Towards uncovering polyomavirus-carrying human cancers

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Towards Uncovering Polyomavirus-Carrying Human Cancers: Pathology and Implications for Tumorigenesis

Faisal Mahdi Klufah

**Towards Uncovering Polyomavirus-Carrying
Human Cancers:**
Pathology and Implications for Tumorigenesis

Faisal Mahdi Klufah

The studies in this thesis were performed in the Pathology Department, Maastricht University Medical Centre+ within the School for Oncology and Developmental Biology (GROW) at the faculty of Health, Medicine, and life sciences at Maastricht University. This thesis was financially supported by Albaha University, Saudi Arabia.

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Towards Uncovering Polyomavirus-Carrying Human Cancers: Pathology and Implications for Tumorigenesis

DISSERTATION

To obtain the degree of Doctor at Maastricht University,
on the authority of the Rector Magnificus,
Prof.dr. Rianne M. Letschert
in accordance with the decision of the Board of Deans,
to be defended in public on Wednesday 19th of January 2022, at 10.00 hours

By

Faisal Mahdi Klufah

Born 26th October 1984 in Jazan, Saudi Arabia

Promotores:

Prof. Dr. Axel zur Hausen

Prof. Dr. Ernst-Jan Speel

Co-promotor:

Dr. Véronique Winnepenninckx

Assessment Committee:

Prof. Dr. M.G.J Tilanus (Chairman)

Prof. Dr. Ugo Lionel Moens (The Arctic University of Norway)

Prof. Dr. GA van Koeveringe

Dr. A.J.C van den Brule (Jeroen Bosch Hospital, 's-Hertogenbosch, The Netherlands)

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CHAPTER 1

General introduction

General Introduction

Cancer is one of the leading causes of death in humans worldwide and cancer-related mortality is still globally increasing^{1,2}. In 2018, approximately 18 million new cancer cases have been diagnosed, and cancer accounted for 9.6 million deaths in that same year worldwide¹. Of interest, trends in incidence and mortality rates of tumors reveal wide geographical variations^{1,3}. The most common risk factors for cancer include exposure to chemicals, smoking, alcohol, radiation, and inherited genetic mutations^{1,2}. Amongst many other factors, infectious agents play an essential role in the etiology of cancer^{1,2}. Infections constitute a well-established causative link with an increased cancer risk in humans, and it is estimated that up to 20% of cancers worldwide are associated with high-risk infectious agents^{2,4-6}. However, the etiology of most human cancers is still unknown. Therefore, screening for emerging infectious agents potentially causing or contributing to human tumorigenesis is crucial to effectively avoid and limit exposure to these potential carcinogenic agents. Ultimately, identifying the presence of novel human tumor viruses or other infectious agents in cells and tissues may be an important opportunity for cancer prevention and early detection if studies will prove their causal/functional contribution to the development of pre-malignant lesions and progression to cancer.

Infectious agents

Several lines of evidence have established an association between infectious agents and tumorigenesis. According to the GLOBOCAN data in 2018, nearly 2.2 million cancer cases were related to specific infections⁴. Based on the International Agency for Research on Cancer (IARC) report, eight viruses, three parasites, and one bacterium infectious agent have been classified as carcinogens^{2,7,8}. *Helicobacter pylori* is a gram-negative bacterium causing non-cardia gastric cancer and low-grade B-cell MALT lymphoma⁹. Three macroparasitic infections have been associated with the etiopathogenesis of human cancers, of which *Opisthorchis viverrine* and *Clonorchis sinensis* were found to be associated with cholangiocarcinoma, while *Schistosoma haematobium* infection is the one associated with inducing bladder carcinoma^{6,7}.

Oncogenic viruses play a major role in human cancers and currently eight human viruses have been shown to be oncogenic in humans. Five of these oncogenic viruses are well-known DNA viruses, i.e. Epstein-Barr virus (EBV), hepatitis B virus (HBV), human papillomaviruses (HPVs), Kaposi's sarcoma herpesvirus (KSHV), and Merkel cell polyomavirus (MCPyV)^{2,7,8}. The remaining three are RNA viruses, i.e. the human T-lymphotropic virus-1 (HTLV-1), hepatitis C virus (HCV), and the human immunodeficiency virus type 1 (HIV-1)^{6-8,10,11}. A more detailed list of these human tumor viruses and the related human cancers is shown in Table1.

Viruses and tumors

The significance of tumor viruses in human carcinogenesis has been studied intensely, and the discovery and characterization of oncogenic viruses paved the way to understand the etiopathogenesis, diagnosis, and treatment of a variety of human malignancies². It is of particular interest to both identify and study potential human tumor viruses to develop potential vaccines, treatments, and prevention plans. It is generally accepted that infection with a potential human tumor-virus alone is not enough to cause human cancers. Additional predisposing factors appear to be involved, such as immunosuppression or environmental factors.

Viruses as direct or indirect carcinogens

Oncogenic viruses exert their oncogenic potential either by direct or indirect contribution to carcinogenesis Figure 1^{12, 13}. To date, five human oncogenic DNA-viruses have been identified, four of which are directly involved in the tumorigenesis, e.g. by the expression of viral oncoproteins that deregulate cellular tumor suppressor pathways^{2, 8, 12, 13}. A defined set of viral proteins of EBV plays a direct role in the cellular transformation to Burkitt's lymphoma through targeting cellular suppressor genes^{14, 15}. The direct role of HPV replication in oncogenesis is well established by the integration of the viral genome into the cell, and the viral early genes E6 and E7 have the oncogenic property to transform cells¹⁶⁻²⁰. The closely related KSHV genome encodes oncogenic proteins to target cellular tumor suppressor proteins that may induce Kaposi sarcoma (KS)^{21, 22}. MCPyV is the most recently identified DNA-tumor virus, which was found to be clonally integrated into the Merkel cell carcinoma (MCC) genome and carries tumor-specific mutations within the large tumor antigen (LTAg)^{10, 11, 23}. HBV is the only known DNA-virus that is indirectly promoting the development of hepatocellular carcinoma (HCC) through induction of chronic inflammation²⁴⁻²⁷.

Next to these DNA-tumor viruses, three RNA viruses have been identified to be associated with human carcinogenesis. HTVL-1 is a retrovirus linked directly to the tumorigenesis of aggressive T-cell lymphoma (ATL)²⁸. Tax is the known HTVL-1 oncoprotein that plays key role in the transformation of human T-lymphocytes to ATL²⁹. HCV and HIV-1 are the other two RNA-tumor viruses that induce human cancer by indirect mechanisms via the interaction between viral proteins, environmental factors, inflammation and/or hereditary risk factors Figure 1^{12, 25, 30-34}.

