isolate HPV-16 DNA from cervical cancer biopsies[31] and did the same with HPV-18 the following year[7]. These discoveries initiated research in determining what role HPV played in the development of cervical cancer. In 2008, zur Hausen was awarded the Nobel Prize in Physiology or Medicine for his contribution to research involving HPV and cervical cancer.

According to the International Agency for Research on Cancer (IARC), there are 15 HPV types which are considered carcinogenic (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, -82)[85]. Since HPV-16 and HPV-18 are the most prevalent types

in found in cervical cancer, vaccines have been developed to protect against infection with these types. Currently, there are two prophylactic vaccines on the market: Gardasil and Cevarix. Both vaccines protect against infection with HPV-16 and HPV-18, but only Gardasil has additional protection against two low-risk types associated with genital warts, HPV-6 and HPV-11[80]. A vaccine protecting against 9 high-risk HPV types is undergoing clinical trials[51].

1.2. HPV Classification

The Papillomaviridae family is made up of 189 papillomaviruses (PVs) infecting humans, non-human mammals, birds and reptiles[3]. As their name states, these viruses are responsible for the formation of papillomas (or warts) on keratinised cutaneous or mucosal epithelia[18]. The L1 gene, which encodes for the major capsid protein, is the most conserved gene of the genome and it is therefore used to phylogenetically classify PVs[3, 18]. Based on L1 sequences, PVs can be further classified into genera. Currently, there are 29 PV genera and between different genera there exist less than 60% sequence homology in the L1 gene[18]. Genera are further broken down into species which share between 60-70% sequence homology in the L1 gene[18]. For each species a "type species" is chosen. Usually this type species is the most studied PV type and is considered to have prototype characteristics of the species[3]. The PV types contained within a species share between 71-89% sequence homology in the L1 gene[18]. Furthermore, HPV types can be further classified into variants[12, 72]. Variants are distinguished by mutations found in the sequences of their E6 gene and/or the long

control region[72]. It is proposed that some variants are more oncogenic than others. HPV variants will be discussed in section 2.0.

The HPVs are contained within 5 genera named alpha-, beta- gamma-, mu- and nu- papillomaviruses[3]. HPV types in the beta, gamma, mu and nu genera mostly infect the keratinised epithelium of the skin, often causing benign and self-limiting lesions (skin warts)[3]. These species are rarely associated with cancer in healthy individuals[28]. However, in individuals afflicted with epidermodysplasia verruciformis (EV), infection with beta-PVs, could lead to the development of skin cancer[38]. EV is a rare genetic disease in which individuals are very susceptible to persistent beta-PV (HPV-5, -8, -9, -12, -14, -15, -17, -19, -25, -36, -38, -47 and -50) infections[38]. This results in life long outbreaks of disseminated verrucae-like lesions[38]. Approximately half of all EV patients will develop cutaneous malignancies later in life[38]. This occurs mainly on sun exposed areas of the skin[17, 38]. Malignant transformation of EV lesions has mainly been linked to infections with HPV-5 and -8[38, 90]. DNA from these two types have been found in 90% of EV cancers[38]. In addition to this, HPV-5 DNA has been found in cutaneous lesions of psoriasis patients and HIV patients have also been found to suffer from EV-like lesions[38,127,56].

The alpha-papillomaviruses mostly contain HPVs affecting the keratinised epithelia of the anogenital or oral mucosae and can be classified as high or low risk[28,137]. Table 1 lists some of the alpha-papillomavirus species and their high- or low-risk classifications.

Table 1 – Classification of Alpha-papillomaviruses*

Genus	Species	Type Species	Other Papillomavirus types	High- or Low- -Risk Classification
Alpha-papillomavirus	4	HPV 2	HPV 27 HPV 57	Low
	7	HPV 18	HPV 39 HPV 45 HPV 59 HPV 68 HPV 70	High
	9	HPV 16	HPV 31 HPV 33 HPV 35 HPV 52 HPV 58 HPV 67	High
	10	HPV 6	HPV 11 HPV 13 HPV 44 HPV 74	Low

* - modified from de Villiers *et al.* [18]

Low risk HPV types cause benign and self-limiting infections of the cervix and other genital mucosae[137]. HPV-6 and -11 are the cause of 90% of anogenital warts[54] and more rarely, are the cause of recurrent respiratory papillomatosis (RRP)[80,75]. RRP is a chronic disease where papillomas can be found throughout the aerodigestive tract, but most commonly in the larynx[22]. The onset of RRP can either be in childhood or adulthood. RRP acquired during childhood is often due to the vertical transmission of HPV during childbirth and is much more aggressive than the adult form, which can be acquired through sexual contact[22]. RRP can cause significant health problems, as the

airway is obstructed by the papillomas and must be removed by surgery. This being said, infections with low-risk types rarely progress to malignant transformation.

High-risk types are much more likely to cause dysplastic lesions which can progress to cancer[125,137,74, 85,5,8]. Initially, any HPV type that could be directly isolated from cancer patient biopsies was considered to be high-risk and thus any types that were phylogenetically related were also considered to be high-risk[30]. After the discovery that E6 and E7 genes were important for malignancy[82], E6 and E7 genes of different HPV types which were experimentally shown to immortalize or transform human keratinocytes were also considered to be high-risk types[3]. Coincidentally, the majority of the high-risk HPV types belong to either species 7 or 9 of the alpha-papillomaviruses[18]. Epidemiological studies determined the risk of cervical cancer of different HPV types by calculating the odds ratio in healthy women and cancer patients[6, 86]. They found that HPV types were classified as high or low-risk along the same lines as the phylogenetic classification. An analysis by Munoz *et al.* (2003) confirmed that the epidemiologic classification of HPV types correlated with phylogenetic classification[85].

On the evidence of the phylogenetic and epidemiological results, the IARC classifies HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, -82 as high-risk types, HPV- 26, -53, -66 as probable high-risk types and HPV-6, -11, -40, -42, -43, -44, -54, -61, -70, -72, -81 as low-risk types[85]. It is important to note that while HPV infection is most notably associated with cervical cancer, HPV has also been found in numerous other cancers[54]. These include: anogenital cancers (vulvar, vaginal, penile,

anal)[19, 23, 26, 53, 100], upper aerodigestive tract cancers (oral, tonsil, neck)[1, 40] and skin cancers[52].

1.3. HPV Genome

HPV has a circular double stranded DNA (dsDNA) genome that is approximately 8000 base pairs (bp) in length (Figure 1)[54]. It can be divided into 3 regions: the non-coding region, the early region and the late region.

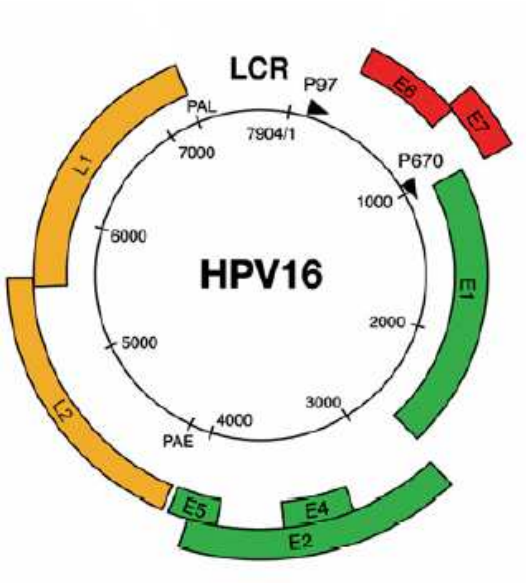

Figure 1 – HPV genome. HPV-16's 7904bp genome is represented by the black circle. The genome can be divided into 3 regions: 1) the long control region (LCR) which contains the p97 promoter and important binding sites for replication and transcription factors, 2) the early region which includes the E1, E2, E4, E5 E6 and E7 genes, and 3) the late region which includes the L1 and L2 genes. The genome contains two promoters, p97 and p670 which are marked by black arrows. The p97 promoter is used in the early stages of infection, while the p670 promoter is used in later stages of infection, when cells have started to differentiate. All the viral genes are encoded on one strand of DNA. Following expression, mRNA is alternatively spliced and polyadenlylated using either the early polyadenlyation site (PAE) or the late polyadenylation site (PAL) to eventually give all the viral proteins[28] (Modified from: Doorbar, *Clinical Science*, 2006[28])

The non-coding region, also known as the upstream regulatory region (URR) or the long control region (LCR) is the most variable region of the genome[12]. It does not encode any genes however it does contain many binding sites for viral proteins which regulate gene expression and DNA replication[12]. It contains four binding sites for the viral protein E2, which is an important transcription regulator; and one binding site for the viral protein E1, in close proximity to the DNA origin of replication[54]. It also contains the p97 promoter which is used in the early stages of infection and from which early gene mRNAs can be transcribed[28].

The early region encodes the E1, E2, E4, E5, E6 and E7 proteins. These proteins are all involved in replication, transcription, and oncogenesis of HPV[9, 11, 12, 48, 54, 78,34,10]. The E1 protein is a DNA helicase which unwinds viral DNA during replication[54]. The E1 protein is recruited by E2 in order for it to bind to its binding site near the origin to initiate DNA replication[9, 27, 7834]. E2 is also involved in transcription regulation, especially for the E6 and E7 genes. It is proposed that at low levels E2 activates transcription, whereas at high levels, E2 represses transcription[54,10]. It is also able to simultaneously bind to a copy of the viral genome and chromosomes during mitosis, ensuring that each daughter cell receives a copy of the viral genome[78,122]. The E4 protein is usually expressed at later stages of infection and is important in the expression of the late genes[54]. It may also play a role in virus release as it disrupts the cytoskeleton of infected cells which would allow the easy rupture of cells containing newly synthesized virus[12,29]. The E5 protein has been proposed to enhance the transforming properties of E6 and E7 by stimulating cell growth[9, 11, 112]. E5 is able to upregulate the expression of epidermal

growth factor receptors, and reduce their turnover, allowing infected cells to continue to be stimulated by growth factors[24]. E6 and E7 are both oncoproteins that play a role in deregulating the cell cycle, resulting in the immortalization and transformation of the infected cells[45, 48, 80]. Their role in HPV's oncogenicity will be discussed in further detail in section 1.5.

The late region encodes the structural capsid proteins L1 and L2. The L1 protein is the major capsid protein, while the L2 protein is the minor capsid protein. The L1 protein is involved in the initial target cell receptor binding[96]. The L2 protein, which only has a portion exposed on the capsid surface, may also be involved in the later stages of virus entry[96]. It is also believed to be involved in binding viral DNA during capsid formation and viral assembly[136]. It has been found that the L1 protein (alone or together with L2) can self-assemble into virus-like particles (VLPs)[60, 61] which are very immunogenic. Thus, L1 VLPs are the basis for the two vaccines currently in use: Gardasil and Cevarix[80].

1.4. HPV Life Cycle

HPV is a non-enveloped virus which has an icosahedral capsid[12]. It has a specific tropism for keratinized epithelial cells and its life cycle is linked to the differentiation of infected cells[54]. Initially, HPV gains access to the basal cell layer via a small wound[80]. HPV target cell binding and entry is not yet fully understood. It is not yet known what specific cell receptor is used by HPV to bind and enter the basal cells. In a model proposed by Raff *et al.* (2013), L1 first binds to heparin sulfate proteoglycans (HSPGs) found on the

host cell surface. Growth factor receptors, such as epidermal growth factor receptors or keratinocyte growth factor receptors are activated. After binding to HSPG, L1 undergoes a conformational change, such that the L2's N-terminus is further exposed. This exposes L2 to cleavage by furin, which causes another conformational change. Following this, it is believed that a secondary receptor is bound. It has been proposed that L1 binds to α6 integrin, while L2 binds to annexin A2 heterotetramer (A2t). After binding to a secondary receptor, the virus is then endocytosed by a mechanism yet to be determined.

Once the virus has uncoated, the viral DNA migrates to the cell nucleus where its genome is replicated. The E1 and E2 proteins help maintain the genome as a low copy number episome[12]. Since the virus does not encode its own polymerase, it depends entirely on host cell machinery to carry out replication. In the lower basal cell layer, E6 and E7 are also expressed to drive infected cells to divide. While differentiating cells would normally exit the cell cycle, in the presence of HPV, E6 and E7 continue being expressed and keep the infected cells in an actively dividing state[80]. As the basal cells start to differentiate and move upwards in the epithelium layer, the virus also begins to replicate at a high rate[80]. In addition to this, expression of E1 and E2 increases and thus genome amplification and transcription increase. As the cells begin to reach the top epithelium layers, E4 is expressed, which is needed for expression of late viral proteins and virus release. As L1 and L2 are expressed, new virions begin to assemble[12]. Newly synthesized viruses are released as the cells are shed from the uppermost layer of the epithelium[80].

1.5. E6 and E7 Proteins

HPV's oncogenicity is mainly due to two virally encoded oncoproteins, E6 and E7. Although their intended function is to allow infected cells to continue to replicate, they both contribute greatly to HPV's ability to progress from an asymptomatic infection to cervical cancer. Early studies showed that when cells were transfected with different mutants of whole HPV-16 genomes, the mutants in which the E6 and E7 genes were interrupted were not able to transform primary human keratinocytes[82], while mutations affecting the other HPV proteins did not have an effect on transformation. Further to this, other studies showed that when cells are transfected with only the E6 and E7 open reading frames (ORFs) they are capable of immortalizing and transforming human keratinocytes, confirming that E6 and E7 are sufficient for transformation[2, 45, 48]. It was also found that E7 alone is capable of immortalizing cells, but that the presence of E6 drastically increases immortalization efficiency[45]. These studies also confirmed that low-risk types are not capable of immortalization or transformation. If we consider the different protein interactions in which both proteins participate, we can begin to understand how the E6 and E7 proteins both play an important role in the development of cervical cancer.

The E7 protein is 13 kDa in size and its main interacting protein is the retinoblastoma (Rb) tumor suppressor protein[80]. The Rb protein regulates the transition from G1 to S phase in the cell cycle by interacting with E2F transcription factors. Normally, Rb is bound to E2F's transactivation domain[80]. When Rb is phosphorylated by

cyclin-dependent kinases (CDKs), it dissociates from E2F, which goes on to activate the transcription of S-phase specific genes[80]. During HPV infection E7 binds Rb, leaving E2F free to activate transcription. This results in the uncontrolled entry of infected cells into S-phase and therefore, continuous proliferation of infected cells. In addition to this, the E7 protein of high-risk types, tags Rb for proteasomal degradation via the ubiquitin dependent pathway[11].This degradation of Rb is a mechanism which ensures that HPV infected cells remain in the cell cycle. Another manner in which E7 can disrupt the normal cell cycle, independent from Rb, is by interacting with other factors which control the progression of the cell cycle, such as cyclin-dependent kinase inhibitors (CKIs)[35, 134]. CKIs such as p21 and p27 negatively regulate CDKs, causing G1 cell cycle arrest. E7 has been shown to interact with p21 and p27[35, 134], such that they cannot interact with CDKs and therefore cell cycle arrest does not occur. Mutational studies of the E7 protein have found that the C-terminus is important for p21 binding, whereas the N-terminus is important for Rb binding[64]. Munger *et al.* (1991) showed that it was the differences in the N-terminal tails of high- and low-risk E7 proteins that determined the difference in their biological activity[84]. When comparing the activities of high-risk and low-risk E7 proteins, it has been found that in general, the low-risk E7 protein interacts with its binding partners less efficiently than the high-risk E7 protein[64]. Munger *et al.* (1989) and Gage *et al.* (1990) confirmed that high-risk HPV E7 protein could bind Rb more efficiently than low-risk HPV E7 protein[36, 83]. In experiments done by Funk *et al.* (1997), it was found that low-risk E7 was not able to prevent p21 inhibition of CDK2 activity[35]. Demers *et al.* (1994) found cells expressing high-risk HPV16 E7 protein were

able to overcome induced growth arrest, while those that expressed low-risk HPV6 E7 could not[21]. This confirmed that low-risk HPV16 E7 could not interact as efficiently with cell cycle control factors to overcome cell cycle arrest. In 2002, Helt *et al.* established that both Rb and p21 must be inactivated in order for cells to overcome cell cycle arrest[49, 79].

The E6 protein is 18kDa in size and its main interacting protein is the tumor suppressor protein, p53[103]. As a result of the disruption caused by E7, p53 levels begin to increase in HPV infected cells[58]. This is due to the "trophic sentinel response" [33, 81] which is a cellular defense to eliminate cells that have the potential to become transformed[32, 33], as is the case with HPV16 E7 containing cells. Normally, p53 plays an important role in cell cycle regulation, as well as apoptosis. When DNA damage occurs, p53 can halt the cell cycle so that the DNA can be repaired before replication occurs[39]. On the other hand, if replication has already taken place or too much damage has occurred, p53 can push the cell towards apoptosis by upregulating the expression of proapoptotic proteins PUMA and NOXA[20]. Both proteins are involved in the initiation of apoptosis[20]. To counteract the increase in p53 and prevent the apoptosis of cells affected by E7, the E6 protein interacts with p53 and inhibits its function. However, before this occurs HPV E6 must first associate with the cellular E6 associated protein (E6-AP). This E6-E6-AP complex binds to p53 and E6-AP serves as an ubiquitin-protein ligase, allowing p53 to become ubiquitinated[102]. Once this occurs, p53 is subsequently degraded via the ubiquitin dependent pathway[104]. This allows for an uncontrolled cell cycle, as was seen with E7. Studies have found that both high-risk and low-risk E6 proteins are capable of

binding p53, although similar to E7, high-risk E6s bind with higher affinity[68]. In addition to this, only high-risk types are capable of inducing p53 degradation[79].

A study by Crook *et al.* (1991) identified the regions of the HPV16E6 protein that are important for p53 binding and degradation[16]. When they mutated amino acids in the C-terminal half of the E6 protein that were found to be conserved in high-risk types to amino acids found in low-risk types, they found that this did not have an effect on p53 binding. On the other hand, when they constructed truncated HPV16 E6 mutants, they found that these mutants were severely impaired in p53 binding. This indicated that the C-terminus was essential for p53 binding. Additional mutational studies found that the region from amino acids 110 to 115 was necessary for p53 binding. Since none of the mutants which changed amino acids to those found in low-risk types did not have effect on p53 binding, they tested and confirmed that HPV6 and HPV 11 E6 were capable of binding p53, however, they still could not degrade p53. This led to the conclusion that p53 binding is necessary, but not sufficient to promote p53 degradation. To identify what regions of HPV16 E6 are necessary for p53 degradation they made two chimeric E6 proteins. One consisted of the N-terminal 60 amino acids of HPV6 E6 fused to the C-terminal 92 amino acids of HPV16E6 (6/16E6), while the other consisted of the N-terminal 59 amino acids of HPV16E6 fused to the 90 C-terminal amino acids of HPV6E6 (16/6E6). Both mutants were able to bind p53, but only the 16/6E6 chimera could degrade p53; therefore they concluded that the N-terminus was important in targeting p53 for degradation. Additional mutational studies found that amino acids 9 to 13 and a conserved region in high-risk types from amino acids 45-49 were both important in

directing p53 degradation. Furthermore, other studies looking at the p53 protein have shown two possible binding sites for the E6 protein; one within the core structure, and the other at the C-terminal domain of the p53 protein[70]. Binding to the core site of p53 is essential for degradation. High-risk E6's are capable of binding both sites, while low-risk E6's only bind the C-terminal site, thus further explaining the difference in their capabilities in inducing p53 degradation.

1.6. E6 Protein; additional protein interactions

It is said that E7 is important for immortalization of the infected cell, but that E6 pushes the cell towards full transformation[120]. As mentioned previously, E7 is successful at immortalizing cells; however in conjunction with E6 it has a much stronger immortalization capacity. A study by Song *et al.* (2000) found that in E7 transgenic mice, the tumors that formed were primarily benign. Whereas the tumors that formed in E6 transgenic mice were primarily malignant, indicating that E6 was important for promoting malignancy[109]. If we look at additional protein interactions in which E6 participates, we can begin to see how E6 pushes cells into malignancy.

The E6 protein can interfere with another apoptosis pathway which is p53 independent, by interacting with the Bak protein[114, 115]. Bak belongs to the Bcl-2 family of proteins. Proteins in this family play an important role in apoptosis as they regulate the activation capases[114,15]. Bak induces the release of apoptotic protein cytochrome c, which will then go on to activate caspase-9, eventually resulting in apoptosis of the cell[15]. Thomas & Banks (2001) showed that both high- and low risk E6's can bind to Bak.

They also showed that both types were capable of Bak degradation and of preventing Bak-induced apoptosis[115]. They did note that in all their experiments, the high-risk type E6s always resulted in a stronger interaction. Since the Bak proteins are degraded through their interaction with E6, cells infected with HPV are able to overcome another mechanism capable of inducing apoptosis and continue to replicate.

High-risk E6 has also been shown to alter keratinocyte differentiation by targeting the Notch signalling pathway. The Notch signalling pathway was shown to be involved in keratinocyte growth arrest and differentiation[97]. With elevated levels of the Notch1 protein, it was found that there was an increase in p21 expression, which is involved in induction of cell cycle arrest. They also found that elevated levels of Notch 1 coincided with the expression of early differentiation markers. Yugawa *et al.* (2007) showed that Notch 1 expression is regulated by p53[131,132]. Therefore, Notch 1 expression can be indirectly down-regulated by E6's degradation of p53[39, 132]. As a result, the infected cells remain in the cell-cycle and continue to actively divide, instead of exiting the cell cycle to differentiate.

Another protein interaction exclusive to the high-risk E6 proteins, is the MAGUK (Membrane Associated Guanylate Kinase) protein family, which are involved in maintaining cell polarity and signal transduction pathways[120]. Interaction of the MAGUK proteins with high-risk E6, results in degradation[37, 41, 65, 87, 119]. Since, only high-risk E6 proteins are capable of interacting with these proteins it is proposed that this

interaction contributes to E6's oncogenicity[120]. These interactions will be discussed in further detail in section 3.0.

Finally, high-risk E6 is able to extend the life span of cells by preventing telomere shortening. Telomeres are tandem repetitive sequences which are found at the end of chromosomes[105]. Each time a cell divides, telomeric repeats are lost, and eventually this loss leads to replicative senescence[105,39]. Therefore, this is the reason cells normally have a finite lifespan. To counteract the shortening of telomeres and extend the life of cells, the enzyme telomerase is able to add six base pair repeats to the end of telomeres (to replace the repeats loss during cell division)[108]. Telomerase has two components: an enzymatic human telomerase reverse transciptase (hTERT) which is the catalytic subunit, and an RNA component, which is used as template for synthesizing the six base pair repeat[105]. It has been found that E6 is able to activate telomerase activity[63]. Further work has shown that E6 is able to induce transcription of the hTERT and that an upregulation of hTERT expression correlates an increase in telomerase activity[123]. The continued presence of telomerase allows the lifespan of cells to become infinite. Active telomerase has been found to be essential for cellular transformation[44].

Experiments done by Hahn *et al.* concluded that expression of hTERT along with two oncogenes (simian virus 40 large T oncogene (large-T) and an oncogenic allele of H-ras (ras)) was sufficient to convert normal human keratinocytes and fibroblasts into tumorigenic cells[44]. These two oncogenes are capable of inactivating p53 and pRb (large-T) and disrupting normal cell growth and differentiation via the mitogen response

pathway (oncogenic ras). Interestingly, E6 and E7 proteins from high-risk HPV are able to alter or disrupt the above mentioned pathways through E6's activation of hTERT, E6 and E7's degradation of p53 and pRb and E6's interaction with Notch 1. Therefore, high-risk HPV E6 is able to affect elements which are sufficient to cause tumorigenesis of infected cells.

1.7. Progression into Cervical Cancer

Progression of a HPV infection to cervical cancer is a rare occurrence and it is a process which may take many years. Key events must occur in order for cancer to develop. First, HPV must be able to persist in the host. In the basal cell layer, HPV's life cycle does not cause cell death and throughout the entire viral life cycle there is relatively low expression of viral proteins[39, 132]. This makes it difficult for the host's immune system to detect the HPV infection. Throughout the course of infection, it is found that both the high-risk E6 and E7 proteins interact with host elements such as interferon regulatory factors (IRFs), which results in the suppression of the antiviral response controlled by these factors[132, 92, 99]. With persistence there is continued expression of viral proteins (i.e. E6 and E7), allowing upregulated activity of these proteins that will push infected cells to become immortalized and eventually become fully transformed. Along with persistence, integration of the HPV genome into the host genome occurs[121]. No specific regions into which the HPV genome integrates consistently have been identified to date, however integration occurs more often near common fragile sites[80, 132,121]. The integrated viral DNA always includes the p97 promoter

and the E6 and E7 genes[80, 132]. The remaining parts of the genome may be present, disrupted, mutated or completely deleted. The E2 gene which normally represses E6 and E7 expression is usually disrupted or completely removed. This results in uncontrolled and continuous expression of E6 and E7, allowing for an increase in their activity and immortalization of cells[132]. It has been suggested that E6 and E7 mRNAs that are expressed from integrated copies are much more stable and cells expressing E6 and E7 from integrated copies imparts a selective growth advantage[80]. This would allow for the replication and survival of infected cells which are more likely to become malignant.

Since E6 and E7 are constantly being expressed, and p53 and Rb are depleted, the chance that mutations are introduced into the genome is increased. These mutations may increase cell replication or improve HPV's ability to persist in the host. Finally, another element which contributes to progression to cervical cancer is the induction of host genomic instability by the E6 and E7 proteins. E7 is able to induce centrosome over-duplication and E6 allows the infected to cell to continue to replicate despite having centrosome abnormalities[132]. This could lead to aneuploidy, thereby accelerating malignant progression. In addition to this, E6 and E7 have been shown to induce the formation of anaphase bridges, which could result in chromosome abnormalities such as rearrangement, deletions or additions[132]. At this point, infected cells would be considered fully transformed. Fully transformed cells have an infinite life-span, increased migration capacity and are no longer anchorage dependent[89].

A review by Hanahan & Weinberg (2000) outlined a list of six characteristics which are acquired by cells during the development of tumorigenic cells in human cancers. These characteristics are: 1) self-sufficiency in growth signals, 2) insensitivity to growth-inhibitory signals, 3) evasion of programmed cell death, 4) limitless replicative potential, 5) sustained angiogenesis and 6) tissue invasion and metastasis[46]. If we look at HPV infected cells, they are self-sufficient in growth signals by continuing to stay in the cell cycle with a down regulation of Notch 1[131]. Through the degradation of pRb[11], HPV infected cells are no longer sensitive to inhibitory growth signals. HPV infected cells are able to evade apoptosis through degradation of p53 and Bax[103, 114]. Finally, HPV infected cells have limitless replicative potential through the induction of telomerase[63, 123]. While the two remaining characteristics may develop over an extended period of time, we can see that through HPV E6 and E7 protein interactions, cells infected with high-risk HPV would have most of the characteristics listed above. Therefore, given the rare occurrence of persistence and the abilities of HPV's oncoproteins to produce tumorigenic cells, it is evident how an HPV infection can become carcinogenic. In 2011, Hanahan & Weinberg updated their review to include two emerging hallmarks of cancer, i.e. deregulation of cellular energetics and evasion of immune destruction and two enabling characteristics, i.e. genome instability and tumor promoting inflammation[47]. As previously mentioned, E6 and E7 are capable of interacting with IRFs and therefore suppressing the antiviral response[92, 99]. In addition to this, during the increased replication of E6 and E7, mutations are introduced and both proteins are able to induce genomic instability[132]. These two characteristics reinforce the fact that HPV is

able to fulfill many of the characteristics considered to be essential in order for cancer to develop.

1.8. Rationale

While HPV has been extensively studied with respect to its viral protein interactions and differing oncogenic potential amongst different HPV types, little is known about *what* makes a specific HPV type or variant more carcinogenic than others. This project aimed to study two possible determinants of HPV-16 oncogenicity: HPV variants and the E6's interaction with MAGUK proteins.

Previous studies have proposed that different HPV variants are more oncogenic than others and that they are found more frequently in cervical cancer cases. Therefore, the goal was to study the viral sequences of specific regions of cervical cancer samples, which were HPV-16 positive, to determine if any mutations could be associated with an increased risk of cervical cancer. As a control, HPV-16 positive non-cancer samples from the general population in Manitoba were also sequenced to serve as a non-cancer control. It was hypothesized that mutations which confer a higher oncogenic potential would be found more commonly in cervical cancer specimens

In the second part of this work, the oncogenic role of the E6 PDZ-binding domain of HPV-16 was examined. Since only high-risk HPV E6 proteins are capable of interacting with the MAGUK proteins, this indicates that these interactions may be important in determining HPV's oncogenicity. Therefore, the goal was to determine which features of the high-risk E6 protein are important for E6-induced MAGUK protein degradation, more specifically with a focus on MAGI-1 degradation. It was hypothesized that specific

regions of the high-risk E6 will be required for E6-induced MAGUK protein degradation and would therefore be key determinants of HPV tumorigenesis.

1.9. Objectives

1) Sequence the LCR and E6 and E7 genes of Manitoba cervical cancer and Manitoba HPV-16 positive non- cancer samples to determine if any mutations can be associated with an increased risk of developing cervical cancer

2) Determine which regions of the HPV16 E6 protein are important for E6-induced MAGI-1 degradation (since this interaction may contribute to HPV oncogenicity) with the construction of different HPV16E6 mutants.

2.0. CHAPTER 1: HPV VARIANTS

2.1. Summary:

To look for mutations that may be associated with an increased risk of cervical cancer, the LCR and E6 and E7 ORFs of seventy-five archival cervical cancer samples from Manitoba and thirty-seven HPV-16 positive non-cancer samples were sequenced. The cervical cancer sequences of each region were compared to those of Manitoba HPV-16 non-cancer samples to distinguish which mutations were exclusive to cervical cancer. These sequences were subsequently aligned with the reference sequences (obtained from the National Centre for Biotechnology Information (NCBI)) of known HPV-16 variants. Phylogenetic analysis was used to identify the variants using published reference sequences.

This analysis of cervical cancer samples did not reveal any variant or specific mutations that were significantly associated with cervical cancer specimens in Manitoba. These results suggest that no high oncogenicity HPV-16 variants are circulating in Manitoba and that any one of the HPV-16 variants confer the same risk of malignant progression. This conclusion differs from previous studies which showed that HPV-16 variants with higher oncogenic potential were overrepresented in clinical cancer cases.

2.2. Introduction

HPV types can be further classified into variants[12, 72] and they are distinguished from one another by mutations found in the sequences of their E6 gene or the LCR. By definition, variants differ from each other by about 2% in coding genes and 5% in the LCR[98]. Variants of HPV-16 and HPV-18 were first studied in 1993. Ho *et al.* studied HPV-16 and found that the phylogenetic analysis of samples of from 25 different ethnic groups or geographical locations gave rise to 48 different variants[50]. They were able to construct a phylogenetic tree from LCR sequences that had five principal branches and were named for the geographical location from which most of the samples in that grouping were found. These variants groups were named: European, Asian-American, East-Asian, African 1 & 2[50]. However, there was considerable overlap in the geographical distribution of the variants. A similar study was conducted by Ong *et al.* (1993) for HPV-18 and three distinct branches could be seen: European, African and East Asian/American Indian[91]. Both these studies suggested that HPV evolved within the different populations and accumulated common mutations which are now common to each variant[72]. In 2012, Cornet *et al.* undertook a larger study of variant typing, in which 953 HPV-16 samples from 27 countries were studied. They used sequences from the E6 gene and LCR and they were able to distinguish 9 different HPV16 variants: European, Asian, African 1a, African 1b, African 2a, African 2b, North American, Asian American 1 and Asian American 2[14]. Since these variants carry mutations found in key regions of the genome involved in viral regulation (LCR) and carcinogenesis (E6) it has been suggested

that different variants may have different biological outcomes and that some variants may be more oncogenic than others.

Early studies first found that certain variants were more prevalent in cases of high grade lesions (precancerous lesions) or cervical cancer. In a study by Xi *et al.* (1997) female university students who were positive for HPV16 were grouped into two groups: those with prototype-like HPV16 variants i.e. grouped on the European branch and those with non-prototype HPV16 variants i.e. grouped on branches other than European. This was determined by LCR sequences. These women were then followed and observed for the development of precancerous lesions. It was found that women who were infected with non-prototype variants were 6.5 times more likely to develop precancerous lesions than those infected with prototypic variants[130]. In 1998, Zehbe *et al.* took samples from women with precancerous lesions and from women with cervical cancer and looked for variations in the E6 and E7 genes[133]. For the precancerous lesions, the distribution of E6 prototypic and non-prototypic variants was very uniform (56% non-prototypic, 44% prototypic). However, 94% of the cervical cancer samples had non-prototypic variant E6 sequences. They observed mutations in regions that were important for interactions with p53, as well in regions that could affect interactions with the host immune system. The most frequent variant was that in which the leucine residue at position 83 was mutated to a valine (L83V). They observed that this mutation was found alone or in combination with other mutations in 40% of the precancerous lesions and 88% of the cervical cancer lesions. They suggested that the presence of this mutation could be used to predict the risk of progression to cervical cancer.

The L83V mutation was also found linked to persistence of an HPV infection[43, 73]. Interestingly, a paper by Xi *et al.* (2006) hypothesized that the distribution and persistence of variants may be related to the ethnic background of the individual[129]. The study population consisted of 1025 women residing in the United States who were either HPV-16 or HPV-18 positive. Although different racial groups were identified, the paper put the most emphasis on the Caucasian and African-American populations and their variants because these groups made up most of the study population. They found that African American women were more likely to be infected with an African variant than a European variant and consequently, European women were more likely to be infected with a European variant than an African variant. When they looked at persistence, their results showed that variants appeared to persist more frequently in women whose racial background matched that of the variant, i.e. European variants persist longer in Caucasian women whereas African variants persist longer in African-American women. Other studies looking at persistence concluded that non-European variants were associated with persistence and progression to cervical cancer[124,106].

To determine if variants have different biological functions, studies looked at different properties known to contribute to HPV oncogenicity. Stoppler *et al.* (1996) looked at three HPV-16 E6 variants and their abilities to alter keratinocyte differentiation and induce p53 degradation in comparison to a reference E6 protein[111]. All the variants had mutations in the N-terminus i.e. the region important for p53 binding, with respect to the prototype E6 protein[16]. In their experiments they found that one variant was enhanced in its ability to alter keratinocyte differentiation compared to

the reference E6 protein whereas, the other two variants had equal or reduced abilities of altering keratinocyte differentiation. Interestingly, performance in this assay correlated with the abilities of the variants to induce p53 degradation. That being said, the variant which had enhanced abilities in altering keratinocyte differentiation also had the greatest ability to induce p53 degradation. A study by Kammer *et al.* (2000) looked at the LCR of different variants and compared the transcriptional activities of their p97 promoters[59]. After sequencing, the variants were found to belong to the African 1 & 2, Asian American and North American (which branches off the Asian American branch[14]) groups. They found that the Asian American and North American variants both had enhanced p97 promoter activity. Mutational studies were able to decipher that increased promoter activity was due to mutations in the 3' end of the LCR. This increase in promoter activity could greatly affect the biological outcome of a variant. More E6 and E7 proteins could be produced and therefore a more aggressive infection and perhaps a faster progression to cervical cancer could result. Another study looking at variants and their differing protein interactions, found that some variants showed comparable activities when they looked at properties such as p53 and Bax degradation. However, there was more variation when looking at their ability to bind the human discs large protein[71] (a proposed human tumor suppressor protein, discussed in section 3.2). Although this study could not find variants which were consistently enhanced or deficient in all interactions assayed, it was still informative in showing that variants can have differing biological activities when compared to that of prototype.

As mentioned previously, non-European variants seem to be more persistent and associated with progression to cervical cancer. This has been narrowed down to the Asian-American (AA) variant being more likely to progress to cervical cancer. A study by Berumen *et al.* (2001) showed that the frequency of the AA variant was 21 times higher in cervical cancer patients than in control patients and the odds ratio of cervical cancer associated with AA variants was higher than that of cervical cancer associated with European variants[4]. The mutation L83V which is found frequently in cervical cancer E6 protein is one of the mutations found in AA variants. In a study by Richard *et al.* (2010) an AA variant and a European variant in the presence of E7 were compared in their ability to immortalize and transform primary human foreskin keratinocytes (PHFKs)[98]. They found that the AA variant had a faster doubling time and that AA variants grew thicker raft cultures. However, when they looked at p53 degradation and telomerase activation, they were comparable. In *in vitro* transformation assays, only the AA variant was able to transform cells. In 2012, the same group looked at AA E6 activity without the presence of E7 compared to prototype E6[89]. They found that E6 alone could extend the lifespan of the cells and similar to the previous study, AA had a faster doubling time and could transform cells *in vitro*. In addition to this, they found that cells that had the AA variant had increased migratory capabilities compared to the prototype.

All of these studies confirmed that differences in biological functions can be observed between different variants. Therefore, it would be useful if specific mutations/ variants could be linked with the development of cervical cancer. During early detection

of HPV infection, the knowledge of which variant an individual is infected with could be helpful in determining the probability that infection could progress to cervical cancer.

In this study, the LCR and, when possible, the E6 and E7 genes of 75 HPV16 isolates from a study of archival cervical cancer specimens in Manitoba were sequenced, in order to identify possible variants or mutations associated with an increased risk of cancer.

2.3. Materials and Methods

2.3.1. Sample Population

Archival cervical cancer samples were obtained as a part of a study conducted in collaboration with the Cadham Provincial Laboratory in Manitoba. This study analyzed the HPV types present in cervical cancer specimens going as far back as 25 years, from all over Manitoba. Five hundred forty specimens were typed using an in-house Luminex typing method that detects 46 genital HPV types. Three hundred sixty-three samples were found to be HPV-16 positive. Seventy-five of those samples were used in this study.

As a control, non-cancer archival HPV-16 positive cervical specimens from Manitoba sent to the Viral Exanthemata and Sexually Transmitted Diseases Section at the National Microbiology Lab for confirmatory HPV typing were used.

2.3.2. DNA extraction of Paraffin Embedded Tissue Specimens

DNA was extracted from paraffin embedded tissue specimens by Cadham Provincial Lab using the following protocol: Buffer (50 mM KCl, 10 mM Tris- HCl pH 8.3, 2.5 mM MgCl2, 0.1 mg/ml gelatin, 0.45% Nonidet P40, 0.45%Tween 20, proteinase K) was added to specimen and incubated in a 65-70°C water bath for 2 hours. The protein kinase A was then inactivated by boiling the sample for 10 minutes. The paraffin was left to solidify and the aqueous phase was used as template for PCR reactions.