Viral infections and subsequent viral latency precede the development of human cancers years before clinical detection. Latent infections cause chronic inflammatory reactions due to the immune response to chronically persisting exogenous viral antigens, which are not sufficiently eradicated by the immune system and thus results via acute inflammation in chronic inflammation^{35, 36}. Reactive oxygen radicals can be produced years later

Figure 1: Models of direct and indirect viral carcinogenesis in humans. ROS; reactive oxygen species. (created in BioRender.com)

following chronic inflammation and may trigger modifications in the host nucleic acids and subsequently induce transformation, as it is known for HBV and HCV viruses^{35, 37}. Persistent infections with HBV or HCV viruses are responsible for most HCC cases in HBV and HCV endemic regions due to the indirect effects of chronic inflammation and progressive fibrosis^{24-26, 38}. For instance, HCV virus is not detected in the cancer cells that may develop after many years of infection but is often found in the lesions adjacent to the hepatocellular carcinoma³⁰⁻³³. Some other tumor viruses are incapable of transforming cells by itself, and rather serve as a cofactor for some malignancies, such as postulated for HIV-1³⁹. Infection with HIV-1 is not sufficient for obtaining malignant transformation. However, co-infection of HIV-1 and other viruses, e.g. KSHV, apparently play an important role in the initiation of neoplastic transformation³⁴.

Table 1: Viruses linked to human cancers

Virus genome	Oncogenic virus	Virus family (taxonomy)	Year identified	Approximate genome size (Kb)	Mechanism of tumorigenesis *	Human malignancy associated to viral infection	Ref.
DNA	EBV	Herpesviridae	1964	174	Direct	Burkitt lymphoma, nasopharyngeal carcinoma, Hodgkin's lymphoma, non-Hodgkin lymphoma, extranodal NK/T-cell lymphoma, and gastric carcinoma	(2, 15)
	HBV	Hepadnaviridae	1965	3	Indirect	Hepatocellular carcinoma	Non-Hodgkin lymphoma and cholangiocarcinoma (2, 27)
	HPV	Papillomaviridae	1983	8	Direct	Carcinoma of the cervical, anus, vulva, vagina, penis, oral cavity, oropharynx, and tonsil	Cancer of the larynx (2, 19)
RNA	KSHV	Herpesviridae	1994	137	Direct	Kaposi sarcoma and primary effusion lymphoma	Multicentric Castleman's disease (2, 22)
	MCPyV	Polyomaviridae	2008	5	Direct	Merkel cell carcinoma	- (2, 11)
	HTVL-1	Retroviridae	1980	9	Direct	Adult T-cell leukemia/lymphoma	- (2, 28)
	HCV	Flaviviridae	1989	9.6	Indirect	Hepatocellular carcinoma and non-Hodgkin lymphoma	Cholangiocarcinoma (2, 25)
	HIV-1	Retroviridae	1981	9.6	Indirect	Hodgkin and non-Hodgkin lymphoma, Kaposi's sarcoma, cancer of cervical, anus, and conjunctiva	Cancer of genital tract (vagina, vulva, and penis), non-melanoma skin cancer, and hepatocellular carcinoma (2, 39)

* Mechanism of tumorigenesis as described in Figure 1
EBV: Epstein-Barr virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HIV-1: human immunodeficiency virus type 1; HPV: Human papillomavirus; HTVL-1: Human T-lymphotropic virus-1; KSHV: Kaposi's sarcoma herpesvirus; MCPyV: Merkel cell polyomavirus

Polyomaviruses

In this thesis, the focus will be on polyomaviruses (PyVs), which are small, unenveloped, circular and double-strand DNA viruses with a size range between 4.5–5.4 kilobase (kb) that belong to the family of polyomaviridae, Figure 2^{40–42}. The PyV family has been significantly expanded in the last decade and consists of more than 102 species capable of infecting mammals, birds, and fish as their host^{41, 43, 44}. The murine polyomavirus (MuPyV) was the firstly discovered polyomavirus in 1953, which proved to induce different types of tumors in animal models^{45, 46}. In 1953, Eddy *et al.* showed that injection of MPyV in mice, hamster, and rat newborns lead to different neoplasms^{47, 48}. In 1960, Sweet and Hilleman discovered the simian vacuolating virus 40 (SV40) in rhesus monkey kidney cells⁴⁹. SV40 was shown to be an oncogenic virus in animal models, but although SV40 DNA was found to be present in many different human tumors, it was neither considered a human polyomavirus nor as a human carcinogen^{8, 50}. Polyomaviruses have been shown to be strongly oncogenic in heterologous animal models, which point to the possibility that PyVs play a role in human diseases, although yet no decisive role has been proven^{45, 51, 52}.

Human Polyomaviruses

JC polyomavirus (JCPyV) and BK polyomavirus (BKPyV) were the first human polyomaviruses isolated from patients in 1971^{53, 54}. JCPyV was isolated from brain tissues and has been shown to cause progressive multifocal leukoencephalopathy (PML) in immunosuppressed patients⁵⁴. BK was isolated from the urine of a renal allograft recipient and has been shown to cause hemorrhagic cystitis, also in immunosuppressed

Figure 2: General features of polyomavirus. Circular double-strand DNA genome with non-enveloped capsid. The early gene contains small and large tumor antigens, while the late gene contains the three capsid structural proteins VP1, VP2, and VP3. (created in BioRender.com)

patients⁵³. JCPyV and BKPyV have been shown to induce cancer in rodent models and have the ability to target the tumor suppressor protein p53 in a similar way as SV40^{51, 52}.

Since 2007, 13 new PyVs have been isolated from human specimens, and 10 of them were confirmed to be human polyomaviruses (HPyV's) Table 2. In 2007, two new members of the HPyVs family were identified, i.e. the Karolinska Institute polyomavirus (KIPyV) and the Washington University polyomavirus (WUPyV). Both KIPyV and WUPyV were isolated from nasopharyngeal aspirate (NPA) specimens of lower respiratory tract disease in children^{55, 56}. Yet, no human disease has been associated with one of the two viruses^{55, 56}.

In 2008, the fifth HPyV called Merkel cell polyomavirus (MCPyV) was discovered and linked to the pathogenesis of Merkel cell carcinoma (MCC)⁵⁷. MCC is an extremely aggressive, relatively rare non-melanoma skin cancer of unknown cellular origin, affecting especially elderly and immunosuppressed patients^{11, 58}. MCPyV DNA was found to be present and clonally integrated into the tumor DNA in approximately 80% of MCCs. In addition, in MCC the large tumor antigen (LTag) gene proved to carry point mutations introducing stop codons, leading to expression of a truncated helicase domain^{10, 11}. This renders a deficient virus replication and thus promotes the mutated MCPyV supertransforming properties. In particular, the binding of truncated LTag to the retinoblastoma protein (pRB) maintains the replication capacity in MCPyV-positive cell lines Figure 3^{42, 59}. In addition, MCPyV tumor antigen has been described to bind and inhibit the cell cycle regulator gene p53 gene to induce proliferation⁶⁰.

Consequently, MCPyV has been categorized as "Group 2A carcinogen: probably carcinogenic to humans" carcinogen by the International Agency for Research on Cancer (IARC) in 2012⁸. Of interest, MCPyV-positive MCC seems to have a better response to treatment and a favorable survival rate compared to MCPyV-negative MCC, which has been shown to be associated with UV damage and with a very high tumor mutational burden⁶¹⁻⁶⁵. The finding of MCPyV in chronic lymphocytic leukemia (CLL) patient specimens, with and without a 246bp deletion in the helicase region of the LTag, provided more evidence of the likely association of MCPyV with CLL⁶⁶, as did the finding of MCPyV in neoplastic B cells e.g., in CLL cells. This opened the spectrum for further exploration of possibly MCPyV-associated human cancers⁶⁶⁻⁶⁸.

In 2010, three new HPyVs were discovered. Human polyomavirus 6 (HPyV6) and human polyomavirus 7 (HPyV7) were isolated simultaneously from healthy skin swabs using a modified rolling circle amplification (RCA) technique. The complete HPyV7 genome was found to share about 68% of HPyV6 nucleotides⁶⁹. In addition, trichodysplasia spinulosa-associated polyomavirus (TSPyV) was isolated from plucked facial spines of a

Figure 3: Schematic diagram showing MCPyV-induced Merkel cell carcinoma tumorigenesis. (created in BioRender.com)

heart transplant patient. TSPyV was found to be associated with a very rare skin condition called trichodysplasia spinulosa (TS) in an immunocompromised patient⁷⁰. These newly discovered HPyVs raised the question about their potential role in the etiopathogenesis of other human diseases and cancers. Recently, literature reported the seroprevalence and prevalence of HPyV6 and 7 DNA in neoplastic diseases, such as melanoma, as well as in non-neoplastic diseases, such as chronic tonsillitis⁷¹⁻⁷⁵.

In 2011, Human Polyomavirus 9 (HPyV9) was identified from different human specimens. Scuda et al. detected HPyV9 from a serum sample of a kidney transplant patient who was receiving an immunosuppressive treatment⁷⁶. In addition, Sauvage et al. detected HPyV9 from a cutaneous swab obtained from an MCC patient⁷⁷. However, several studies screened for HPyV9 in different tumor specimens, and no evidence was found for the presence of HPyV9 in diverse skin cancers including basal cell carcinoma, squamous cell carcinoma, keratoacanthoma, microcystic adnexal carcinoma, atypical fibroxanthoma, actinic keratosis, and mucosal melanoma^{78, 79}. Also, HPyV9 could not be detected in other neoplasms, such as CLL and glioblastoma^{80, 81}. Thus, no evidence of clinical relevance has been shown so far for the presence of HPyV9 in either serum or skin.

Later in 2012, both Malawi polyomavirus (MWPpyV) and St Louis polyomavirus (STLPyV) were isolated from fecal samples of healthy children⁸²⁻⁸⁴. Furthermore, MWPpyV (also known as HPyV10) was identified in the skin of a patient with warts,

hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome ⁸⁵. In addition, the prevalence of HPyV10 (MWPyV) was reported in diverse specimens other than stool ⁸⁶⁻⁸⁸. Rockett et al. reported the presence of MWPyV in respiratory secretions of both symptomatic patients (no further details were reported) and healthy individuals ⁸⁶. In addition, MWPyV was detected in the tonsil and adenoid lymphoid tissues of immunocompetent children ^{87, 88}. MWPyV tumor antigen (TAg) was found to bind with tumor suppressor proteins p53, pRB, and PP2A, however, the TAg was unable to promote cellular proliferation ⁸⁹. Likewise, STLPyV was isolated from stool specimens as well as other specimens including tonsillar tissues and the skin of condylomas patients ^{88, 90}.

In 2013, human polyomavirus 12 (HPyV12) was initially isolated from liver specimens, then confirmed to be present in the colon and rectum specimens of patients with malignancy ⁹¹. HPyV12 has no definitive association with a human disease yet. Indeed, a very similar sequence of HPyV12 was found to be commonly present in shrews ⁹². It has been suggested that detecting HPyV12-positive human specimens was due to external artifact sources, such as laboratory contamination ⁹²⁻⁹⁴. This is supported by the findings that HPyV12 was not detected in diverse specimens from different diseases ^{93, 95-97}. Therefore, the International Committee on Taxonomy of Viruses (ICTV) excluded HPyV12 from the list of HPyVs ⁴⁴.

In 2014, New Jersey polyomavirus (NJPyV) was isolated from vascular endothelial cells of a pancreatic transplant patient ⁹⁸. Indeed, this patient was reported to be sick two weeks after the exposure to the floodwaters of hurricane Sandy ⁹⁸. NJPyV tissue tropism and its role in human diseases are still not definitive. In addition, serological seroprevalence does not support that NJPyV is indeed infecting humans, as it is also not detected by metagenomic or serologic analyses in humans ⁹⁴. Of interest, NJPyV shows a closely related (70–80%) sequence to a primate polyomavirus, i.e. the chimpanzee polyomavirus (ChPyV) ⁹⁹.

In 2017, a new polyomavirus was identified in human skin swabs and named Lyon IARC PyV (LIPyV) ¹⁰⁰. Yet, similarly to HPyV12 and NJPyV, LIPyV has not been detected by metagenomic analyses in human specimens ⁹⁴. However, LIPyV was detected in cat faces and found in human specimens most likely due to contamination ¹⁰¹. Recently, in 2019, Quebec polyomavirus (QPyV) was isolated from the stool of human patients by metagenomic analysis ⁹⁴. So far, only one study tested the presence of QPyV in urine, CSF, nasopharyngeal aspirates, and plasma specimens obtained from diverse non-neoplastic diseases, and revealed that QPyV-DNA is detected at a low frequency in the urine specimens of SLE patients, pregnant women, and multiple sclerosis patients ¹⁰². However, no other data are available about QPyV and thus further confirmation of this virus in human tissues is required.