2.3.3 PCR amplification of the HPV-16 LCR and E6 and E7 ORFs from Manitoba Cervical Cancer & Manitoba HPV-16 non-cancer Samples

The primers listed in Table 2 were used to amplify each of the regions. The first round primers for LCR amplification were designed by Ho *et al* (1993)[50] , whereas the second round LCR and E6 and E7 primers were designed by the author. They were synthesized by the DNA Core Facility at the National Microbiology Lab in Winnipeg, Manitoba. The LCR was amplified using a nested PCR, while both the E6 and E7 ORFs were amplified by conventional PCR.

Table 2 – Primers used to amplify HPV-16 LCR, E6 and E7 ORFs

Region of HPV-16 genome	Primer name	Primer sequence
LCR	HPV16LCRR1-F	5'-CACCTACTAATTGTGTTGTGG-3'[50]
	HPV16LCRR1-R	5'-GTTTGCACACACCCATG-3'[50]
	HPV16LCRR2-F	5'-GGGGTACCTCGGTTGCATGCTTTTTGGC-3'
	HPV16LCRR2-R	5'-GGTCTAGACGGTTTGCACACACCCATGT-3'
E6	HPV16E6-F	5'-TAACCGAAATCGGTTGAACCGAAA-3'
	HPV16E6-R	5'-TTCATGCAATGTAGGTGTATCTCC-3'
E7	HPV16E7-F	5'-ATGTCTTGTTGCAGATCATCAAG-3'
	HPV16E7-R	5'-CATCCCGTACCCTCTTCCCCAT-3'

A plasmid containing the entire HPV16 genome was used as a positive control. All PCR reactions used 5ul of extracted DNA, 1mM $MgCl_2$, 200µM dNTPs (Invitrogen, Burlington,

Ontario), 10µM of each primer, 2.5U units of Taq DNA Polymerase (Invitrogen) and 10X PCR buffer (Invitrogen) diluted to 1X in a 50ul reaction. A Verti 96-well Thermal Cycler (Applied Biosystems/Life Techonolgies, Ontario) was used to carry out all PCR amplifications under the following conditions: initial denaturing step of 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. A final elongation step was done at 72°C for 7 minutes. All PCR products were kept at 4°C. Gel electrophoresis was used to confirm amplification of the correct insert. Following confirmation the PCR product was purified by running the sample through an Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-30 membrane (Millipore, MA, USA). Purified Sample was sent to the DNA Core Facility (National Microbiology Lab, Winnipeg, Manitoba) for sequencing. Both strands of DNA were sequenced.

2.3.4. Sequence Analysis and Phylogenetic Tree Construction

Sequences obtained were assembled using the SeqMan Pro software (DNASTAR Lasergene 10 Core Suite). Once all the contigs were assembled, they were put into the MEGA5[113] program, where they were trimmed to 364bp for the LCR, 477bp for the E6 gene and 297bp for E7. An alignment of the sequences was produced using the Clustal W option after which, a phylogenetic tree (Neighborhood-Joining Tree) was generated from the aligned sequences. The following GenBank accession numbers were used as reference sequences for each variant: AF472508 (African Type 1), AF472509 (African Type 2), AF02678 (Asian-American), AF534061 (East-Asian) and AF536179 (European).

Sequences were put through NCBI's nucleotide Basic Local Alignment Search Tool (BLAST) and compared to sequences in the Nucleotide collection (nr/nt) database.

2.4. Results

2.4.1. Analysis of the LCR in Cervical Cancer and Manitoba HPV-16 Non-Cancer Samples

Sequences of the LCR were analyzed first. The full length LCR is 832bp in length, however, only a 364bp portion of the LCR was analyzed, as this region was found to be the most variable[50] and it is most commonly used to define HPV-16 variants. This corresponds to the region from 7478-7841 of the complete HPV-16 genome. The sequences were aligned with reference sequences of the LCRs of each HPV16 variant and a Neighbour-Joining Tree was produced.

Of the 363 samples which were HPV-16 positive, the LCRs of seventy-five samples were analyzed. Nineteen variants were found; a list of mutations found in each sequence can be found in Table 3. The position of each mutation is given as its position in the whole HPV-16 genome. Also found in Table 3 are the accession numbers of the top results obtained when the sequence was put into BLAST.

Table 3 – Mutations Found in LCR sequences of Cervical Cancer Samples

Sample Number (Number of identical samples)	Mutation(s)	BLAST results (Accession Number of Top Result, Percent Identity)
36(1)	A7482G, C7568T, G7766A	KF466837.2, 99%
51	C7568T, G7797A	KF466839.2, 99%
57(1)	C7568T, A7728C	KF466801.2, 100%
58(2)	G7507C, A7519G, C7568T, C7790G	KF466769.2, 99%
59(1)	G7550A, C7568T	KF466819.2, 100%
62	C7568T, C7790A	KF466839.2, 99%
77	G7507C, C7568T, T7711G, A7828C	KF466828.2, 99%
83(4)	C7568T, T7711G, A7828C	KF466828.2, 99%
429	C7568T, C7784T, A7799C, G7840A	KF466839.2, 99%
449	C7568T, G7797C	KF466824.2, 100%
461	C7568T, C7667T, C7687A, A7727C, T7741G, C7762T, C7784T	KF466526.2, 99%
495	C7568T, C7579A	KF466839.2, 99%
510	A7483C, G7487A, A7505G, C7568T, C7667T, C7687A, A7727C, C7762T, C7784T	KF466533.2, 100%
533	C7568T, A7776G, A7781T, C7784T	KF466839.2, 98%
538	G7550A, C7568T, A7793C, C7794G	KF466819.2, 99%
551(15)	A7519G, C7568T	KF466837.2, 100%
552	A7519G, C7568T, A7791T	KF466837.2, 99%
572(1)	A7519G, C7568T, C7790T	KF466789.2, 100%
575(31)	C7568T	KF466839.2, 100%

Two large clusters of identical sequences were found when the sequences were aligned and put into a phylogenetic tree. The first cluster consisted of 32 samples which all had a C→T mutation at position 7568. The second cluster of 16 samples had mutations in positions 7519 (A→G) and 7568 (C→T). When these sequences were put into BLAST they were found to be 100% identical to sequences which were described previously. Similarly, when the other variants were put into BLAST they were 98-100%

identical to sequences which were previously described[14]. In regards to variant distribution, the majority of the samples (73 samples, 97%) belong to the European/East Asian group, while the remaining samples (2 samples, 3%) belong to the Asian American group. There were two sequences, 533 and 429 that did not group within any of the variant groups. When these sequences were put into BLAST, 533 was found to be 98% identical to a European variant, while 429 was 99% identical to an Asian variant.

As a control, 37 Manitoba HPV-16 samples from non-cancer cases were analyzed. Ten variants were found; a list of the mutations found in each sequence can be found in Table 4. Similar to the cervical cancer samples, the position of each mutation is given as its position in the entire HPV-16 genome. Also found in Table 4 are the accession numbers of the top results obtained when the sequence was put into BLAST.

Table 4 - Mutations Found in LCR sequences of Manitoba HPV-16 Non- Cancer Samples

Sample Number (Number of identical samples)	Mutation(s)	BLAST results (Accession Number of Top Result, Percent Identity)
71236(2)	C7568T, T7734G	KF466839.2, 99%
72920(1)	G7550A, C7568T	KF466819.2, 100%
HPV-08-315	A7519G, C7568T, G7797A, A7799C, T7812A, T7818A,	HQ644272.1, 99%
HPV-08-376(3)	A7483C, G7487A, C7568T, C7667T, C7687A, A7727C, T7741G, C7762T, C7784T	KF466526.2, 100%
HPV-08-377	A7519G, C7568T, C7790T	KF466789.2, 100%
HPV-08-504	C7568T, G7573A	AY453867.1, 100%
HPV-10-208	A7483C, G7487A, C7568T, C7667T, C7687A, C7762T, C7784T, G7824A, G7832T, A7835C, A7837G	KF466627.2, 100%
HPV-10-441	A7519G, C7568T, T7792A	KF466837.2, 99%
HPV-11-13(6)	A7519G, C7568T	KF466837.2, 100%
HPV-11-33(15)	C7568T	KF466839.2, 100%

Two large clusters of identical sequences were present. The first cluster consisted of 16 samples which all had a C→T mutation at position 7568. The second cluster of 7 samples had mutations in positions 7519 (A→G) and 7568 (C→T). When all the sequences were put into BLAS, they were found to be 99-100% identical to sequences which were described previously[14,107,13]. In regards to variant distribution, the majority of the samples (32 samples, 86%) belong to the European/East Asian group, four samples belong to the Asian-American group and one sample was in the African 2 variant group.

All the cervical cancer and Manitoba HPV-16 non-cancer samples were assembled into one tree to look at the overall distribution of the sequences (Figure 2).

Table 5 lists all the mutations found in all the LCR sequences and their distribution in the two sample populations.

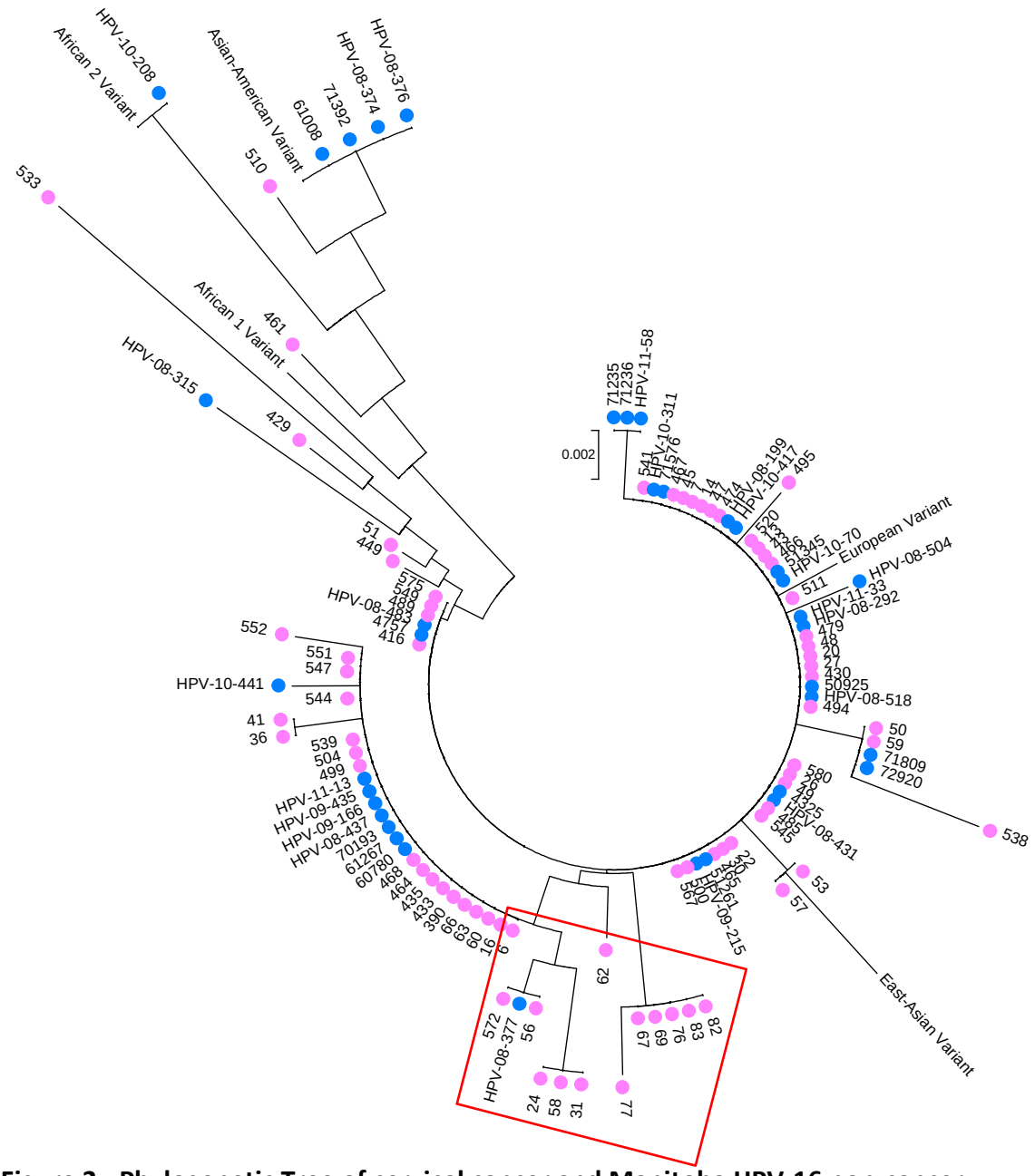

Figure 2 - Phylogenetic Tree of cervical cancer and Manitoba HPV-16 non-cancer Samples based on LCR Sequences. All cervical cancer and Manitoba HPV-16 non-cancer samples were combined into one phylogenetic tree to look at the distribution of the different sample groups. Samples highlighted with a blue circle are Manitoba HPV-16 non-cancer Samples; samples highlighted with a pink circle are the cervical cancer samples. Both sample groups are dispered similarly thoughout the phylogenetic tree. Sequences enclosed in the red box are those which contained mutations which were exclusive to cervical cancer samples.

Table 5 – Distribution of Mutations in LCR sequences of Cervical Cancer and Manitoba HPV-16 Non-Cancer Samples

Mutation	Number of Cervical Cancer Samples with mutation	Number of Manitoba HPV-16 Non- cancer Samples with mutation
A7482G	2	0
A7483C	1	5
G7487A	1	5
A7505G	1	0
G7507C	4	0
A7519G	22	10
C7568T	75	37
G7573A	0	1
C7579A	1	0
C7667T	2	5
C7687A	2	5
T7711G	6	0
A7727C	2	4
A7728C	2	0
T7734G	0	3
T7741G	1	4
G7750A	2	2
C7762T	2	5
G7766A	2	0
A7776G	1	0
A7781T	1	0
C7784T	4	5
C7790A	1	0
C7790T	2	1
C7790G	3	0
A7791T	1	0
T7792A	0	1
A7793C	1	0
C7794G	1	0
G7797A	1	1
G7797C	1	0
A7799C	1	1
T7812A	0	1
T7818A	0	1
G7824A	0	1
A7828C	6	0
G7832T	0	1
A7835C	0	1
G7840A	1	0

When looking at the mutations found in all the LCR sequences, four mutations were found exclusively in cervical cancer sequences. These mutations were G7507C, T7711G, C7790G, A7828C and they were present in 4, 6, 3 and 6 cervical samples, respectively. Interestingly, the samples which had these mutations all grouped together on the phylogenetic tree (Figure 2). However, when the BLAST sequences which were matched with these sequences were further analyzed, the E6 genes of the BLAST sequences were all identical to the prototype. This suggests that these samples would not be more oncogenic than the prototype virus.

This being said, for the most part, each sample group showed a similar distribution throughout the phylogenetic tree. The two most common mutations which were found in the LCR of cervical cancer samples were also found to be the most common in the Manitoba HPV-16 samples. This also proved to be true with other mutations found in the LCR of cervical cancer samples. Thus, in our analysis of the LCR, no mutations could be associated with an increased risk of cervical cancer.

2.4.2. Analysis of the E6 gene sequences in Cervical Cancer and Manitoba HPV-16 Non-Cancer Samples

To further analyze sequences of cervical cancer samples, the E6 open reading frame (ORF) was investigated next. Experiments have shown E6's role and importance in HPV's oncogenicity[63, 82, 103, 109, 114, 123, 131]. In addition to this, E6 variants are capable of having different biological activities[71, 89, 98, 111]. Therefore, it would be interesting to determine if any common mutations in this region could be found in the cervical cancer samples. Similar to the previous section, E6 cervical cancer sequences were compared to E6 sequences from general Manitoba HPV-16 samples. The entire E6 gene (477bp) was sequenced (positions 83-559 in the HPV genome). The sequences were aligned with E6 reference sequences of each HPV16 variant and a Neighbour-Joining phylogenetic tree was produced. It is important to note that not all the cervical cancer samples which were sequenced in the previous section could be sequenced for the E6 ORF. This was due to either a lack of sample or failure in amplification because of DNA degradation.

The E6 genes of 26 cervical cancer samples were sequenced and analyzed. Nine variants were found; a list of mutations found in each sequence can be found in Table 6. The position of each mutation is noted as its position from the start of the E6 ORF. Also included in Table 6 are the amino acid substitutions which result from the mutations in the E6 sequence, as well as the accession numbers of the top results obtained when the sequence was put into BLAST.

Table 6 - Mutations Found in E6 sequences of Cervical Cancer Samples

Sample Number (Number of identical samples)	Mutation(s)	Amino Acid Substitution(s)	BLAST results (Accession Number of Top Result, Percent Identity)
7(2)	---		AF536179.1, 100%
31(1)	G49A, A294T	G17R, Q98H	AB663704.1, 99%
41	G49A, G350T, G543A	G17R, V90L, R154K	AB818691.1, 99%
57	G350T	V90L	AB818691.1, 99%
461	G49A,G63T,T204A, A207G, C253T, A450G	G17R, H85Y	JQ004098.1, 100%
466	G49A, A145G, G461A	G17R, I49V, R154K	AB663704.1, 99%
479	G49A,A80G	G17R, Q27R	AB663704.1, 99%
533(3)	G49A	G17R	AB663704.1, 100%
552(11)	G49A, G268T	G17R, V90L	AB818691.1, 100%

The most common mutation, which was found in 22 (85%) samples, was a G→A mutation at position 49. This resulted in a G→R amino acid change at position 17. The second most common mutation which was found in 14 (54%) samples was a G→T mutation at position 268. This resulted in a V→L amino acid change at position 90. A cluster of 12 identical samples had both of the above mentioned mutations. When the sequences were put into BLAST, they were either 99 or 100% identical to E6 sequences which were previously described[55,67]. All but one sample was grouped as European/East-Asian.

The E6 genes of 23 Manitoba HPV-16 samples from non-cancer cases were sequenced and analyzed. Seven variants were found; a list of mutations found in each sequence can be found in Table 7. The position of the each mutation is noted as its position from the start of the E6 ORF. Also included in Table 7 are the amino acid

substitutions which result from the mutations in the E6 sequence, as well as the accession numbers of the top result obtained when the sequence was put into BLAST.

Table 7 - Mutations Found in E6 sequences of Manitoba HPV-16 Non-Cancer Samples

Sample Number (Number of identical samples)	Mutation(s)	Amino Acid Substitution(s)	BLAST results (Accession Number of Top Result, Percent Identity)
HPV-08-86	---	---	AF536179.1, 100%
HPV-08-376(2)	G49A, G63T, T204A, A207G, C253T, A450G	G17R, Q21H, H85Y	JQ004098.1, 100%
HPV-10-208	T27C, G49A, G50T, C61G, T204A, A207G, C253T, G268T, A321G	G17I, Q21E, H85Y, V90L	KC904909.1, 100%
HPV-10-417	T27C,G49A	G17R	JQ067944.1, 100%
HPV-10-441(8)	G49A, G268T	G17R, V90L	AB818691.1, 100%
HPV-11-33(6)	G49A	G17R	AB663704.1, 100%
HPV-11-58	G268T	V90L	AB818691.1, 99%

The most common mutation, which was found in 21 (91%) samples, was a G→A mutation at position 49. This resulted in a G→R amino acid change at position 17. The second most common mutation which was found in 11 (48%) samples was a G→T mutation at position 268. This resulted in a V→L amino acid change at position 90. Two clusters of identical sequences were present. The first had 9 samples, which had both of the mutations mentioned above. The other cluster was of 7 samples, which had only the G49A mutation. When the sequences were put into BLAST, all but one sequence was 100% identical to E6 sequences that were previously described[55, 67, 101]. The majority of

the samples belong to the European/East-Asian group, while 3 were in the Asian-American group and one was in the African 2 group.