Table 2 Polyomaviruses isolated from human and associated diseases

#	Polyomavirus isolated from human	Genome Size (bp)	Isolated from	Associated disease	Year of discovery	Reference
1	BK polyomavirus (BKPyV)	5141	Urine	Nephropathy and hemorrhagic cystitis	1971	(53)
2	JC polyomavirus (JCPyV)	5130	Brain tissue and urine	Progressive multifocal leukoencephalopathy	1971	(54)
3	Karolinska Institute polyomavirus (KIPyV)	5040	Respiratory tract	Not defined	2007	(55)
4	Washington University polyomavirus (WUPyV)	5229	Respiratory tract	Not defined	2007	(56)
5	Merkel cell polyomavirus (MCPyV)	5387	Merkel cell carcinoma	Merkel cell carcinoma	2008	(11)
6	Human polyomavirus 6 (HPyV6)	4926	Skin swabs	Pruritic skin eruption in immunocompromised	2010	(69)
7	Human polyomavirus 7 (HPyV7)	4949	Skin swabs	Pruritic skin eruption in immunocompromised	2010	(69)
8	Trichodysplasia spinulosa polyomavirus (TSPyV)	5232	Plucked facial spines	Trichodysplasia spinulosa and pilomatrix dysplasia	2010	(70)
9	Human polyomavirus 9 (HPyV9)	5026	Skin, blood and urine	Not defined	2011	(76, 77)
10	Malawi polyomavirus (MWPyV)/ MX polyomavirus (MXPpyV)/ HPyV10	4939	Fecal samples and wart	Not defined	2012	(82, 83, 85)
11	St Louis polyomavirus (STLPyV)	4776	Fecal samples	Not defined	2012	(84)
12	Human polyomavirus 12 (HPyV12)	5033	Liver and gastrointestinal tract	Not defined	2013	(91)
13	New Jersey polyomavirus (NJPyV)	5108	Vascular muscle endothelium	Not defined	2014	(98)
14	Lyon IARC PyV (LIPyV)	5269	Skin swab	Not defined	2017	(100)
15	Quebec polyomavirus (QPyV)	4973	Stool	Not defined	2019	(94)

The aim and outline of this thesis:

The etiopathogenesis and risk factors of many neoplasms are still unclear. In the last decade, the human polyomaviruses family has been expanded to 12 HPyVs **Figure 4**. Yet, MCPyV is the only human tumor virus of the HPyVs family, and this raised significant attention to the potential role of other novel HPyVs in human tumorigenesis.

The aim of this thesis was to investigate and assess the potential contribution of known and novel HPyVs in a spectrum of human cancers. A broad spectrum of molecular techniques was used to test for the presence of these HPyVs. **Chapter 2** reviews the current evidence to evaluate a possible role of the two closely related viruses HPyV6 and 7 in the etiopathogenesis of neoplastic human diseases. In **chapter 3**, the prevalence of HPyVs in cholangiocarcinoma (CCA) tissues was investigated by screening for HPyV using diverse molecular techniques, including degenerate and virus-specific DNA PCR, FISH, RISH, and IHC. In **chapter 4**, the focus on the relation of BK virus-positive urinary cytology (presence of Decoy cells) to urothelial cell carcinoma (UCC) of the urinary bladder was assessed. In addition, the relation of positive BKPyV in the urine of UCC to a previously given intravesicular BCG or mitomycin immunotherapy treatment was evaluated. In **chapter 5** it was investigated, if MCPyV plays a major role in the

Figure 4: A Brief timeline showing the years of discovery of human polyomaviruses

etiopathogenesis of human epithelial thymic tumors. For this purpose, diverse molecular techniques were applied to screen for MCPyV in a clinicopathologically well-characterized thymoma cohort, in which our research group has shown previously a high prevalence of HPyV7. In **chapter 6**, the presence of three different tumor viruses, i.e. MCPyV, human papillomavirus (HPV), and Epstein-Barr virus (EBV), were assessed in a unique large cohort of non-smokers and non-drinkers with head and neck squamous cell carcinoma (HNSCC) was performed. **Chapter 7** discusses the findings and implications of this thesis and future perspectives about the role of HPyVs in human tumorigenesis. In addition, to address the overall strengths and limitations of methods and techniques of HPyVs screening. Finally, we provide a summary and the overall conclusions of our findings.

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CHAPTER 2

2

Emerging Role of Human Polyomaviruses 6 and 7 in Human Cancers

Faisal Klufah, Ghalib Mobaraki, Dan Liu, Raed A. Alharbi, Anna Kordelia Kurz, Ernst Jan M. Speel, Véronique Winnepenninckx, and Axel zur Hausen

ABSTRACT

Background

Currently 12 human polyomaviruses (HPyVs) have been identified, 6 of which have been associated with human diseases, including cancer. The discovery of the Merkel cell polyomavirus and its role in the etiopathogenesis in the majority of Merkel cell carcinomas has drawn significant attention, also to other novel HPyVs. In 2010, HPyV6 and HPyV7 were identified in healthy skin swabs. Ever since it has been speculated that they might contribute to the etiopathogenesis of skin and non-cutaneous human cancers.

Main body

Here we comprehensively reviewed and summarized the current evidence potentially indicating an involvement of HPyV6 and HPyV7 in the etiopathogenesis of neoplastic human diseases. The seroprevalence of both HPyV6 and 7 is high in a normal population and increases with age. In skin cancer tissues, HPyV6- DNA was far more often prevalent than HPyV7 in contrast to cancers of other anatomic sites, in which HPyV7 DNA was more frequently detected.

Conclusion

It is remarkable to find that the detection rate of HPyV6-DNA in tissues of skin malignancies is higher than HPyV7-DNA and may indicate a role of HPyV6 in the etiopathogenesis of the respected skin cancers. However, the sheer presence of viral DNA is not enough to prove a role in the etiopathogenesis of these cancers.

KEYWORDS

HPyV6, HPyV7, Merkel cell polyomavirus, oncogenic viruses, cutaneous tumors, viral persistence, tumor virus.

Background

Polyomaviruses (PyV's) comprise a family of non-enveloped viruses containing a small genome of approximately 5.0 kbp in size of circular double-stranded DNA and are capable of infecting mammals and birds ^{1,2,3}. PyV's are strongly oncogenic in heterologous animal models, suggesting that next to human also non-human polyomaviruses could possibly play a role in human cancers ^{4,5,6,7}. To date, fifteen novel PyV's have been isolated from different human specimens ⁸. In 2019, the International Committee on Taxonomy of Viruses (ICTV) reported only twelve human polyomaviruses (HPyVs) after having HPyV12 excluded, which was shown to be infecting shrews ^{9, 10}. In addition, the Lyon IARC polyomavirus which initially was isolated from human skin swab in 2017 is also found in cats feces ¹¹. Recently in 2019, Ondov et al. isolated the Quebec PyV from a stool sample of one patient through the MinHash algorithm ¹². Yet it still has not been listed as a human polyomavirus by the ICTV ⁹. Six of the identified HPyVs have been associated with human diseases, including cancer ^{3, 8, 13, 14}.