All the cervical cancer and Manitoba HPV-16 E6 sequences were assembled into one tree to look at the overall distribution of the sequences (Figure 3). Table 8 lists all the amino acid substitutions in the E6 sequences and their distribution in the two sample populations.

Table 8 – Distribution of Amino Acid Substitutions in E6 sequences of Cervical Cancer and Manitoba HPV-16 Non-Cancer Samples

Amino Acid Substitution	Number of Cervical Cancer Samples with mutation	Number of Manitoba HPV-16 Non- cancer Samples with mutation
G17I	0	1
G17R	21	20
Q21E	0	1
Q21H	0	3
Q27R	1	0
I49V	1	0
H85Y	1	4
V90L	14	11
Q98H	2	0
R154K	2	0

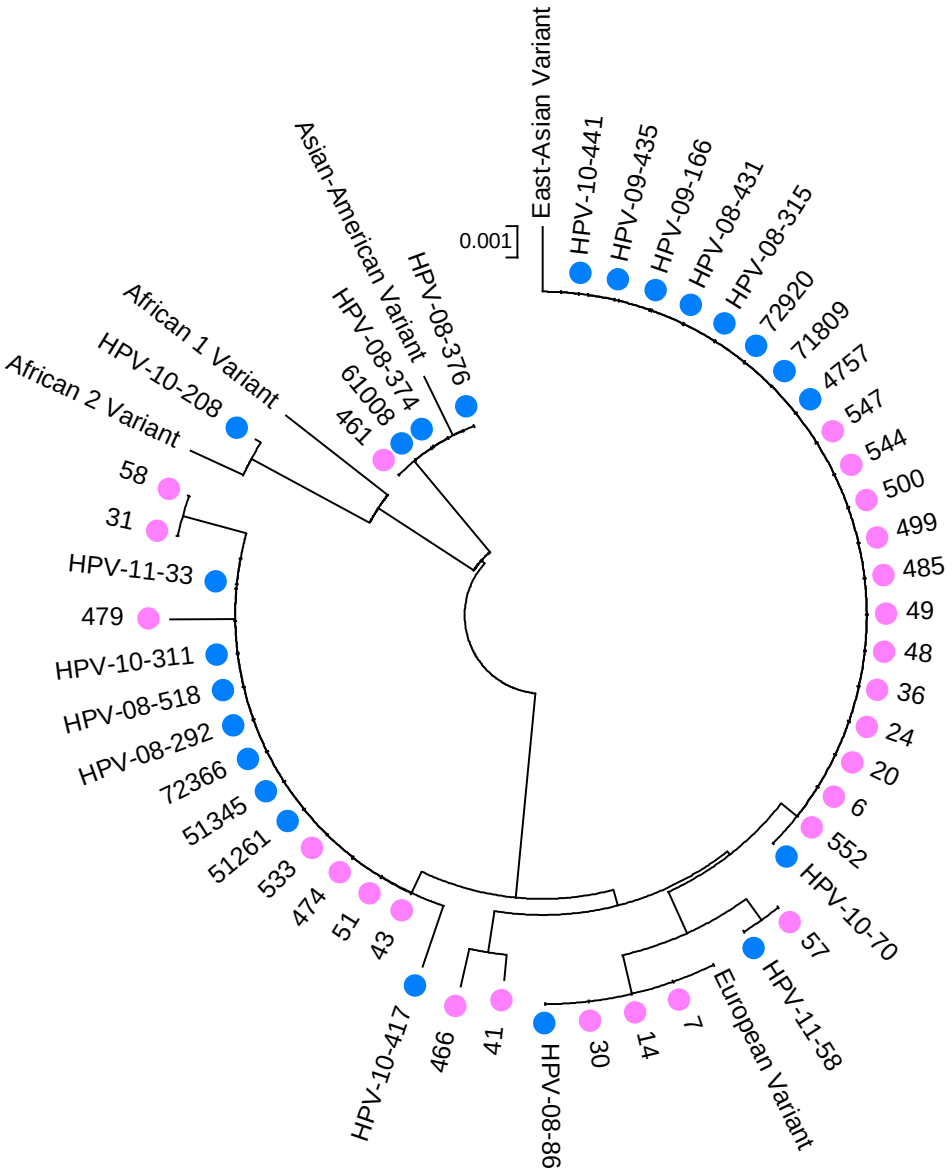

Figure 3 - Phylogenetic Tree of cervical cancer and Manitoba HPV-16 Non-cancer Samples based on E6 Sequences. All cervical cancer and Manitoba HPV-16 Non-Cancer E6 sequences were combined into one phylogenetic tree to look at the distribution of the different sample groups. Samples highlighted with a blue circle are Manitoba HPV-16 non-cancer samples; samples highlighted with a pink cricle are the cervical cancer samples. Both sample groups are dispered similarly thoughout the phylogenetic tree.

Each sample group showed a similar distribution throughout the phylogenetic tree. This is similar to what was observed for the LCR sequences. Again, the most common mutations were observed in both the cervical cancer and Manitoba HPV-16 samples. Any other mutations found only in cervical cancer specimens were only present in one or two samples. Therefore, in this analysis of the E6 ORF of cervical cancer samples, no mutations were associated with cervical cancer.

2.4.3. Analysis of the E7 gene sequences in Cervical Cancer and Manitoba HPV-16 Non-Cancer Samples

The last region of the HPV-16 genome that was analyzed was the E7 gene. Since this region encodes the other oncogenic HPV-16 protein, any mutations in this region could again affect HPV's biological activity. Similar to the previous sections, E7 cervical cancer sequences were compared to E7 sequences from Manitoba HPV-16 non-cancer samples. The entire E7 gene (297bp) was sequenced, which corresponds to positions 562-858 of the entire HPV genome. The sequences were aligned with E7 reference sequences of each HPV16 variant and a Neighbour-Joining phylogenetic tree was produced. Similar to the E6 analysis, not all the samples for which the LCR was sequenced could be sequenced for the E7 gene. This was due to either a lack of sample, or failure in amplification because of DNA degradation.

The E7 genes of 20 cervical cancer samples were sequenced and analyzed. Five variant sequences were found; a list of mutations found in each sequence can be found in Table 9. The position of each mutation is noted as its position from the start of the E7 ORF. Also included in Table 9 are the amino acid substitutions that result from the mutations in the E7 sequence, as well as the accession numbers of the top result obtained when the sequence was put into BLAST. All the samples were either 99 or 100% identical to E7 sequences which were previously described. The majority of the samples were identical to the prototype (European) E7 gene. Only one sample was identical to the Asian-American variant.

Table 9- Mutations Found in E7 sequences of Cervical Cancer Samples

Sample Number (Number of identical samples)	Mutation(s)	Amino Acid Substitution(s)	BLAST results (Accession Number of Top Result, Percent Identity)
390	T126C	---	KC736931.1, 99%
461	T171, T228C, T234G	---	JQ004098.1, 100%
468	G119T	G40V	KC736931.1, 99%
545	C151A, G223T	N51H, Y75D	KC736931.1, 99%
547(15)	---	---	KC736931.1, 100%

The E7 genes of 22 Manitoba HPV-16 samples from non-cancer cases were sequenced and analyzed. Four variants were found; a list of mutations found in each sequence can be found in Table 10. The position of each mutation is noted as its position from the start of the E7 ORF. Also included in Table 10 are the amino acid substitutions that result from the mutations in the E7 sequence, as well as the accession numbers of the top result obtained when the sequence was put into BLAST. All of the sequences were 100% identical to E7 sequences that were previously described. The majority of the samples were identical to the prototype (European) E7 gene. Four samples were identical to the Asian-American E7 gene.

Table 10- Mutations Found in E7 sequences of Manitoba HPV-16 Non-Cancer Samples

Sample Number (Number of identical samples)	Mutation(s)	Amino Acid Substitution(s)	BLAST results (Accession Number of Top Result, Percent Identity)
71235	C151A	N51H	KC736931.1, 100%
HPV-08-376 (3)	T171C, T228C, T234G	---	JQ004098.1, 100%
HPV-10-417(15)	---	---	KC736931.1, 100%
HPV-11-33	T117C	---	JX073664.1, 100%

All the cervical cancer and Manitoba HPV-16 sequences were assembled into one tree to look at the overall distribution of the sequences (Figure 4). Each sample group showed a similar distribution throughout the phylogenetic tree. Table 11 lists all the amino acid substitutions in the E7 sequences and their distribution in the two sample populations.

Overall the E7 ORF was less variable than the LCR and E6 ORF, as most of the samples were identical to the prototype (European) E7. Similar to results obtained from the LCR and E6 sequence analysis, no mutations were exclusive to the cervical cancer samples in the E7 ORF.

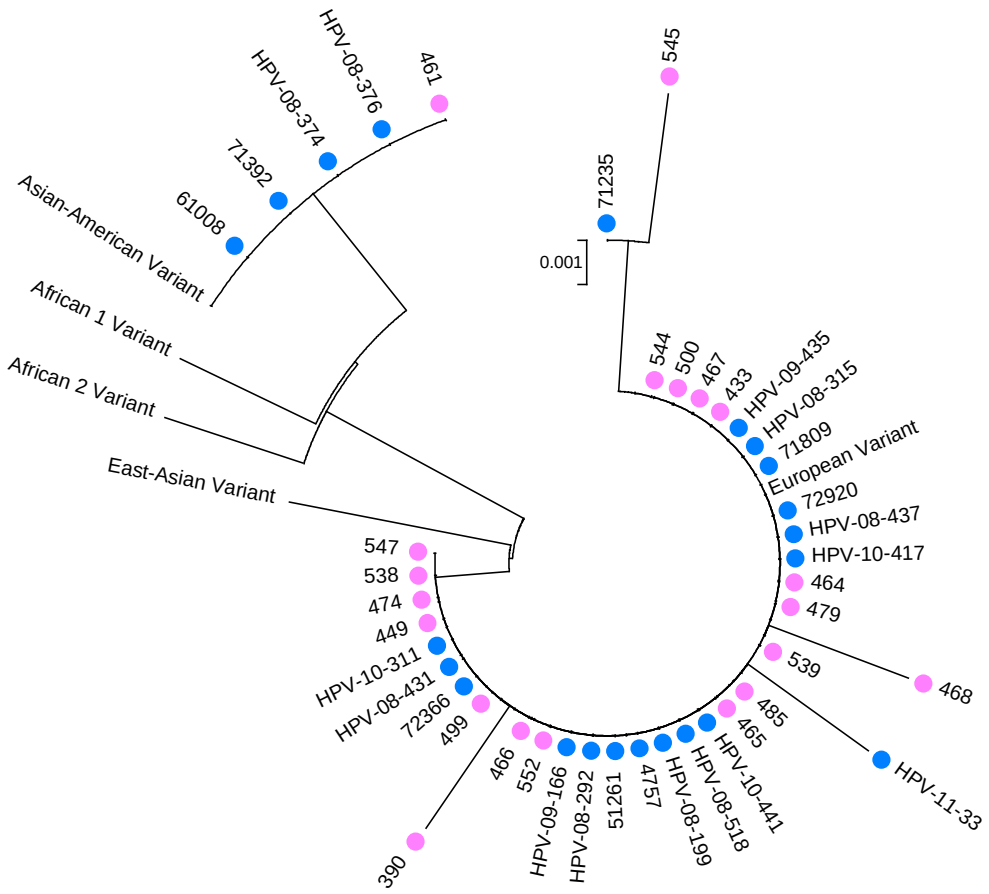

Figure 4 - Phylogenetic Tree of cervical cancer and Manitoba HPV-16 Non-Cancer Samples based on E7 Sequences. All cervical cancer and Manitoba HPV-16 non-cancer E7 sequences were combined into one phylogenetic tree to look at the distribution of the different sample groups. Samples highlighted with a blue circle are Manitoba HPV-16 non-cancer samples; samples highlighted with a pink circle are the cervical cancer samples. Both sample groups are dispered similarly thoughout the phylogenetic tree.

Table 11 – Distribution of Amino Acid Substitutions in E7 sequences of Cervical Cancer and Manitoba HPV-16 Non-Cancer Samples

Amino Acid Substitution	Number of Cervical Cancer Samples with mutation	Number of Manitoba HPV-16 Non- cancer Samples with mutation
G40V	1	1
N51H	1	0
Y75D	1	0

2.5. Discussion

The goal of this work was to determine if any mutations in HPV-16 could be associated with the development of cervical cancer. The analysis consisted of sequencing the LCR, and the ORFs of the E6 and E7 genes of samples obtained from cervical cancer patients in Manitoba. These regions were chosen in particular because of their contribution to viral regulation and oncogenicity. Therefore, any differences in sequence could result in enhancement or impairment of viral expression, and ultimately, the biological outcome of infection. In the case of the cervical cancer samples studied here, any mutations exclusive to cervical cancer could be important in determining a variant's ability to progress to cervical cancer. To determine which mutations are exclusive to cervical cancer, sequences of HPV-16 positive samples from non-cancer cases from Manitoba were also examined. The non-cancer samples will show which mutations are normally found in Manitoba HPV-16 positive samples. Therefore, any mutations found in both cervical cancer and Manitoba HPV-16 non-cancer cannot be considered to be associated with HPV's carcinogenicity. The analysis performed here found that no mutations in any of the regions studied were associated with cervical cancer. Many of the mutations found in the cervical cancer samples could also be found in the Manitoba HPV-16 samples. There were four mutations in the LCR which were exclusive to cervical cancer samples. However, since these samples represent a small proportion of the cervical cancer sample population, these mutations cannot be confirmed as being associated with an increased risk of cervical cancer. In addition to this, when the BLAST sequences that were matched with these sequences

were further analyzed, it was found that the E6 genes of the BLAST sequences were all identical to prototype. Thus, this suggests that these samples would not be more oncogenic than the prototype virus. Despite this, other observations can be made in regards to the variants present in both sample sets examined.

When samples from both sample sets were put into BLAST, they were all 98-100% identical to sequences which were previously described[13, 55, 67, 101, 107]. This shows that in this study of Manitoba HPV-16 positive samples no unique sequences were found in any of the regions and that these sequences are comparable to those found world-wide.

In previous studies, the L83V mutation in the E6 ORF alone or in combination with other mutations was associated with progression to cervical cancer[130,133]. In the study presented here, this same mutation was identified as L90V. This is because in some studies, the beginning of the E6 ORF is considered to be at the second methionine. Nonetheless, looking at the E6 sequences in this study, this mutation was found in both cervical cancer and Manitoba HPV-16 samples. Also, for these findings to be in agreement with other studies, the majority of the cervical cancer samples should have this mutation. In the study by Zehbe *et al.* (1998), 88% of the cervical cancer lesions contained the L83V mutation. However in the study presented here, approximately half of the cervical cancer (54%) and half of the Manitoba HPV-16 (48%) have this mutation. Indicating that in this analysis, the L90V mutation cannot be associated with an increased risk of cervical cancer.

The most abundant variant present in the two sample sets examined was the European variant. This is in agreement with previous studies that also found the European variants were the most common in their study populations[14, 124, 130]. In an unpublished study done on HPV-16 variants in the Northwest Territories in Canada, the European variant was the most abundant (212 samples out of 244 sequenced). Some studies have stated that non-European variants are more prevalent in cervical cancer[106, 124], with others focusing specifically on the Asian-American variant[4, 89, 98]. Berumen *et al.* (2001) found that in their study population of cervical cancer samples, the Asian-American variants occurred at a higher frequency than the European variant[4]. In the cervical cancer samples examined here, only one sample was grouped as Asian-American, whereas in the Manitoba HPV-16 samples 4 samples were Asian-American. Therefore in this study, Asian-American variants did not occur at a higher frequency in cervical cancer samples and therefore, is not associated with cervical cancer in Manitoba.

One of the limitations of this study was that sequences for all the regions of every sample were not obtained. However, the few samples with sequences obtained for all of the regions studied showed that sequencing of all the regions may not be necessary to be able to determine to which variant group a sample belongs. For example, the sample HPV-08-376 (Manitoba HPV-16 sample) sequences for all regions studied were obtained. In each of the separate gene alignments and classifications, HPV-08-376 was consistently classified as Asian-American. This indicates that perhaps only one region needs to be sequenced in order for a variant to be classified. However,

this may only be the case for variants that are not European/East Asian. Since the East-Asian variants, branches off from within the European branch, there may be some discrepancies when classifying a variant from one region. For example, in the cervical cancer set, sample 500 was classified as European from the LCR and E7 sequence, but classified as East-Asian from the E6 sequence. Therefore, further analysis may be required for those variants that are classified as European using only one region. Despite this, the trees for each region are congruent with one another and only one region needs to be sequenced for the variant typing of future samples.

Although this study did not find any mutations that could be associated with cervical cancer, it provided an idea of the distribution of HPV-16 variants found in Manitoba cervical cancer samples. Also, it showed that there are no highly oncogenic variants circulating in Manitoba. It would be interesting to sequence a larger number of cervical cancer samples in order to get a better idea of which variants are present in Manitoba. This would also present the opportunity to see if the four mutations, which were found to be exclusive to cervical cancer samples in the LCR, can be found in other cervical cancer samples. Assuming that these mutations are found to be exclusive in cervical cancer, these mutations could serve as markers of HPV-16 variants which are more likely to progress into cervical cancer. When a woman is found to be HPV-16 positive, the LCR could be sequenced. If these mutations are detected, a woman would be observed more closely for persistence of the infection and development of pre-cancerous lesions. This would be done in the hopes of preventing the development of cervical cancer.

Furthermore, although according to their DNA sequences, the cervical cancer samples may not differ drastically from non-oncogenic HPV DNA, it would be interesting to see how some of these variants perform in functional assays like those described in section 2.2. As all these variants did progress to cervical cancer, they must have some advantage over non-oncogenic variants and this should be explore. These experiments could show that only some of the activities are upregulated in these variants. However, this could also tell us which activities are more important with respect to determining HPV oncogenicity.

3.0. CHAPTER 2: E6 AND MAGUK PROTEINS

3.1. Summary:

To determine which regions of the HPV16E6 protein are important in induction of MAGI-1 degradation, several HPV16E6 mutants were constructed. These mutants were *in vitro* translated and the *in vitro* translated proteins were used in individual MAGI-1 degradation assays. With these degradation assays, the MAGI-1 degradation abilities of wild-type HPV16E6 and HPV6E6 proteins were confirmed. By removing the PDZ-binding domain from HPV16E6 and in a separate experiment, adding it to the C-terminus of HPV6E6, this region was found to be necessary but not sufficient to induce MAGI-1 degradation. Using chimeric HPV16/6 E6, it was demonstrated that domains along the entire HPV16E6 protein are needed for MAGI-1 degradation. Finally, it was determined that mutations that affect p53 degradation also affect MAGI-1 degradation, suggesting that the degradation of both proteins occurs through a common mechanism.