In 2008, Merkel cell polyomavirus (MCPyV) has been linked to the etiopathogenesis of the majority (i.e. 80%) of the highly aggressive neuroendocrine Merkel cell carcinoma (MCC) ^{15,16,17}. Already in 2012 MCPyV has been categorized as a group 2A carcinogen by the International Agency for Research on Cancer (IARC) ¹⁸. MCPyV has also been detected in non-neoplastic B cells (e.g. reactive hyperplasia and normal lymph node) and neoplastic B cells (e.g. chronic lymphocytic leukemia cells,) suggesting a role for MCPyV in B-cell neoplasias ^{15, 19,20,21,22}.

In 2010, HPyV6 and HPyV7 were identified from healthy skin swabs using the rolling circle amplification technique ²³. The HPyV7 genome shares approximately 68% sequence identity with HPyV6 ²³. As these novel HPyVs were discovered in the skin, their presence in the skin and non-cutaneous human cancers, similar to MCPyV has been studied intensively ^{24,25,26,27,28,29,30,31}. Additionally, HPyV6 and 7 proteins have been suggested to play a key role in the binding and deactivation of p53 ³². The oncogenic role of HPyVs in general has been reviewed extensively ^{3, 7, 13, 33,34,35,36}. By reviewing sero-epidemiological and tumor-pathological evidence, we here focus on the possible oncogenic role of HPyV6 and 7 in human cancers, and in particular their direct or indirect contribution to tumorigenesis.

Seroprevalence of HPyV6 and HPyV7 in healthy individuals

Several studies have assessed HPyV6 and HPyV7 serum antibody levels across a broad range of age groups of various populations ^{23, 37,38,39,40,41}. In 2010, 95 sera from blood donors were tested for the seroprevalence of HPyVs and revealed higher seropositivity for HPyV6 (69%) than HPyV7 (35%) using direct enzyme-linked immunosorbent assays

(ELISA) directed against virus-like particles (VLP) based on viral protein 1 (VP1) capsid²³. Likewise, by using ELISA against VP1-VLP, another study estimated the seroprevalence of age groups ranging from 20 years and older and concluded that the prevalence of HPyV6 increased with age and was higher than that of HPyV7 (80–92% vs 60–80%, respectively)³⁷. The HPyVs seroprevalence of 1050 Dutch blood donors aged from 18 to 69 was determined and showed that the prevalence of both viruses increased with age, with 84 and 72% seropositivity for HPyV6 and HPyV7 VP1 antibodies, respectively³⁸. Nicol et al. assessed the seroprevalence of both viruses in 828 subjects across different age groups starting from age one using VLP-based direct ELISA. Their study found that HPyV6 (76%) was more prevalent than HPyV7 (53%) across all age groups³⁹. Noteworthy, by performing Luminex xMAP technology to detect VP1 antibodies for both viruses were detected in the early infancy group up to an age of 6 months with high frequency (80.1% for HPyV6 and 58.1% for HPyV7, respectively). On the other hand, a decline in the prevalence of both viruses was found for children age 6 months to 14 years old group⁴⁰.

Altogether, both HPyV6 and 7 were found to be ubiquitous and to infect all age groups, and HPyV6 seroprevalence is slightly higher than HPyV7. Seroprevalence significantly increases with age, and a substantial proportion of individuals 50 years of age and older has tested positive for HPyV6 and HPyV7. Approximately 52–93% of humans are seropositive for HPyV6, whereas 33–84% are seropositive for HPyV7 according to the seroprevalence studies Table 1^{23, 37,38,39,40, 42, 43}. Summarizing the overall age-specific seropositivity of HPyV6 and HPyV7 using ELISA or Luminex platform-based method was shown in (Fig. 1).

Fig. 1 Summarizing age-specific seroprevalence overall positivity of HPyV6 and HPyV7 using ELISA or Luminex platform-based methods to analyze antibodies against virus-like particles and/or glutathione-S-transferase consisting HPyV6 and 7 viral protein

Table 1 Seroprevalence of HPyV6 and HPyV7 antibodies in serum using ELISA or Luminex platform

Source of the specimen	Age (y)	Samples n	HPyV6 Positive n (%)		HPyV7 Positive n (%)		Assay	Reference
			VLP	GST	VLP	GST		
18–65 (y) Blood donors. < 18 or > 65 (y) From discarded lab samples	1–4	48	18 (37.5)		5 (10.4)		ELISA	[39]
	5–9	69	40 (57.9)		23 (33.3)			
	10–14	92	65 (70.6)		41 (44.6)			
	15–19	89	55 (61.8)		32 (36)			
	20–29	49	43 (87.8)		22 (44.9)			
	30–39	73	49 (67.1)		31 (42.5)			
	40–49	105	89 (84.8)		62 (59.1)			
	50–59	95	78 (82.1)		64 (67.4)			
	60–69	100	88 (88)		68 (68)			
	70–79	52	46 (88.5)		42 (80.8)			
	≥ 80	56	55 (98.2)		48 (85.7)			
Blood donors	20–29	96	82 (85.4)	77 (80.2)	58 (60.4)	68 (70.8)	ELISA	[37]
	30–39	129	114 (88.4)	111 (86)	92 (71.3)	106 (82.2)		
	40–49	52	48 (92.3)	48 (92.3)	32 (61.5)	40 (76.9)		
	50–59	24	22 (91.7)	21 (87.5)	16 (66.7)	19 (79.2)		
	> 59	5	4 (80)	4 (80)	3 (60)	4 (80)		
Serum obtained for routine lab tests	0–0.5	31		25 (80.6)		18 (58.1)	Luminex platform	[40]
	0.6–1.9	63		14 (22.2)		12 (19)		
	2–3	62		21 (33.9)		12 (19.4)		
	4–5	58		26 (44.8)		19 (32.8)		
	6–7	58		29 (50)		21 (36.2)		
	8–9	70		44 (62.9)		27 (38.6)		
	10–14	55		31 (56.4)		21 (38.2)		
	15–19	59		38 (64.4)		25 (42.4)		
	20–29	59		39 (66.1)		30 (50.8)		
	30–39	64		41 (64.1)		35 (54.7)		
	40–49	54		34 (63)		33 (61.1)		
	50–59	58		46 (79.3)		36 (62.1)		
	60–69	54		50 (92.6)		45 (83.3)		
	> 70	54		50 (92.6)		46 (85.2)		
Blood donors	18–29	208		156 (75.7)		117 (56.8)	Luminex platform	[38]
	30–39	209		175 (84.5)		154 (74.4)		
	40–49	208		173 (83.6)		155 (74.9)		
	50–59	215		184 (85.6)		154 (71.6)		
	60–69	210		187 (89.5)		169 (80.9)		
Blood donors		95	66 (69)		33 (35)		ELISA	[23]
SCCs transplanted cases		110		65 (59)		57 (51.8)	ELISA and Luminex platform	[42]
Non-SCCs transplanted cases		207		137 (66.2)		85 (41)		
Lung cancer		511	393 (76.9)		333 (65.2)		Luminex platform	[43]
Controls		508	400 (78.7)		334 (65.8)			