3.2. Introduction

As described in the introduction above, there are protein interactions exclusive to high-risk E6 proteins that are proposed to contribute to high-risk E6's oncogenicity[63, 114, 115, 123, 131]. An additional feature unique to the E6 protein of high-risk HPV is the ability to interact with a family of proteins called the Membrane Associated Guanylate Kinases (MAGUK)[119]. MAGUK proteins are scaffolding proteins found mostly at cell-cell junctions[25,93]. They have a core structure of a PDZ binding domain, a SH3 domain and a GUK (guanylate kinase) domain. Through these domains they are able to interact with several different proteins and aid in the assembly of protein complexes[25]. Their roles in cell junctions are to act as scaffolds to maintain the structure of cell-junctions or they can recruit and organize other proteins to allow the transduction of signals between cells[25]. Only a subset of MAGUK proteins have been found to interact with high-risk E6 proteins and they are able to do this via the PDZ domain of the MAGUK protein[120]. The PDZ domain is named for the first three proteins in which the domain was found, PSD95/Dlg/ZO-1[25]. This domain is a stretch of 80-100 amino acids which recognize the sequence: X-T/S-X-V, usually found at the C-terminus of their target protein[120]. All high-risk E6 proteins have conserved this sequence at their C-terminus (Figure 5).

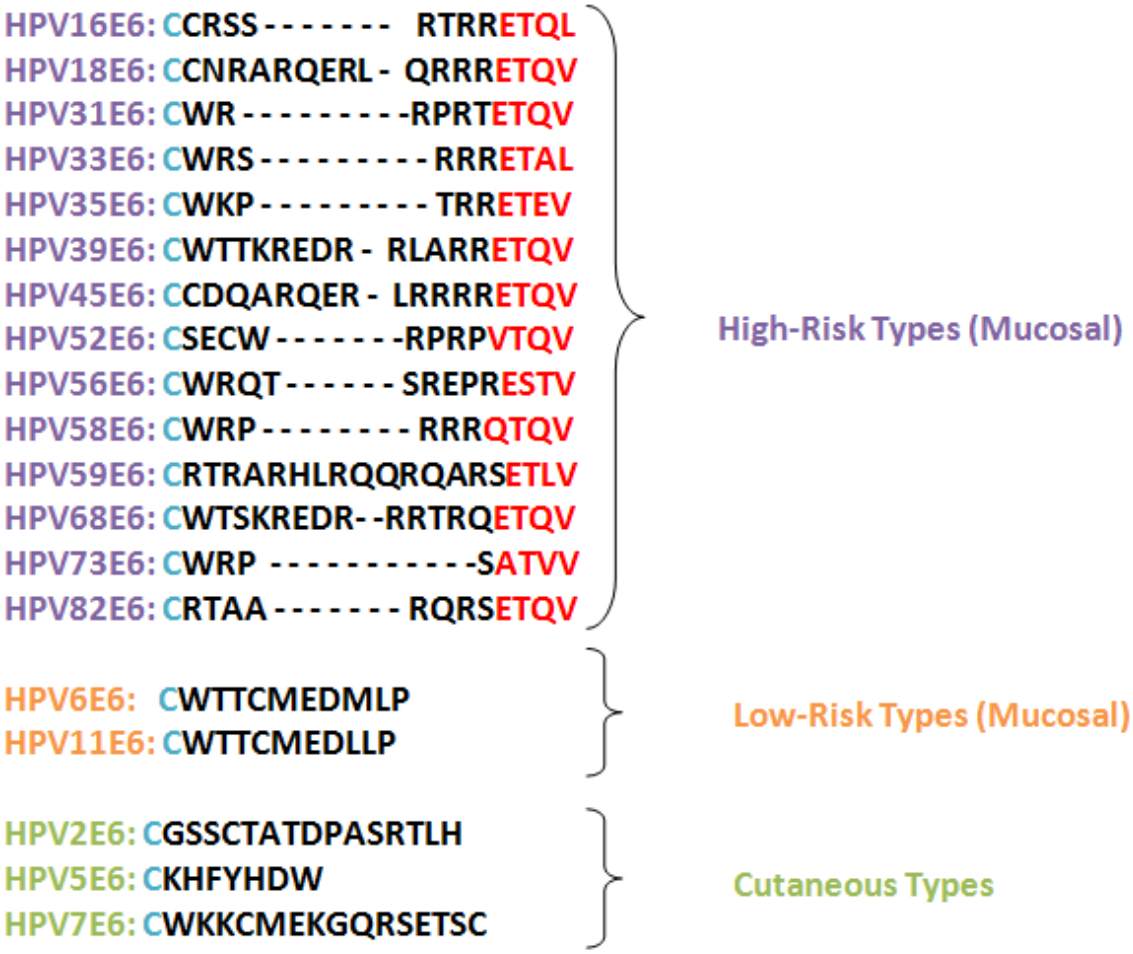

Figure 5 – C-terminal sequences of E6 proteins from different HPV types. Sequences from different HPV types were trimmed beginning at a conserved cysteine residue. The high–risk HPV types have a conserved sequence highlighted in red. This is known as the PDZ-binding domain used to interact with PDZ domain containing proteins. Low-risk, as well as cutaneous types, do not have this PDZ-binding domain and are therefore not capable of these interactions.

Therefore, it is due to the presence of this PDZ-binding domain in high-risk E6 proteins and lack thereof in the low-risk or cutaneous E6, that this interaction is suggested to be connected to high-risk E6's oncogenicity[120]. As a result of the interaction between E6 and MAGUK proteins, the MAGUK proteins are subjected to proteasomal degradation via the ubiquitin-dependent pathway[120], similarly to what happens for p53.

There are a few MAGUK proteins which have been identified as binding partners of high-risk E6 proteins. Most extensively studied are hDLG, hSCRIB, and MAGI-1, -2, -3. hDLG and hSCRIB were the first two MAGUK proteins found to interact with the HPV E6 protein[37, 62, 87]. They are both human homologues of tumor suppressor proteins found in *Drosophila melanogaster* and they are found at tight junctions of cells. In *Drosophila,* mutations in the genes encoding Dlg and Scrib results in the disruption of cell organization and differentiation[62, 87]. Since these proteins have such an effect in *Drosophila*, it is proposed that these proteins may also be tumor suppressors in humans. Both hDlg and hScrib have been found to be degraded in the presence of the HPV E6 protein. Following the discovery of the interaction of E6 with the above proteins, studies aimed to identify other MAGUK proteins which also interact with E6 and this led to the discovery of MAGI-1,-2,-3. These proteins are also found at tight junctions and their degradation is also induced by E6 [41, 65, 119].

Studies looking at these interactions have found that some E6 proteins from different HPV types interact more efficiently with the MAGUK proteins than others. A study by Pim *et al.* (2000) showed that HPV18E6 was more efficient at targeting Dlg than HPV16E6[95] and Thomas *et al.* (2001) found this was also true for the MAGI-1 protein[118]. They found that the difference in affinity was due to the difference in the terminal amino acid, which is valine for HPV18 and leucine for HPV16 (Figure 5). HPV18E6 has the perfect consensus sequence for binding to the PDZ domains. A study by Zhang *et al.* (2007) solved the crystal structure of E6 bound to the PDZ domains of MAGI-1 and hDlg and found that the leucine residue of HPV16E6 weakens interactions between E6 and

the PDZ domain which explains why HPV16E6 has a weaker affinity[135]. This study also showed that it is not only the four peptides (X-T/S-X-V) on the C-terminus of E6 that are required for PDZ domain binding. They showed that the arginine residues found upstream are also essential. Further to this study, Thomas *et al.* (2008) found that mutations in this upstream region which were found to affect the degradation of one MAGUK protein could also have no effect on another. Therefore, they proposed that binding specificities of different HPV types could be determined by the other residues found upstream of the PDZ-binding domain and this could be used to predict the binding specificities of other E6 proteins[117]. It is important to note that binding of MAGUK proteins by E6 does not ensure they are targeted for degradation, as was demonstrated by Pim *et al.* (2002)[94]. In their experiments, they added the last seven amino acids of HPV16E6 (TRRETQL) to low-risk HPV types and found that this gave them the ability to bind to hDlg and MAGI-1[94]. However, only hDlg degradation could be observed in degradation assays using the mutated low-risk E6 proteins.

Additional studies have looked at what mechanism is used to degrade the MAGUK proteins. HPVE6 is capable of degrading p53 via an ubiquitin-dependent pathway through its interaction with the ubiquitin ligase E6-AP, therefore scientists questioned if this also occurred with MAGUK proteins. The results of several studies are conflicting. Matsumoto *et al.* (2006) found that E6-AP is required for MAGUK protein degradation of Dlg *in vitro* and in murine and human cells[77]. Kuballa *et al.* (2007) showed that the ability of HPV16E6 mutants to degrade p53, hDlg and hScrib correlated with their ability to interact with E6-AP[66]. In contrast, studies by Massimi *et al.* (2008) and

Grm & Banks (2004) found that in E6-AP null conditions (E6-AP null mutant cell line, or following an E6-AP immunodepletion assay, respectively), MAGUK proteins were still degraded in the presence of the E6 protein[42, 76]. This indicates that perhaps another ubiquitin ligase was present. A study by Thomas *et al.* (2001) which looked at the degradation of p53, MAGI-1 and hDLG induced by different E6 proteins made an important note; the time it took to degrade p53 and MAGI-1 was similar, however the time it took degrade hDlg was longer, suggesting that they are degraded using two different mechanisms[118]. The question of which ubiquitin ligase is used to induce the degradation of MAGUK proteins has yet to be answered.

The importance of the interaction of E6 and MAGUK protein has been studied using mutants of E6. A study by Kiyono *et al.* (1997) found that rat fibroblasts (3Y1) transduced with a mutant E6 that lost the ability to interact with hDlg remained as a monolayer culture and cobblestone shaped. In contrast, wild-type E6 and mutants that retained the ability to interact with Dlg became spindle-like cells and were able to grow as multilayered sheets of cells. They transduced these same mutants into mouse fibroblasts, and then injected the cells into nude mice were they found that interactions with hDlg correlated with the development of tumors[62]. Nguyen *et al.* (2003) found that transgenic mice that expressed an E6 protein lacking the PDZ-binding domain did not display epithelial hyperdysplasia – a feature of cellular transformation. These results suggested that the presence of a functional PDZ-domain was important for the transformation of cells and that interaction with hDlg was important in the process[88].

Experiments done in human keratinocytes showed similar results. Watson *et al.* (2003) showed that cells transduced with wild-type E6 or a mutant, which had increased Dlg degradation activity, showed a more elongated and fibroblast-like morphology. These cells also did not have tight contact with neighbouring cells[126]. Spanos *et al.* (2007) found that cells transduced with E6 lacking a PDZ-binding domain could not grow as well as cells transduced with wild-type E6 in anchorage independent growth experiments[110]. Lee & Laimins (2004) found that in the context of the whole genome a mutation of E6's PDZ-binding domain had an effect on growth rate of the cells and altered the morphology of differentiating cells[69].

It has been proposed that the biological significance of the interaction of E6 and MAGUK lies in the fact that the MAGUK proteins play an important role in tight junctions and this is abolished when degradation of the proteins occurs due to the presence of the E6 protein. Disruption of the tight junctions could result in the loss of cell-cell adhesion and cell polarity. Without the presence of these proteins in the cell junctions, important signal transduction pathways may be interrupted, affecting cell proliferation and differentiation. All of these factors could potentially play a role in E6's ability to push infected cells into malignancy.

The goal of this study was to determine what features of HPV-16 E6 protein are important for E6-induced degradation of the MAGI-1 protein since this interaction may contribute to its oncogenicity.

3.3. Materials & Methods

3.3.1. Cloning of HPV E6 Genes into an Expression Vector

3.3.1.1. Generation of E6 Inserts by Polymerase Chain Reaction (PCR)

Plasmids made in-house containing the HPV6 genome or the HPV16 E6 genome of a splice donor mutant were used as template DNA for PCR reactions. Although the plasmid containing the HPV16 E6 gene had a mutation which abolished the E6 splice donor site, it will be referred to as "wild-type". Abolishment of the E6 splice site ensures only full length E6 protein will be produced. A MAGI-1 cDNA clone purchased from Origene (Rockville, Maryland) was also used as template DNA in the PCR reaction to generate the MAGI-1 insert to be cloned. Primers found in Table 12 were designed by the author and used to amplify the various E6 wild-type and mutant genes, as well as the sequence encoding the MAGI-1 protein. The primers were synthesized by the DNA Core Facility at the National Microbiology Laboratory in Winnipeg, Manitoba. The sense primers include a BamHI restriction site at the 5' end, whereas the antisense primer include a NotI restriction site. These restrictions sites were used to clone the insert into the expression vector. Additional nucleotides were also added to each primer to improve the restriction digestion reaction.

Table 12 – Primer Sequences for HPV-6, HPV-16 and MAGI-1 Constructs

Name of Construct and Brief Description	Primer name Sense, Antisense	Primer sequence*
HPV16E6SM - Wild-type HPV16E6 with mutation at splice donor site	HPV16E6SM-F	5'-*ATC GCA* GGA TCC ATG CAC CAA AAG AGA ACT G-3'
	HPV16E6SM-R	5'-*GTA CTA* GCG GCC GCT TAC AGC TGG GTT TCT CTA CG-3'
HPV16E6SMNT - Above construct with PDZ-binding domain removed	HPV16E6SMNT-F	5'-*ATC GCA* GGA TCC ATG CAC CAA AAG AGA ACT G-3'
	HPV16E6SMNT-R	5'-*GTA CTA* GCG GCC GCT TAA CAA GAC ATA CAT CGA CCG GT-3'
HPV6E6 – wild-type HPV6E6	HPV6E6-F	5'-*ATC GCA* GGA TCC ATA TAA ACC AGC CCT AAA T-3'
	HPV6E6-R	5'-*GTA CTA* GCG GCC GCG GGT CTG GAG GTT GCA GGT CTA AT-3'
HPV6E6+PDZ – above construct with HPV16E6 PDZ-binding domain added	HPV6E6+PDZ-F	5'-*ATC GCA* GGA TCC ATA TAA ACC AGC CCT AAA T-3'
	HPV6E6+PDZ-R	5'-*GTA CTA* GCG GCC GCT TAC AGC TGG GTT TCT CTA CGT GTG CAG TGT AGG CAG CGA CC-3'
P53deg+PDZ - HPV16E6 ubiquitination domain (first 59 amino acids) with PDZ-binding domain added	P53deg+pdz-F	5'-*ATC GCA* GGA TCC ATG CAC CAA AAG AGA ACT G-3'
	P53deg+pdz-R	5'-*GTA CTA* GCG GCC GCT TAC AGC TGG GTT TCT CTA CGT GTT GGA TTC CCA TCT CTA TAT A-3'
MAGI-1 – cDNA clone of MAGI-1	MAGI-1-F	5'-*ATC GCA* GGA TCC GCC ACC ATG TCC AAA GTG ATC CAG-3'
	MAGI-1-R	5'-*GTA CTA* GCG GCC GCT CAC TTC CGG AAC ACC TTG-3'

*- Italicized nucleotides are added to improve restriction digestion. Underlined nucleotides are recognized by either BamHI or NotI

All PCR reactions used 1ng DNA template, 1.25mM $MgCl_2$, 200µM dNTPs (Invitrogen), 10µM of each primer, 2.5U units of Taq DNA Polymerase (Invitrogen) and 10X PCR buffer (Invitrogen) diluted to 1X in a 50ul reaction. A Verti 96-well Thermal Cycler (Applied Biosystems) was used to carry out all PCR amplifications under the following conditions: initial denaturing step of 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. A final elongation step was done at 72°C for 7 minutes. All PCR products were kept at 4°C.

Following PCR amplification, the amplicon was run on a 1.5% low-melt agarose gel for approximately 1 hour at 95V. The gel was then visualized under ultraviolet light to confirm the presence of the correct size band. The band was cut out of the gel and the DNA was extracted from the gel piece using the QIAEX II Gel extraction Kit (Qiagen, Ontario, Canada).

3.3.1.2. DNA Ligation into pcDNA3.1 (+) expression vector

The pcDNA3.1 (+) vector was chosen because of the presence of a T7 promoter which is necessary for *in vitro* translation. Inserts are cloned downstream from this promoter. The purified DNA inserts and pcDNA3.1 (+) were digested for 3 hours at 37°C in a 40µl reaction which included 20 units each of BamHI and NotI restriction enzymes, one times NEB3 Buffer (100mM NaCl, 50mM Tris-HCl, 10 mM $MgCl_2$, 1mM dithiothreitol, pH 7.9) and 5µg of BSA (New England Biolabs, ON, Canada). Following digestion, products were run on a low-melt agarose gel and DNA was extracted as described in section 3.3.1.1. Ligation of insert to vector was done in a 3:1 ratio using

10U of T4 DNA ligase (Invitrogen) and 1X DNA Ligase Reaction Buffer (250mM Tris-HCl, 50mM $MgCl_2$, 5mM ATP, 5mM dithiothreitol, 25% (w/v) polyethylene glycol-8000) in a total volume of 20µl. The ligation reaction was incubated at room temperature for 1 hour.

3.3.1.3. Transformation

Ligated vectors were transformed into One Shot TOP10 Chemically Competent *Escherichia coli* cells (Invitrogen). Frozen cells were thawed on ice for approximately 5 minutes. Ligated vector (5µl) was added to the cells and mixed gently. The mixture was incubated on ice for 30 minutes then heat-shocked at 42°C for 60 seconds. The tubes were then put back on ice for two minutes after which, 250µl of room temperature SOC Media was added. Cells were then incubated horizontally in a shaking incubator at 37°C with mixing at 250rpm for 1 hour. Transformed cells (100ul) were plated on warmed Luria-Bertani (LB) ampicillin medium (0.01g/ml tryptone, 0.005g/ml yeast extract, 0.01g NaCl, 100ug ampicillin) and incubated at 37°C overnight.

3.3.1.4. PCR Screening of Transformant Colonies

Individual colonies were picked, using a 10µl pipette tip and put into 20µl of nuclease free water (Invitrogen). Extra caution was taken to only take half the colony for PCR screening. The samples were boiled at 99°C in a Thermomixer R (Eppendorf, Mississauga, Ontario) for 10 minutes and then centrifuged at 10000rcf for 5 minutes (Centrifuge 5424, Eppendorf). The supernatant from centrifuged sample (5µl) was used as template for PCR to confirm the presence of the desired insert. The same PCR

conditions outlined in section 3.3.1.1. were used and the primers were dependent on which insert was to be confirmed. The resulting PCR product was run on a 1.5% agarose gel to visually confirm presence of the insert.

3.3.1.5. Plasmid Purification

The remaining half of the colonies which were found to contain the insert were picked and grown overnight in 5ml of LB broth ampicillin at 37°C with shaking at 250rpm. The bacteria were pelleted by centrifugation at 6800rcf at room temperature for 3 minutes. Plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen). Purified clones were sequenced by the DNA Core Facility (National Microbiology Lab, Winnipeg, Manitoba) to ensure no mutations were introduced. Sequences were aligned with reference sequences from GenBank using MegAlign (DNASTAR Lasergene 10 Core Suite). Successful clones were stored in 80% glycerol at -80°C. In addition to this, clones were grown up in 100ml of LB ampicillin broth in order to further purify the plasmids using an EndoFree Plasmid Maxi Kit (Qiagen).