ELISA enzyme-linked immunosorbent assays, GST glutathione-S transferase, SCC squamous cell carcinoma, VLP virus-like particles, (y) years old

In general, HPyV6 and 7 infections seem to be clinically rather asymptomatic, irrespective of the age at which the infection occurs. Increasing seroprevalence of these viruses is clearly age-related and reaches its peak in individuals beyond 40 years of age, possibly indicating a relationship of HPyV6 and 7 infections with immunosenescence. Regarding the methodology, the previous studies reported the seroprevalence of HPyV6 and HPyV7 in the sera obtained from blood donors or routine lab tests in various studies. Most of the studies used ELISA or Luminex platform antibody-binding assay methods to detect the antigen containing the structural viral capsid protein of the targeted viruses fused with VLP or glutathione S-transferase (GST) proteins Table 1. Moreover, one study assessed the agreement and sensitivity of both VLP and GST-based ELISA methods in detecting seropositivity ³⁷. The results showed only minor discrepancies in the seroprevalence comparing these two detection methods ³⁷. Kamminga et al. reported high seropositivity of HPyV6 and 7 by using a Luminex xMAP assay directed against the VP1 capsid of HPyVs, similar to other ELISA-based studies ^{37, 39,40,41}.

Seroprevalence of HPyV6 and HPyV7 in cancer patients

Only a very limited number of studies have reported seroprevalence data of HPyV6 and 7 in cancer patients. Madeleine et al. recently reported that the seroprevalence of HPyV6 in immunosuppressed skin cancer patients (59.1%) was lower compared to healthy controls (66.2%), whereas the seroprevalence of HPyV7 in the same patient cohort was slightly elevated (52%) compared to the control group (41%) ⁴². It is important to note that the ELISA GST detection method used by Madeleine et al. yielded a much lower seroprevalence compared to other studies using the same technique. Similarly, serum from non-smoking-related lung cancer patients showed positivity for HPyV6 (76.9%) and HPyV7 (65.2%), which was comparable to the seroprevalence of these viruses in normal population age groups older than 50 years ⁴³. HPyV6 and 7 seropositivity data and results, including methodology, are summarized in Table 1.

In the past, studies on the seroprevalence of other human tumor viruses, e.g. Epstein-Barr virus or human papillomaviruses (HPV), have helped to indirectly indicate whether or not these viruses are involved in the etiopathogenesis of human cancers ^{44,45,46,47,48}. Moreover, the infection by small DNA tumor viruses at an early age, such as high-risk HPV, has been identified as a potential cause of human cancer decades after infection ^{47,48,49}. Also, HPyV6 and 7 antibodies are already detectable in the sera of healthy individuals at an early age Table 1 indicating that a substantial number of individuals – if not the majority–acquire both viruses already during their early lifetime, which might be a prerequisite for the onset of a possibly associated disease or cancer later in life.

Prevalence of HPyV6 and HPyV7 DNA in non-neoplastic diseases

A limited number of studies have screened for the prevalence of HPyV6- and HPyV7-DNA in non-neoplastic diseases, with very low prevalence across a variety of specimens Table 2. Both HPyVs were reported in Kimura's disease—a chronic inflammatory disorder associated with lymphadenopathy—with 4/5 and 1/5 samples found to be positive for HPyV6- and HPyV7-DNA, respectively ⁵⁰. Only HPyV6-DNA was detected in lichen simplex chronicus (1/13 samples), Kikuchi disease, i.e. histiocytic necrotizing lymphadenitis (3/8 samples), dermatopathic lymph-adenitis (1/28 samples), and angiolymphoid hyperplasia with eosinophilia (ALHE) (4/5 samples) ^{24, 54, 55}. In addition, only one study showed the presence of HPyV6-DNA in lymphocytes of lymph nodes tissue from ALHE case by fluorescence in situ hybridization (FISH) ⁵⁴.

Studies conducted on patients with cutaneous diseases have reported the presence of both HPyV6- and 7-DNA in tissue specimens. Three dyskeratotic dermatosis cases tissue revealed 2 positives for HPyV6 and 1 positive for HPyV7, and in previously 12 HPV-induced warts tested showed that 3 positives for HPyV6 and 8 for HPyV7 ^{50, 53}. In addition, HPyV7-DNA was found in all three biopsies of pruritic patients, thus the diagnostic term HPyV 7-associated pruritic rash has been introduced in transplantation medicine ^{51, 52}. The higher prevalence of both viruses in skin disease samples could be attributed to their high occurrence in the skin of healthy individuals. However, the viral load and prevalence of both viruses seem to increase in the skin of elderly people, in particular immunosuppressed individuals, which may lead to associated skin disorders ⁶⁵.

The prevalence of HPyV6- and 7-DNA in non-neoplastic non-cutaneous diseases appears to be much lower compared to non-neoplastic skin diseases. Our group reported the presence of HPyV7-DNA in (3/8; 40%) of non-malignant thymic hyperplasia ³¹. Other studies detected HPyV6-DNA in (12/226; 5.3%) of tonsillitis tissue specimens Table 2 ^{56,57,58}. The detection rate of both viruses was found to be slightly higher in tissue specimens in contrast to body fluids specimens ^{24, 31, 50,51,52,53,54, 56,57,58,59,60,61, 63, 64}. For instance, in cerebrospinal fluid, HPyV6-DNA was detected in (1/14; 7.1%) samples from HIV patients with leukoencephalopathies and (1/135; 0.7%) samples from patients with meningoencephalitis ^{61, 62}. In addition, (5/91; 5.5%) bile fluid samples from acute gallstone cholangitis patients tested positive for HPyV6 ⁶⁴. In comparison, there was a low detection rate (<2%) of both viruses in nasopharyngeal aspirates specimens collected from respiratory symptomatic patients ^{59, 60}. Similarly, the presence of HPyV6 and 7 was uncommon (<1%) in the feces obtained from patients with gastrointestinal illness for routine lab investigation ⁵⁹. Overall, the prevalence of HPyV6 and HPyV7 in formalin-fixed-paraffin-embedded (FFPE) and fresh frozen biopsies were elevated compared to liquid biopsy specimens, in which the prevalence of HPyV6 and HPyV7 was 1.1 and 0.2%, respectively Table 2.