3.3.2. Construction of HPV16.6 and HPV6.16 Mutants

To construct the HPV16.6 mutant, purified PCR products of HPV16E6SM and HPV6E6 were digested with 20U of NdeI (New England Biolabs, Ontario, Canada) for 3 hours at 37°C in a 40µl reaction which included one times NEB4 Buffer 50mM potassium acetate, 20 mM Tris-acetate, 10mM magnesium acetate, 1mM dithiothreitol, pH 7.9). The N-terminal digestion product of HPV16E6SM (199bp) and the C-terminal digestion product of HPV6E6 (278bp) were extracted from a 1.5% low-melt agarose gel as

described in section 3.3.1.1. Following DNA purification, the two components were mixed in a 1:1 ratio with 10U of T4 DNA ligase (Invitrogen) and 1X DNA Ligase Reaction Buffer (250mM Tris-HCl, 50mM $MgCl_2$, 5mM ATP, 5mM dithiothreitol, 25% (w/v) polyethylene glycol-8000) in a total volume of 20µl for a ligation reaction. The ligation reaction product was used as template in a PCR reaction identical to that described in section 3.3.1.1. using the HPV16E6SM-F and HPV6E6+PDZ-R primers. The amplicon was then ligated into pcDNA3.1 (+), transformed into One Shot TOP10 Chemically Competent *Escherichia coli* cells and resulting plasmid was purified as described in previous sections.

To construct the HPV 6.16 mutant, purified PCR product of the HPV16E6SM insert was digested with 20U NdeI as described above. Purified PCR product of the HPV6E6 insert was digested with 20U of BsmI (New England Biolabs) for 3 hours at 65°C in a 40µl reaction, which included 1X NEB4 Buffer (50mM potassium acetate, 20 mM Tris-acetate, 10mM magnesium acetate, 1mM dithiothreitol, pH 7.9). The C-terminal digestion product of HPV16E6SM (278bp) and the N-terminal digestion product of HPV6E6 (111bp) were extracted from a 1.5% low-melt agarose gel as described in section 3.3.1.1. In order to fill the gap of nucleotides missing in HPV6E6 from the BsmI site to the NdeI site, the following oligonucleotides were synthesized by the DNA Core Facility (National Microbiology Lab, Winnipeg, Manitoba):

5’CTGACCACAGCAGAGATTTATTCATATGCATATAAACACCTAAAGGTCCTGTTTCGAGGCGGC TATCCA3’ and

5’TATGGATAGCCGCCTCGAAACAGGACCTTTAGGTGTTTATATGCATATGAATAAATCTCTGCT

GTGGTCAGTG3'. To anneal the oligonucleotides, the oligonucleotides were mixed at a 1:1 ratio and incubated at 95°C for 5 minutes. Then the mixture was left to cool down at room temperature for approximately 1 hour. The annealed oligo was designed so that the 5' end had an overhang of oligonucleotides complementary to the N-terminal digestion product of HPV6E6 (111bp), and the 3' end had an overhang of oligonucleotides complementary to the C-terminal digestion product of HPV16E6SM (278bp). The three components were mixed in a 1:1:1 ratio with 10U of T4 DNA ligase (Invitrogen) and 1X DNA Ligase Reaction Buffer (250mM Tris-HCl, 50mM $MgCl_2$, 5mM ATP, 5mM dithiothreitol, 25% (w/v) polyethylene glycol-8000) in a total volume of 20µl for a ligation reaction. The ligation reaction product was used as template in a PCR reaction identical to that described in section 3.3.1.1. using the HPV6E6-F and HPV16E6SM-R primers. The amplicon was then ligated into the pcDNA3.1 (+), transformed into One Shot TOP10 Chemically Competent *Escherichia coli* cells and resulting plasmid was purified as described in previous sections.

3.3.3. Construction of 16E6Δ9-13,16E6 t155a_t161a_g166c, 16E6t155a and 16E6a145g Mutants

To construct the 16E6Δ9-13, t155a_t161a_g166c, t155a and a145g mutants the QuickChange Lightening Site-Directed Mutagenesis kit (Agilent Technologies, Mississauga, Ontario) was used on the HPV16E6SM plasmid following manufacturer's instructions. The primers found in Table 13 were designed using the QuickChange Primer design program found on the Agilent company website.

Table 13 – Primer Sequences for 16E6Δ9-13, 16E6t155a_t161a_g166c, 16E6t155a and 16E6a145g Site-Directed Mutagenesis

Name of Construct and brief description	Primer Name	Primer Sequence
16E6Δ9-13 – HPV16E6SM with amino acid residues 9 to 13 deleted	16E6Δ9-13F	5'-GGA CCC ACA GGA GCG ACA GTT ATG CAC AGA GC-3'
	16E6Δ9-13R	5'-GCT CTG TGC ATA ACT GTC GCT CCT GTG GGT CC-3'
16E6t155a_t161a_g166c – HPV16E6SM with the following mutations : T^{155}→A, T^{161}→A, G^{155}→C	t155a_t161a_g166cF	5'-CTG CGA CGT GAG ATA TAT GAC TAT GCT TAT CGG CAT TTA TGC ATA GTA TAT AGA G-3'
	t155a_t161a_g166cR	5'-CTC TAT ATA CTA TGC ATA AAT GCC GAT AAG CAT AGT CAT ATA TCT CAC GTC GCA G-3'
16E6t155a – HPV16E6SM with a T^{155}→A mutation	16E6t155aF	5'-ACT GCG ACG TGA GAT ATA TGA CTA TGC TTT TCG GGA-3'
	16E6t155aR	5'-TCC CGA AAA GCA TAG TCA TAT ATC TCA CGT CGC AGT-3'
16E6a145g– HPV16E6SM with a A^{145}→G mutation	16E6a145gF	5'-ACA GTT ACT GCG ACG TGA GGT ATA TGA CTT TGC TTT TCG-3'
	16E6a145gR	5'-CGA AAA GCA AAG TCA TAT ACC TCA CGT CGC AGT AAC TGT-3'

The mutant plasmids were transformed into One Shot TOP10 Chemically Competent *Escherichia coli* cells and resulting plasmid was purified as described in previous sections.

3.3.4 *In vitro* Translation

Each purified plasmid (1µg) was *in vitro* translated using the TNT T7/SP6 Coupled Reticulocyte Lysate System (Promega, Madison, Wisconsin) following manufacturer's instructions. This kit allows transcription and translation to take place in one tube. Transcription takes place from the T7 promoter by a T7 RNA polymerase included in the kit, while rabbit reticulocyte lysate is used for the translation of protein (also included in

the kit). Radiolabelled isotopes are used in the reactions and are thus incorporated into newly synthesized protein to allow for detection. Radiolabelled ^{35}S-Cysteine (Perkin Elmer, Ontario, Canada) was used because of the number of cysteine residues found in the inserts.

3.3.4.1. Autoradiography

To confirm and visualize newly synthesized radiolabelled protein, a portion of the *in vitro* translation reaction was run on a SDS-PAGE gel, which was stained, dried and exposed to autoradiography film.

3.3.4.1.1. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Tris-Glycine pre-cast gels (7.5%) (Bio-Rad) were used for all experiments. A 6X solution SDS-PAGE gel loading buffer (3% glycerol, 0.6 g/ml bromophenol blue, 0.6 SDS, 10mM Tris pH6.8 and 50µl/ml β-mercaptoethanol) was diluted to 1X in 5µl of *in vitro* translation reaction and boiled at 99°C for 2 minutes. The boiled samples (5ul) were loaded onto the gel and run in 1X Tris/Glycine/SDS Buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3) at 120 volts for approximately 1 hour.

3.3.4.1.2 Gel Staining & Drying

Protein bands on the SDS-PAGE gel were stained using GelCode Blue Stain Reagent (Fisher) according to the manufacturer's instructions. GelCode Blue uses the colloidal properties of Coomassie G-250 dye for protein staining. The stained gel was

dried using the DryEase Mini-Gel Drying System (Invitrogen) following manufacturer's instructions.

3.3.4.1.3. Autoradiograph

The dried gel was exposed to Amersham Hyperfilm ECL film (GE Healthcare, Quebec, Canada) for 24 hours and developed using a SRX-201A (Konica Minolta, Ontario, Canada) film processor.

3.3.5. Degradation Assay

The degradation assay was adapted from protocols developed by Thomas and Banks[116]. *In vitro* translated E6 protein (24µl) was mixed with 8µl of *in vitro* translated MAGI-1 protein and left to incubate for 1 hour at 30°C. Reaction time-point samples (5µl) were taken from the reaction at the following time points: 0, 15, 30, 45 and 60 minutes, to which 1µl of 6X solution SDS-PAGE gel loading buffer was added.

For the MAGI-1 degradation assays with inhibitory peptides added to the reaction mixture, two peptides made with the following amino acids were synthesized (ThermoFisher Scientific, Rockford, IL, USA): TRRETQL and CCRSSRTRRETQL. Five micrograms of the TRRETQL (173µM) and 2µg (39µM) were added to their respective MAGI-1 + HPV16E6SM degradation reactions.

3.3.5.1. Western Blot

Western blots were used to observe MAGI-1 degradation. Timed samples from the degradation assays were prepared as described in section 2.3.1.1. The boiled

samples (1µl) were loaded onto a 7.5% Tris-Glycine gel and run at 120V for approximately 1 hour. Proteins on the gel were then transferred on to polyvinylidene difluoride (PVDF) membranes (Immobilon-P Transfer Membrane, Millipore). Transfers took place overnight using a Mini Trans-Blot Electrophoretic Transfer Cell (Biorad) with transfer buffer (25mM Tris, 192mM glycine, 20% methanol) at a constant current of 90mA. Transfer membranes were washed with a solution of 5% skim milk in TBS-T (1X TBS Buffer, 0.1% Tween) for 1 hour to block non-specific binding sites. Then the membrane was rinsed 3 times for 5 minutes with TBS-T. A mouse MAGI-1 IgG antibody (Santa Cruz Biolabs, Texas, USA) was used as the primary antibody and was incubated with the membrane at a 1:500 dilution for 3 hours at 4°C. The membrane was rinsed three times for 10 minutes with TBS-T. An antimouse IgG antibody conjugated to horseradish peroxidase (KPL, Maryland, USA) was used as the secondary antibody and was incubated with the membrane at a 1:10,000 dilution for 1 hour. The membrane was again rinsed three times for ten minutes with TBS-T. For chemiluminescent visualization of the protein bands, the Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used. This includes a luminol reagent and a peroxide solution which are mixed in a 1:1 ratio. The mixture is poured over the membrane and left to sit for 1 minute. The membrane is then put in between two sheets of plastic and protein bands are visualized on a VersaDoc 5000MP (Biorad) using the Quantity One analysis software's chemiluminescence channel.

3.4. Results

3.4.1. Cloning of E6 inserts into the pcDNA 3.1 (+) Vector

The inserts were directionally cloned into the pcDNA3.1(+) vector (Figure 6A). This vector was chosen because of the presence of the T7 promoter which is used in the *in vitro* translation process. In addition to this, the ampicillin resistance gene allows for selection of transformants during the cloning process. The MAGI-1 ORF as well as the E6 inserts were cloned downstream of the T7 promoter using the BamHI and NotI restriction enzymes (Figure 6B). The following E6 inserts were constructed: HPV16E6SM, HPV16E6SMNT, HPV6E6, HPV6E6+PDZ, p53deg+PDZ, HPV16.6, HPV6.16, 16E6Δ9-13, 16E6 t155a_t161a_g166c, 16E6t155a and 16E6a145g (Figure 6C). They are described in further detail in the following sections. All clones were sequenced for confirmation.

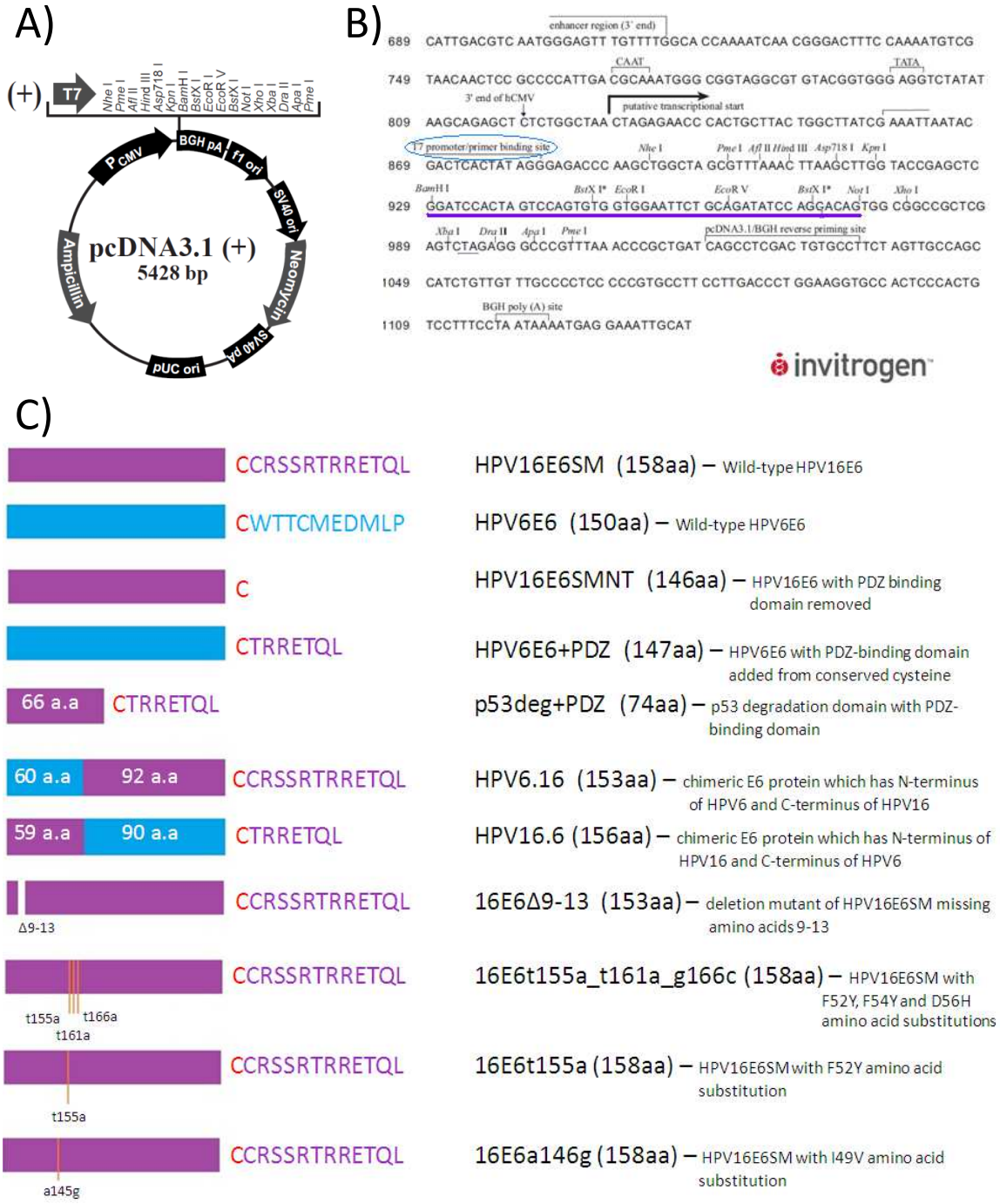

Figure 6 – Schematic representation of pcDNA3.1 (+) vector and inserts. A) pcDNA3.1(+) vector map B) Sequence of the pcDNA3.1(+) multiple cloning site. Highlighted in blue is the T7 promoter binding site, in purple is the region into which MAGI-1 or the E6 inserts are cloned into the vector. These figures were extracted from the pcDNA3.1(+) manual, Invitrogen, www.lifetechnologies.com. C) General Schematic representation and description of the E6 inserts that were constructed and cloned into pcDNA3.1(+).

3.4.2. *In vitro* translation of MAGI-1 and E6 inserts

Following confirmation by sequencing, all the clones were used in individual *in vitro* translation reactions. The *in vitro* translations were done using S^{35} labelled cysteine to allow for detection of synthesized protein. Autoradiography was used to confirm the synthesis of protein (Figure 7).

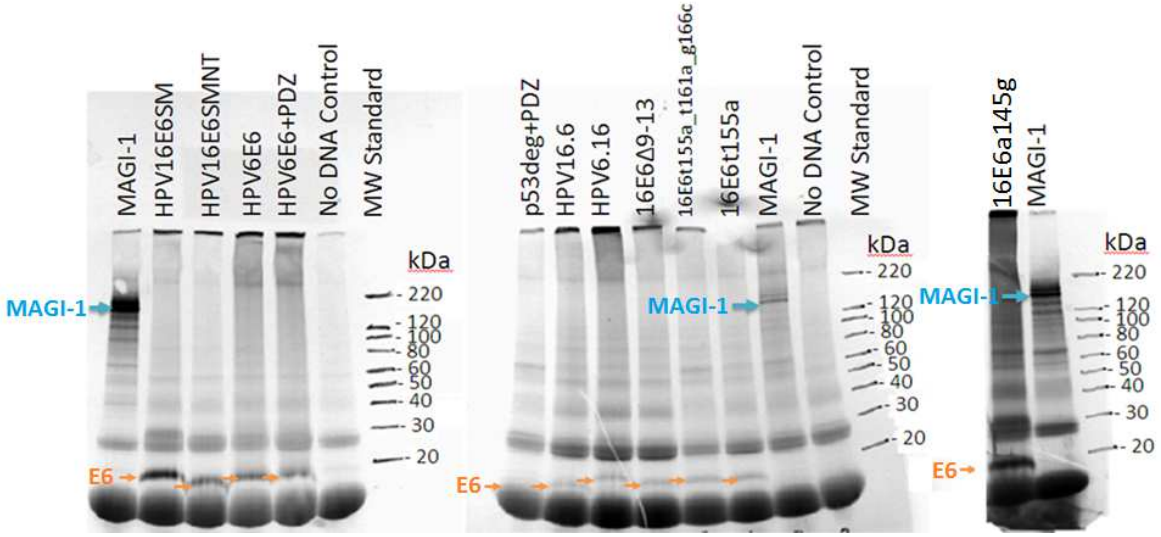

Figure 7 – Autoradiographs of *in vitro* translated proteins. Each *in vitro* translated product (5µl) was run on SDS-PAGE gel. Control reactions containing no DNA were also run. Gels were subsequently stained, dried and exposed to X-ray film for 24 hours. The *in vitro* translated MAGI proteins are highlighted by blue arrows. The *in* vitro translated E6 proteins are highlighted by orange arrows. Magic Mark XP (Invitrogen) was used as the MW standard.