Table 2 Prevalence of HPyV6 and HPyV7 in human non-neoplastic diseases

Non-neoplastic disease	Specimen	Samples n	Method	HPyV6 Positive n (%)	HPyV7 Positive n (%)	Reference
Cutaneous diseases						
Dyskeratosis and parakeratosis	FFPE	3	PCR/qPCR/IHC/ Seq.	2 (66.6)	1 (33.3)	[50]
Lichen simplex chronicus	FF	13	rt-PCR	1 (7.7)	ND	[24]
Pruritic in Cardiac transplant recipient	FFPE	1	IHC/PCR	NA	1 (100)	[51]
Pruritic Rash (lung transplant)	FFPE	2	IHC/PCR/RCA PCR/rt-PCR	NA	2 (100)	[52]
Viral warts	FFPE	12	qPCR	3 (25)	8 (66.7)	[53]
Angiolymphoid hyperplasia with eosinophilia or Kimura disease ^a	FF	1	Shotgun metagenomic/ FISH	1 (100)	NA	[54]
Angiolymphoid hyperplasia with eosinophilia	FFPE	5	qPCR/IHC	4 (80)	0	[55]
Kimura disease	FFPE	5	qPCR/IHC	4 (80)	1 (20)	[55]
Kikuchi disease	FFPE	38	qPCR	3 (8)	0	[55]
Dermatopathic lymphadenitis	FFPE	28	qPCR	1 (4)	0	[55]
Non-cutaneous diseases						
Thymic hyperplasia	FFPE	20	PCR/IHC/FISH	NA	8 (40)	[31]
Chronic tonsillitis & tonsillar hypertrophy	FF	78	Luminex-assay	6 (7.7)	ND	[56]
Chronic tonsillitis & tonsillar hyperplasia	FF	40	qPCR	1 (2.5)	ND	[57]
Tonsillectomy (due to chronic tonsillitis, peritonsillar abscess, or sleep apnea syndrome)	FF and FFPE	108	qPCR	5 (4.6)	1 (0.9)	[58]
Patients with respiratory symptoms	NPA	1232	rt-PCR	2 (0.16)	3 (0.24)	[59]
Respiratory tract infections	NPA	887	rt-PCR	15 (1.7)	ND	[60]
HIV patients with leukoencephalopathies.	CSF	14	NGS/PCR	1 (7.1)	NA	[61]
Meningoencephalitis	CSF	135	qPCR	1 (0.74)	0	[62]
Immunocompromised patients scheduled to receive transplants	NPS, urine, fecal, plasma	32	rt-PCR	3 (9.4)	1 (3.1)	[63]
Acute gallstone cholangitis	Bile fluids	91	rt-PCR	5 (5.5)	NA	[64]
Patients with gastrointestinal illness	Fecal	185	rt-PCR	1 (0.5)	1 (0.5)	[59]

^aALHE case with Kimura disease clinical syndromes

FF fresh frozen tissue, FFPE formalin-fixed paraffin-embedded, FISH fluorescence in situ hybridization, IHC immunohistochemistry, NA not applicable, ND not detected, NGS next-generation sequencing, NPA nasopharyngeal aspirates, NPS nasopharyngeal swab, PCR polymerase chain reaction, qPCR quantitative polymerase chain reaction, rt-PCR real-time polymerase chain reaction, Seq Sanger sequencing

HPyV6 and HPyV7 DNA prevalence in primary cutaneous malignancies

According to the World Health Organization, the incidence of skin tumors has increased over the past decades, approximately 8500 new cases of skin cancers are reported daily in the United States^{66, 67}. Up to date, the presence of the DNA of five HPyVs has been reported in the human skin: MCPyV, HPyV6, HPyV7, HPyV9, and trichodysplasia spinulosa-associated polyomavirus⁴⁰. Of these, only MCPyV has been identified as a novel human tumor virus closely linked to the etiopathogenesis of the majority of MCCs. Since HPyV6 and 7 were isolated from the skin their possible contribution to the etiopathogenesis of skin cancers has been studied intensively^{24,25,26,27,28,29,30, 68}. Numerous studies, summarized in Table 3, have assessed the prevalence of HPyV6- and 7-DNA in primary cutaneous malignancies, including epithelial, neuroendocrine, and lymphoid skin cancers.

One study reported the presence of HPyV6- and HPyV7-DNA (2/47; 4.3%) in malignant melanoma (MM) specimens using rt-PCR²⁵. In non-melanoma skin cancer tissues, both HPyV6- and 7-DNA have also been identified. Four studies have shown the presence of HPyV6-DNA in the most common human skin cancer, i.e. basal cell carcinoma (BCC) Table 3. The detection rate was between 2 to 21%, and one study confirmed the detection of HPyV6 in BCC by FISH^{25, 27, 29, 68}. HPyV7-DNA was identified in 4% of BCC cases by rt-PCR in a single study²⁵. Studies on squamous cell carcinoma (SCC), which is the second most common human cutaneous malignancy, revealed a prevalence between 3 to 38% for HPyV6^{25, 27,28,29, 68}. Other studies tested keratoacanthoma and trichoblastoma for HPyV6-DNA, although no studies have tested trichoblastoma for HPyV7^{27, 68}. HPyV6- and 7-DNA were detected in <2% of tissue biopsies from MCC and <4% of actinic keratosis tissues^{27, 29, 30, 53, 69}. To date, only one study has analyzed verrucous keratosis tissues, and (3/4; 75%) specimens were positive for both viruses⁵³. Another study by Schrama et al. detected HPyV6 in all tested 18 epithelial proliferation specimens (SCC and/or keratoacanthoma obtained from MM patients treated with the BRAF inhibitor vemurafenib) and HPyV7 in 17/18 specimens using rt-PCR combined with immunohistochemistry (IHC) using 6V32 and 6V12 antibodies targeting HPyV6 VP1 and have cross-reactivity with HPyV7²⁶. It is of interest to mention that only very few studies report the usage of IHC to screen for HPyV6 and 7 protein expression, due to the lack of commercially available HPyV6- or 7-specific antibodies. In this respect, one may also consider the use of the commonly PAb416 antibody used in routine laboratories originally directed against the LTag of SV40. PAb416 antibody which detects conserved epitope region of diverse HPyVs including HPyV6 and 7 but not detecting MCPyV which there is a commercial antibody (CM2B4) available to detect its LTag^{72, 73}.