3.4.3. MAGI-1 degradation Assays

3.4.3.1. Confirmation of MAGI-1 degradation abilities of wild-type HPV16 and HPV6 E6 proteins

In order to confirm that *in vitro* translated proteins behave as they should, with respect to their abilities to degrade the MAGI-1 protein, wild-type HPV6 and HPV16 E6 proteins were used in MAGI-1 degradation assays. In theory, HPV6E6 should not be able to induce MAGI-1 degradation since it does not have a PDZ-binding domain, whereas HPV16E6SM should be able to induce the degradation of MAGI-1 due to the presence of the PDZ-binding domain at its C-terminus. Each E6 (HPV16E6SM or HPV6E6) *in* vitro translated protein was incubated with *in* vitro translated MAGI-1 protein for one hour at 30°C. Samples were taken from each reaction at the 0, 15, 30, 45, and 60 minute time points. Time point samples were run on SDS-PAGE gel and Western blot was performed using an anti-MAGI-1 antibody to observe MAGI-1 degradation. First, to ensure that MAGI-1 degradation isn't taking place due to other factors in the reaction, MAGI-1 was mixed with a no DNA *in vitro* translation control reaction. Figure 8A shows that MAGI-1 degradation does not take place due other factors in the *in vitro* translation mix. When HPV6E6 was incubated with MAGI-1, it was not able to induce MAGI-1 degradation, as the MAGI-1 protein remains present at all time points (Figure 8B). In contrast to this, when HPV16E6SM was incubated with MAGI-1, degradation did take place (Figure 8C). At the 15 minute time point, approximately 50% of the MAGI-1 protein remains, and at the 30 minute time point, MAGI-1 is no longer present. This proves that the MAGI-1 degradation assays were successful and that the synthesized wild-type E6 proteins behave as expected.

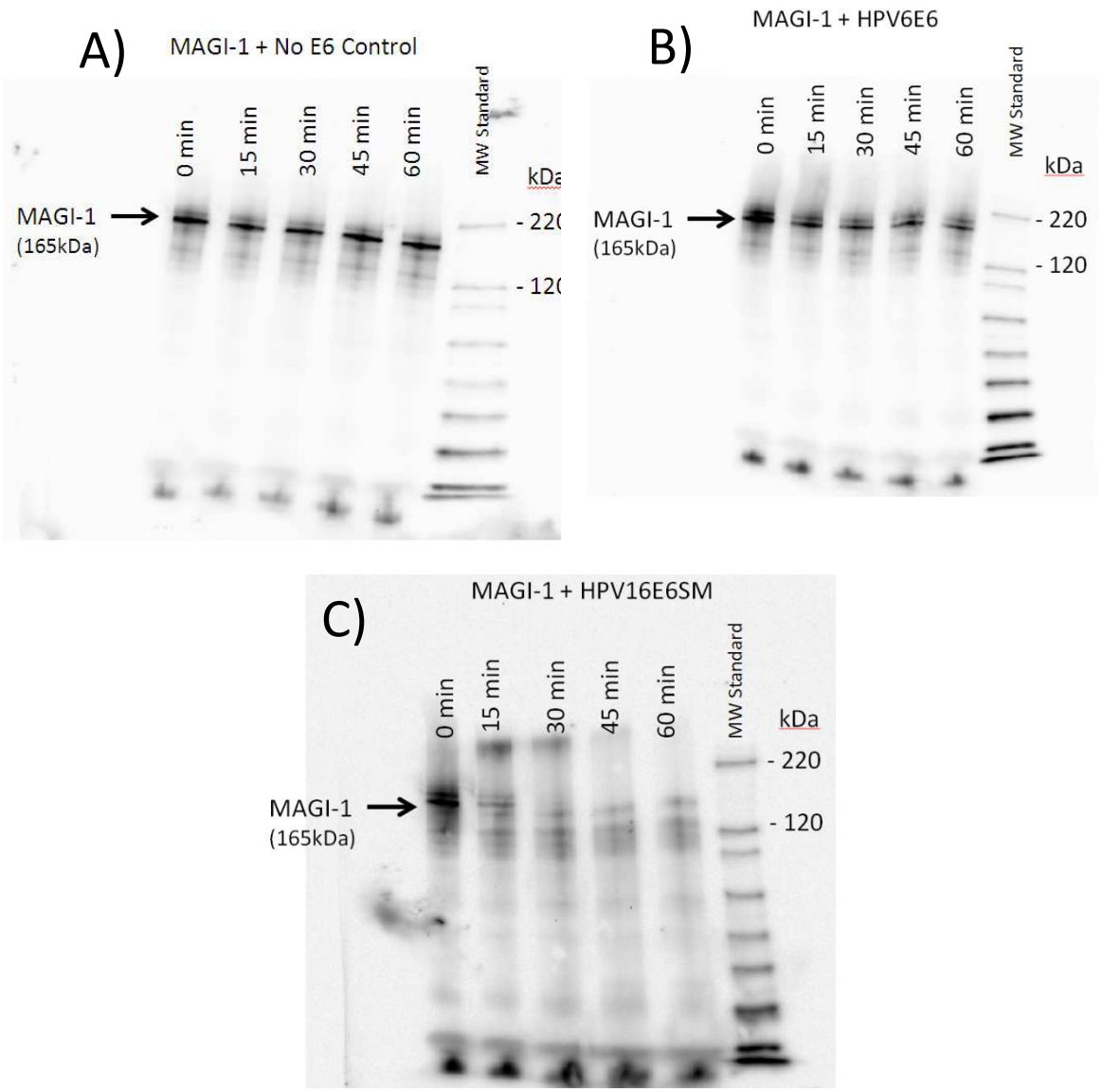

Figure 8 – HPV16E6SM and HPV6E6 MAGI-1 Degradation Assays. *In vitro* translated HPV16E6SM or HPV6E6 was incubated with *in vitro* translated MAGI-1 protein for 1 hour at 30°C. Samples were taken from the reaction at 0, 15 30, 45, and 60 minutes. The time point samples were run on SDS-PAGE gel and Western blot was performed using an anti-MAGI-1 antibody to observe MAGI-1 degradation. Magic Mark XP (Invitrogen) was used as the MW standard. A) MAGI-1 and no E6 control reaction. No MAGI-1 degradation is observed which indicates that MAGI-1 degradation does not occur due to factors present in the *in vitro* translation reaction. B) MAGI-1 and HPV6E6. No MAGI-1 degradation occurs since HPV6E6 does not have a PDZ-binding domain C) MAGI-1 and HPV16E6SM. MAGI-1 degradation occurs due to the presence of a PDZ-binding at the C-terminus of HPV16E6SM.

3.4.3.2. Determination of the importance of the PDZ-binding domain in MAGI-1 degradation

To determine if the PDZ-binding domain is the only region necessary for the induction of MAGI-1 degradation, two mutants were constructed: HPV16E6SMNT and HPV6E6+PDZ. The first mutant removes the PDZ-binding domain from HPV16E6SM by terminating the ORF at the conserved cysteine at residue 146. The second mutant adds the last seven amino acids of HPV16E6SM (TRRETQL) on to HPV6E6 following the conserved cysteine residue. This region was used similarly i.e. added to low-risk or cutaneous types, in other studies[94, 135]. Both mutants were used in MAGI-1 degradation assays. Figure 9A shows that when HPV16E6SMNT is incubated with MAGI-1 it cannot induce its degradation. This suggests that the PDZ-binding domain is necessary for the induction of MAGI-1 degradation. Figure 9B shows that when the PDZ-binding domain is added to HPV6E6 this does not allow it to induce MAGI-1 degradation. Thus, this shows that the PDZ-binding domain of HPV16E6SM is necessary but not sufficient to induce the degradation of MAGI-1.

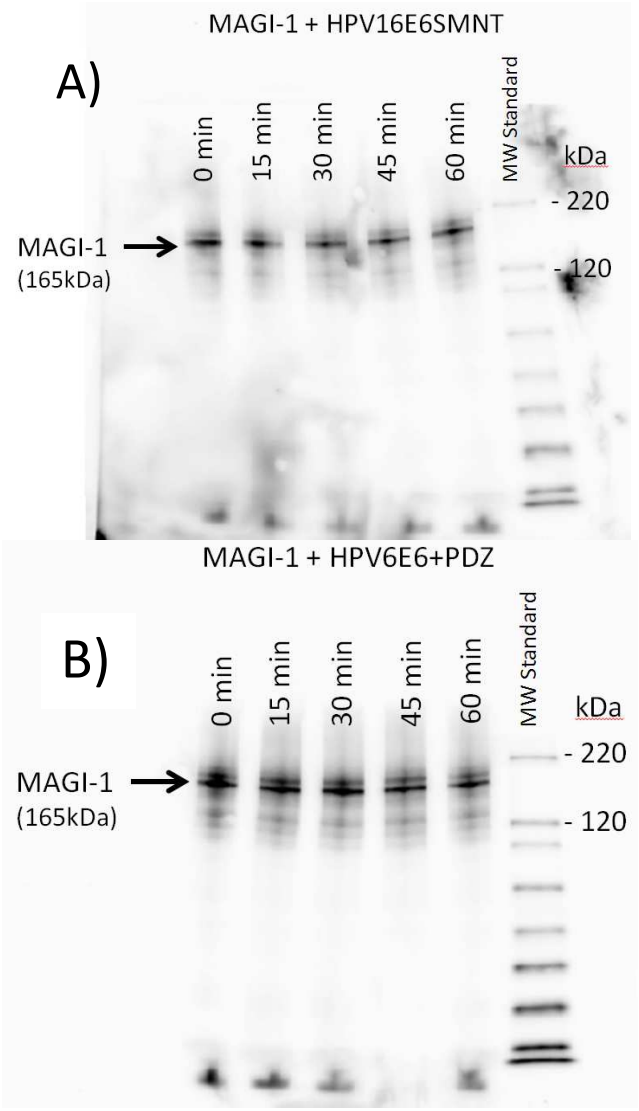

Figure 9 – HPV16E6SMNT and HPV6E6+PDZ MAGI-1 Degradation Assays. *In vitro* translated HPV16E6SMNT or HPV6E6+PDZ was incubated with *in vitro* translated MAGI-1 protein for 1 hour at 30°C. Samples were taken from the reaction at 0, 15, 30, 45, and 60 minutes. The time point samples were run on SDS-PAGE gel and Western blots were performed using an anti-MAGI-1 antibody to observe MAGI-1 degradation. Magic Mark XP (Invitrogen) was used as the MW standard. A) MAGI-1 and HPV16E6SM. No MAGI-1 degradation occurs, indicating that the PDZ-binding domain is essential for MAGI-1 degradation. B) MAGI- and HPV6E6+PDZ. No MAGI-1 degradation occurs when the PDZ-binding domain is added to HPV6E6. This indicates that the PDZ-binding domain is necessary but not sufficient to induce MAGI-1 degradation.

The next goal was to confirm that the PDZ-binding domain of HPV16E6SM must bind to MAGI-1 in order for MAGI-1 degradation to occur. A small peptide TRRETQL was synthesized which was identical to the last seven amino acids of HPV16E6SM i.e. the PDZ-binding domain. This peptide was added to the HPV16E6SM and MAGI-1 degradation reaction at a concentration of 173μM. If the PDZ-binding domain must bind to MAGI-1 for this degradation, this short peptide should compete with the full length HPV16E6SM protein for binding to MAGI-1 and inhibit MAGI-1 degradation. Figure 10A shows the western blot obtained from the time point samples of this reaction. If this is compared to the western blot obtained for the original HPV16E6SM and MAGI-1 degradation reaction in Figure 10B (identical to Figure 8C), the addition of the short peptide was not able to completely inhibit MAGI-1 degradation. In Figure 10B, MAGI-1 is completely degraded by the 30 minute time point, whereas in the reaction containing the short peptide, MAGI-1 is completely degraded by the 60 minute time point. Since complete inhibition of MAGI-1 degradation was not obtained with the above mentioned peptide, another peptide (CRSSRTRRETQL) which had a longer sequence beginning from the conserved cysteine at residue 146 was used. When this peptide was added to the HPV16E6SM and MAGI-1 degradation reaction at a concentration of 39μM, it was able to completely inhibit the MAGI-1 degradation (Figure 10C). This suggests that more than just the PDZ-binding domain reported in literature is required for high affinity binding to MAGI-1.

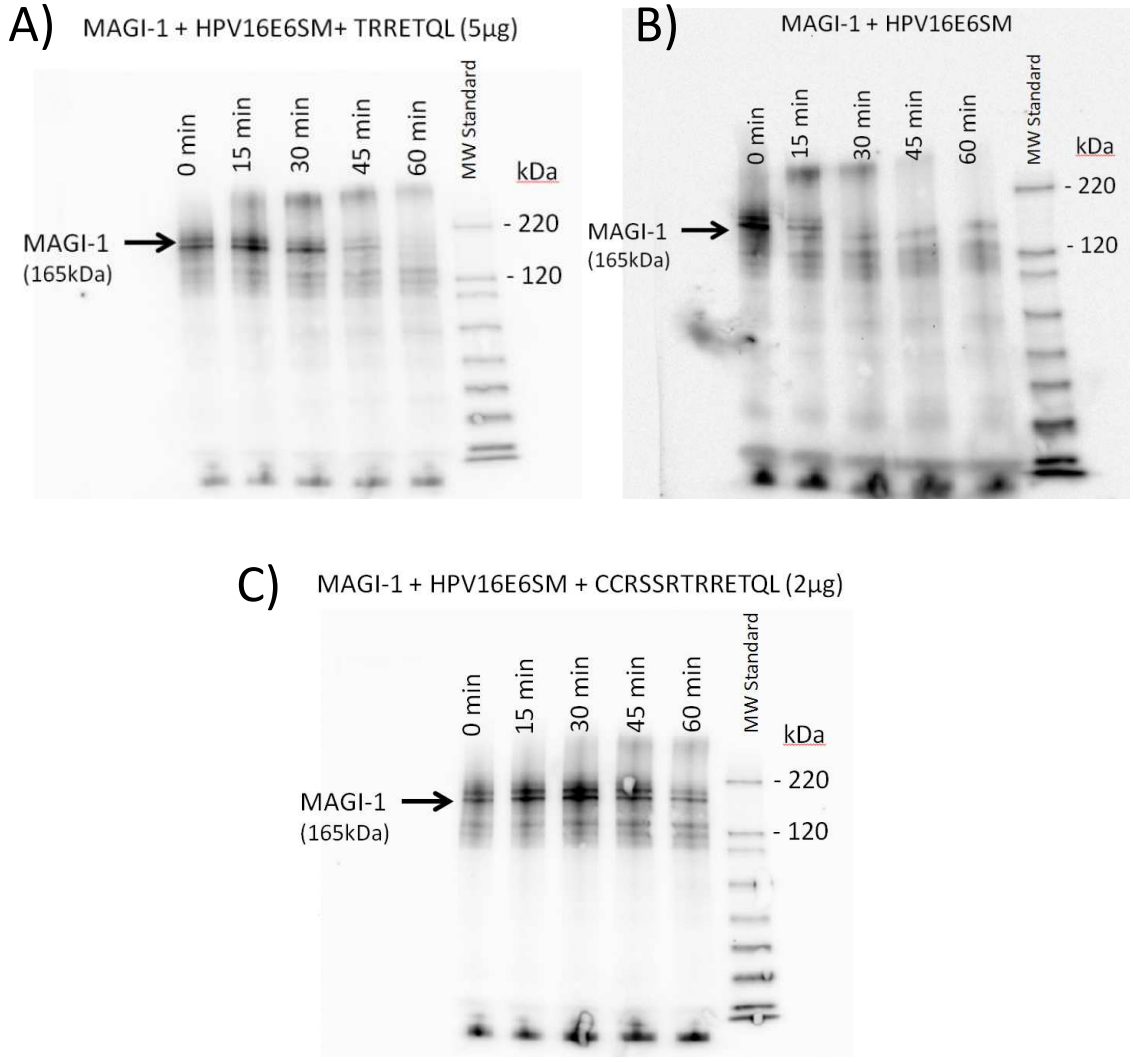

Figure 10 – HPV16E6SM MAGI-1 Degradation Assays with Inhibitory Peptides Added. *In vitro* translated HPV16E6SM was incubated with *in vitro* translated MAGI-1 for 1 hour at 30°C. Either a TRRETQL (5µg) or a CCRSSRTRRETQL (2µg) peptide was added to the degradation reaction. Samples were taken from the reaction at 0, 15, 30, 45, and 60 minutes. The time point samples were run on SDS-PAGE gel and western blots were performed using an anti-MAGI-1 antibody to observe MAGI-1 degradation. Magic Mark XP (Invitrogen) was used as the MW standard. A) MAGI-1 + HPV16E6SM + TRRETQL. Addition of this peptide delayed, but was not able to completely inhibit the degradation reaction. B) MAGI-1 + HPV16E6SM C) MAGI-1 + HPV16E6SM+ CCRSSRTRRETQL. This peptide was able to completely inhibit MAGI-1 degradation.

3.4.3.3. Determination of other regions of E6 which are important for MAGI-1 Degradation

Next, the goal was to determine which regions of E6, other than the PDZ-binding domain, are needed to induce MAGI-1 degradation. Crooks *et al.* studied the regions of E6 that are important for p53 binding and degradation. They found that the N-terminus was required for degradation, while the C-terminus was important for binding[16]. Therefore, the goal was to investigate whether the same regions involved in inducing p53 degradation are also involved in inducing MAGI-1 degradation. To begin, the first 66 amino acids of HPV16E6SM (involved in p53 degradation[16]) were attached the PDZ-binding domain (TRRETQL), which has been used in previous studies[94, 135], to see if this construct was sufficient to induce MAGI-1 degradation. This construct was named p53deg+PDZ. Figure 11 demonstrates that this construct is not sufficient to induce MAGI-1 degradation. This could be due to the fact that this is a truncated protein. Thus, more of the HPV16E6SM protein must be needed to induce degradation.

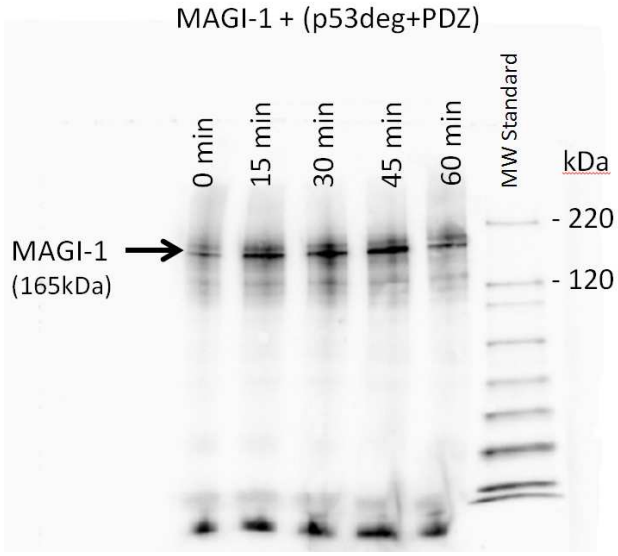

Figure 11 – p53deg+PDZ MAGI-1 Degradation Assay. *In vitro* translated p53deg+PDZ was incubated with *in vitro* translated MAGI-1 protein for 1 hour at 30°C. Samples were taken from the reaction at 0, 15 30, 45, and 60 minutes. The time point samples were run on SDS-PAGE gel and Western blotting was performed using an anti-MAGI-1 antibody to observe MAGI-1 degradation. Magic Mark XP (Invitrogen) was used as the MW standard. No MAGI-1 degradation takes place which indicates that the E6 p53 degradation and the PDZ-binding domains are not sufficient to induce MAGI-1 degradation.

Next, the same chimeric constructs as those used by Crook *et al.* (1991) were synthesized, with the HPV16.6 chimeric protein being slightly modified. The two constructs that were made were named HPV6.16 and HPV16.6. The HPV6.16 construct consisted of the N-terminal 60 amino acids of HPV6E6, the C-terminal 92 amino acids from HPV16E6SM. This mutant will test if the amino acids upstream of the PDZ-binding domain are important in MAGI-1 degradation. Conversely, the HPV16.6 construct consisted of the N-terminal 59 amino acids from HPV16E6SM, the C-terminal 90 amino acids from HPV6E6 including the PDZ-binding domain (the last seven amino acids - TRRETQL) of HPV16E6SM. This construct, being similar to the E6 p53deg+PDZ construct,

will indicate if it is a full length E6 protein or if it is specifically the full length HPV16E6SM protein that is needed to induce MAGI-1 degradation. When these constructs were used in MAGI-1 degradation assay, neither mutant was able to induce MAGI-1 degradation (Figure 12A and 12B), indicating that a full length HPV16E6SM is needed to induce MAGI-1 degradation.

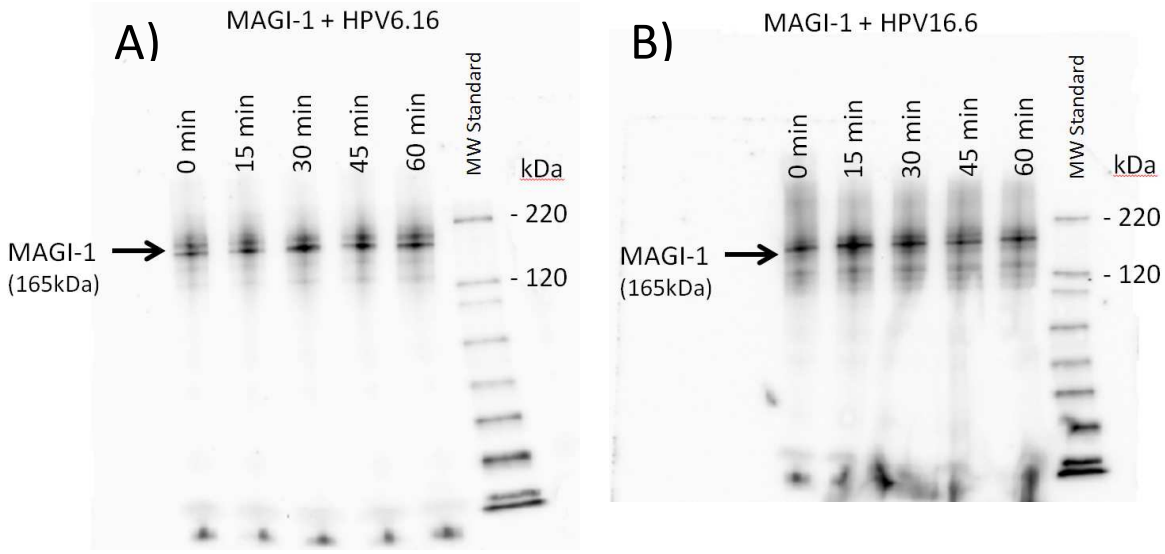

Figure 12 – HPV6.16 and HPV16.6 MAGI-1 Degradation Assays. *In vitro* translated HPV6.16 or HPV16.6 was incubated with *in vitro* translated MAGI-1 protein for 1 hour at 30°C. Samples were taken from the reaction at 0, 15, 30, 45, and 60 minutes. The time point samples were run on SDS-PAGE gel and Western blotted using an anti-MAGI-1 antibody to observe MAGI-1 degradation. Magic Mark XP (Invitrogen) was used as the MW standard. A) MAGI-1 + HPV6.16. B) MAGI-1 + HPV16.6. Both mutants are deficient for MAGI-1 degradation suggesting that the whole HPV16E6SM protein is needed for induction of MAGI-1 degradation.

3.4.3.4. Investigation of Mutations which Affect MAGI-1 Degradation

Lastly, it was investigated if mutations that were found to disrupt p53 degradation also disrupt MAGI-1 degradation. Crooks *et al.* found that the deletion of a specific region in the N-terminus of HPV16E6 abolished p53 degradation. In addition, they found that when they mutated nucleotides in specific regions which are conserved in high risk types to those nucleotides which are found in low risk HPVE6, this also abolished p53 degradation. However, when these mutations were tested individually, they were not able to abolish p53 degradation. The effects of these mutations on MAGI-1 degradation were also examined here. Therefore, 3 different mutants were constructed and used in MAGI-1 degradation assays. First was the 16E6Δ9-13 mutant that had the deletion of amino acids 9 to 13 from HPV16E6SM. When incubated with MAGI-1, this mutant lost its ability to induce MAGI-1 degradation (Figure 13A). The next mutant was named 16E6t155a_t161a_g166c. In this mutant, the amino acids which are conserved in high-risk types at positions 52, 54 and 56 were changed to amino acids that are found in low risk types (F52Y, F54Y and D56H). As shown in Figure 13B, this mutant was also deficient for MAGI-1 degradation. The last mutant, 16E6t155a only contained one of the mutations of the previous construct (F52Y). When Crook *et al.* looked at this mutation, p53 degradation was not disrupted, however here; in the MAGI-1 degradation assay this mutant was still deficient for MAGI-1 degradation (Figure 13C). Therefore, it can be concluded that mutations in HPV16E6 that affect p53 degradation also affect MAGI-1 degradation.

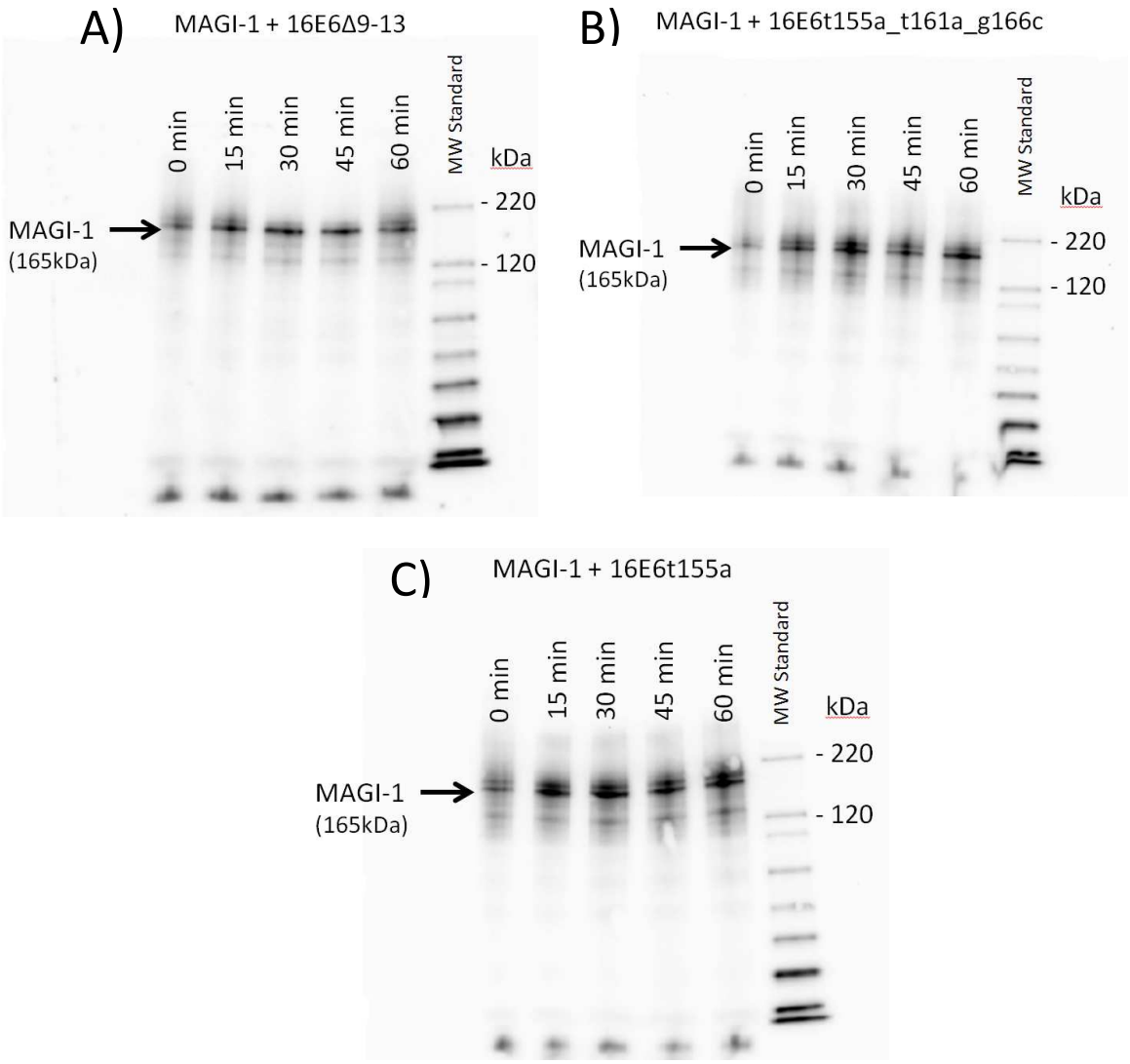

Figure 13 – 16E6Δ9-13, 16E6t155a_t161a_g166c and 16E6t155a MAGI-1 Degradation Assays. *In vitro* translated 16Δ9-13, 16E6t155a_t161a_g166c or 16E6t155a were incubated with *in vitro* translated MAGI-1 protein for 1 hour at 30°C. Samples were taken from the reaction at 0, 15, 30, 45, and 60 minutes. The time point samples were run on SDS-PAGE gel and Western blotting was performed using an anti-MAGI-1 antibody to observe MAGI-1 degradation. Magic Mark XP (Invitrogen) was used as the MW standard. A) MAGI-1 + 16Δ9-13. B) MAGI-1 + 16E6t155a_t161a_g166c. C) MAGI-1 + 16E6t155a. All the mutants were deficient for MAGI-1 degradation. This indicates that mutations that affect p53 degradation also affect MAGI-1 degradation.

Finally, to ensure that the abolishment of MAGI-1 degradation is specific to these mutations and not just any mutation of the N-terminnus, a 16E6a145g mutant was constructed. This mutant has a mutation of A→G at nucleotide position at 145 that results in an amino acid substitution of I49V. This mutation was found in one of the cervical cancer samples in the previous chapter. Figure 14 shows that this mutant still has the ability to induce MAGI-1 degradation despite having a mutation in its N-terminus.

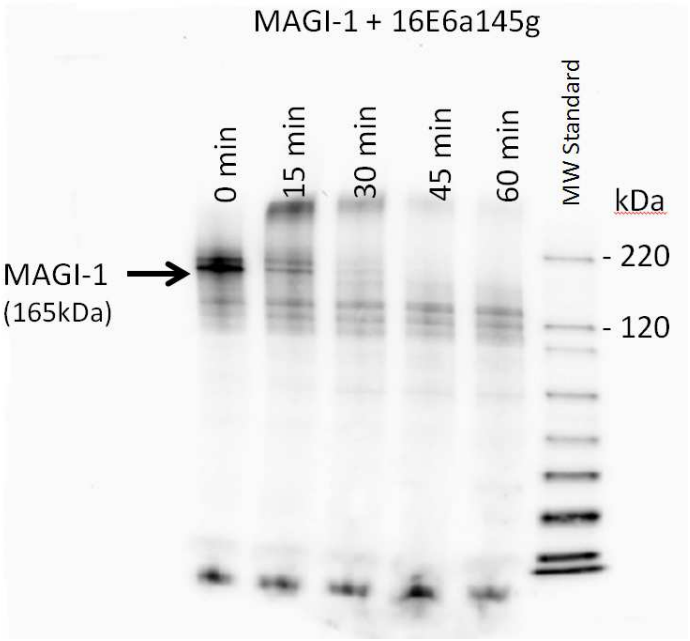

Figure 14 - 16E6a145g MAGI-1 Degradation Assay. *In vitro* translated 16E6a145g was incubated with *in vitro* translated MAGI-1 protein for 1 hour. Samples were taken from the reaction at 0, 15, 30, 45, and 60 minutes. The time point samples were run on SDS-PAGE gel and Western blotted using an anti-MAGI-1 antibody to observe MAGI-1 degradation. Magic Mark XP (Invitrogen) was used as the MW standard. With this mutant MAGI-1 degradation still occurs despite having a mutation in the N-terminus.

3.5. Discussion

In this study, the goal was to identify the regions of the HPV16E6 protein, other than the PDZ-binding domain, that are important for the induction of MAGI-1 degradation. Degradation assays established by Thomas & Banks (2005) were successfully replicated, which allowed the confirmation that wild-type HPV6 and HPV16 E6 constructs have the correct MAGI-1 degradation capabilities i.e. HPV6 cannot induce MAGI-1 degradation, while HPV16E6 can induce degradation. Although, it is the last four amino acids of HPV16E6 (ETQL) which are recognized by PDZ containing proteins, studies have found that residues upstream are also important in PDZ-domain binding. When Zhang *et al.* resolved the crystal structures of MAGI-1 bound to a peptide, which was identical to the last seven amino acids of the HPV-18's C-terminus, they found that arginine residues found upstream of the ETQL amino acids were important for binding the MAGI-1 PDZ domain[135]. In addition to this, Thomas *et al.* (2008) found that the residues upstream can also contribute to the binding specificity of HPV E6 proteins to PDZ domain containing proteins[117]. Interestingly in this study, when a TRRETQL peptide was added to a MAGI-1 + HPV16E6SM degradation reaction, this peptide could not completely inhibit the degradation. Whereas, when a longer peptide CCRSSRTRRETQL was added, it was able to inhibit MAGI-1 degradation completely. This suggested a longer C-terminal sequence of HPV16E6 was required for high affinity binding to MAGI-1 which is contrary to what is currently found in literature. It is possible that the addition of the shorter tail to the C-terminus of HPV6E6, which was done in our experiments for the constructs HPV6E6+PDZ and HPV16.6, may not have allowed for sufficient binding to

MAGI-1, and thus no degradation took place. This may be one aspect of our study that could be repeated with the addition of the longer sequence CCRSSRTRRETQL to the HPV6E6+PDZ and HPV16.6 constructs. This may allow for higher affinity binding of MAGI-1 for these constructs. However, higher affinity binding of MAGI-1 may still not be sufficient to achieve MAGI-1 degradation as was demonstrated by Pim *et al.* in 2002. They found that chimeric low risk proteins which had the PDZ-binding domain (TRRETQL) added to their C-terminus could bind to MAGI-1, however degradation of MAGI-1 could not be observed.

It was also found that the removal of the last 7 amino acids (TRRETQL) from HPV16E6 resulted in the loss of MAGI-1 degradation. However, when the same 7 amino acids were added onto HPV6E6, this did not permit HPV6E6 to induce MAGI-1 degradation. Therefore, it was concluded that the PDZ-binding domain of HPV16E6 is necessary for MAGI-1 degradation, but it was not sufficient to allow the degradation process to occur. Pim *et al.* did similar experiments, which added the HPV18E6 C-terminal tail to low-risk types, and they also observed that addition of this tail did not result in the ability to induce degradation of MAGI-1. However, they also looked at the ability to degrade another MAGUK protein, hDlg, and found that when the low risk-types had the PDZ-binding domain, they were able to degrade the hDlg protein. This suggests that different mechanisms may be used to target and degrade hDlg and MAGI-1.

This idea is further supported by the finding that the whole HPV16E6 protein is needed for MAGI-1 degradation. In attempt to further identify regions, other than the PDZ-binding domain, which are important for the induction of MAGI-1 degradation, chimeric HPV16 and HPV6 E6 proteins were constructed. These constructs were similar to those constructed by Crook *et al*[16]. Their constructs (HPV16/6 and HPV6/16) were used to identify the regions which were important for p53 binding and degradation[16]. Their performance in MAGI-1 degradation assays was tested. It was found that both chimeric proteins could not degrade MAGI-1, therefore indicating that the whole HPV16E6 protein is needed for degradation of MAGI-1. This is in contrast to what was found by Pim *et al.* (2000) when they also used these chimeric proteins in hDlg degradation assays[95].They were able to isolate sequences in the C-terminus that were important for hDlg degradation[95]. They concluded that the mechanisms used to target p53 for degradation are separate from those used to target hDlg. Since it was found that the entire HPV16E6 protein is needed for MAGI-1 degradation, this supports the possibility that there is a different mechanism that leads to MAGI-1 or hDlg degradation.

Although a region important for MAGI-1 degradation in the E6 protein could not be isolated, as it was for p53 degradation, experiments gave results which support the idea that the same mechanisms are used in their degradation. Previous studies are conflicting on whether the same or different mechanisms are used in the degradation of different MAGUK proteins when compared to that of p53. However, in this study mutations which disrupted p53 degradation also disrupted MAGI-1 degradation. This

evidence supports the hypothesis that similar pathways lead to MAGI-1 and p53 degradation. Further experiments will still be needed to test these possibilities.

Since not all HPV types have a PDZ-binding domain, this region is not essential for the HPV life cycle, however, it may be important in the transformation capabilities of high-risk HPV types, since they all have this domain. Therefore, further work is needed in order to determine whether or not the PDZ-binding domain is required for transformation. The next step of this project would be to take the mutants and determine if the PDZ-binding domain or any activity associated with it, i.e. MAGUK protein degradation, is needed for cell transformation. E6 mutants in conjunction with HPV16E7 could be transduced into primary cells. Their capabilities in cell transformation assays would be evaluated and compared with wild-type HPV16 E6 and E7 genes. Perhaps the disruption of MAGUK proteins in cell junctions contributes to the ability of HPV-infected cells to continuously proliferate, which would lead to the development pre-cancerous lesions and eventually cervical cancer.

In addition to this, in finding that the entire HPV16E6 protein was needed to induce the degradation of MAGI-1, it would be interesting to know how the E6 mutants perform in additional degradation assays using other MAGUK proteins such as hDlg, hSRCIB, MAGI-2 and MAGI-3. These experiments may show that different regions of the E6 protein are required to induce the degradation of different the MAGUK proteins. Based on results obtained from these experiments, a clearer insight into which of the MAGUK proteins are processed similarly and/or differently for degradation could be

achieved. Based on their similarity to each other, it could be predicted that MAGI-1, -2 and -3 are processed similarly for degradation. As for what specific mechanism is used for the degradation of MAGUK proteins, this still remains unknown.

4.0. CONCLUSION

This study examined two aspects of HPV oncogenicity. From an epidemiological point of view, HPV-16 variants that were present in a population of cervical cancer samples were studied and it was found that in this sample population, no mutations were found to be indicative of an increased association with cervical cancer. On the other hand, the specific interaction of high-risk HPV E6 protein with the MAGUK protein, MAGI-1 was also observed and it was found that the entire E6 protein was required for E6-induced MAGI-1 degradation.

This study opens up the possibility of studying HPV oncogenicity further. With the cervical cancer samples, it would be interesting to look at how their viral proteins (E6 and E7) perform in functional assays which test factors known to contribute HPV oncogenicity. This could help determine which activities of E6 and E7 determine HPV's oncogenicity. In conjunction with this, it must be determined if the PDZ-binding domain is required for transformation to occur, as this would be a novel contributing factor to HPV oncogenicity. The information gained from these future studies would provide more insight into what makes a specific HPV type more oncogenic than others.

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