Additionally, primary cutaneous lymphomas have been tested for the presence of HPyV6- and 7-DNA: cutaneous T-cell lymphoma (CTCL) patients, and all three studies showed

Table 3 Prevalence of HPyV6 and HPyV7 in primary cutaneous malignancies

Cancer	Specimen types	Samples n	Method	HPyV6 Positive n (%)	HPyV7 Positive n (%)	Reference
MM	FFPE	47	rt-PCR/Seq.	2 (4.3)	2 (4.3)	[25]
AK	FFPE	31	qPCR	1 (3)	ND	[27]
AK	FFPE	92	Seq.	4 (4.3)	NA	[69]
AK	FFPE	2	qPCR	ND	1 (50)	[53]
VK	FFPE	4	qPCR	3 (75)	3 (75)	[53]
BCC	FFPE	18	qPCR	10 (5.5)	ND	[29]
BCC	FFPE	41	qPCR	3 (7)	ND	[27]
BCC	FFPE	50	rt-PCR/Seq.	1 (2)	2 (4)	[25]
BCC	FFPE	109	PCR/Seq./FISH	23 (21.1)	NA	[68]
SCC	FFPE	8	qPCR	1 (12)	ND	[27]
SCC	FFPE	21	qPCR	8 (38)	ND	[29]
SCC	FFPE	52	qPCR	2 (4)	ND	[27]
SCC	FFPE	86	PCR/Seq./FISH	8 (9.3)	NA	[68]
SCC	FFPE	63	rt-PCR/Seq.	2 (3.2)	1 (1.6)	[25]
SCC IN CLL cases who had BMT	FFPE	3	Bead-based multiplex PCR	1 (33.3)	ND	[28]
SCC	FFPE	11	qPCR	4 (36.4)	6 (54.5)	[53]
SCC	FFPE	17 (6 patients)	rt-PCR/IHC	17 (100)	17 (100)	[26]
KA	FFPE	42	qPCR	2 (5)	ND	[27]
KA	FFPE	59	PCR/Seq/FISH	25 (42.3)	NA	[68]
TB	FFPE	45	PCR/Seq	10 (22.2)	NA	[68]
MCC	FFPE	20	qPCR	2 (10)	ND	[29]
MCC	FFPE	28	rt-PCR	1 (3.5)	1 (3.5)	[30]
CTCLs	FFPE	116	rt-PCR	6 (5.20)	1 (0.90)	[70]
CTCLs	FF	71	rt-PCR	13 (18.3)	ND	[24]
CTCLs	FF and FFPE	35	rt-PCR	7 (20)	3 (8.6)	[71]
Total		1072		157/1072 (14.6)	37/681 (5.4)	

AK actinic keratosis, BCC basal cell carcinoma, BMT bone marrow transplantation, CLL chronic lymphocytic leukemia, CTCLs cutaneous T-cell lymphomas, FFPE formalin-fixed paraffin-embedded, FISH fluorescence in situ hybridization, IHC immunohistochemistry, FF fresh frozen tissue, KA keratoacanthoma, MCC Merkel cell carcinoma, MM malignant melanoma, NA not applicable, ND not detected, PCR polymerase chain reaction, qPCR quantitative polymerase chain reaction, rt-PCR real-time polymerase chain reaction, SCC squamous cell carcinoma, Seq Sanger sequencing, TB trichoblastoma, VK verrucous keratosis

that CTCLs were positive (5.20 to 20%) for HPyV6- and (0.90 to 8.6%) for HPyV7-DNA, while no cutaneous B-cell lymphoma was positive for either virus^{24, 70, 71}. Overall, results showed that the occurrence of HPyV6-DNA in CTCL was more frequent than HPyV7-DNA Table 3.

It is remarkable that HPyV6-DNA is far more often detected than HPyV7-DNA in human neoplastic skin diseases (Fig. 2), which may indicate a role for HPyV6 in the etiopathogenesis of these cancers. However, the reported copy loads of HPyV6- and 7-DNA in diverse skin cancers do not clearly support this hypothesis. Studies reported a generally low viral load of HPyV6- and 7-DNA-positive skin cancers which does not reflect the situation of high viral load of MCPyV in MCC^{25, 27, 29, 53, 74}. Nevertheless,

viral load copies of HPyV6 per cell range (0.00014–0.14) and MCPyV (0.0016–0.36) were almost in the same range in non-MCC tumors such as SCC, BCC, and MM, while HPyV7 copy numbers (0.000079–0.0094) were much lower. Moreover, specimens from healthy skin specimens showed the same range of HPyV6-DNA and MCPyV-DNA viral load as non-MCC samples^{25, 27, 29, 53}. Notably, HPyV7-DNA was more often detectable than HPyV6-DNA in non-cutaneous cancers, while HPyV6-DNA was identified more often in skin malignancies Tables 2 and 3.

Fig. 2 Representing a summary of the prevalence of HPyV6-DNA (a) and HPyV7-DNA (b) in tissues of cutaneous malignancies. AK, actinic keratosis; BCC, basal cell carcinoma; CTCLs, cutaneous T-cell lymphomas; KA, keratoacanthoma; MCC, Merkel cell carcinoma; MM, malignant melanoma; SCC, squamous cell carcinoma; TB, trichoblastoma; VK, verrucous keratosis

HPyV6 and HPyV7 in non-cutaneous malignancies

The prevalence of HPyV6 and 7 have also been studied in a variety of non-cutaneous malignancies summarized in Table 4. In tissue specimens, HPyV7- but not HPyV6-DNA was frequently (62%) found in thymic epithelial tumors ³¹. In contrast, HPyV6-DNA was more prevalent (5.4%) in tonsillar SCC ^{57, 58}.

Table 4 Prevalence of HPyV6 and HPyV7 in non-cutaneous other human malignancies

Tumor	Specimen	Samples n	Method	HPyV6 Positive n (%)	HPyV7 Positive n (%)	Reference
BC	FF	54	rt-PCR	1 (2)	1 (2)	[75]
LSCC	FF	7	Luminex platform (Multiplex PCR)	1 (14.3)	ND	[76]
TETs	FFPE	37	FISH/PCR/Seq/IHC	ND	23 (62.2)	[31]
TSCC	FF	38	qPCR	2 (5.3)	1 (2.6)	[57]
TC	FF and FFPE	112	qPCR	6 (5.4)	2 (1.8)	[58]
CCA	FFPE	42	PCR/FISH/RISH/IHC	4 (9.5)	19 (45.2)	[77]

BC breast cancer, *CCA* cholangiocarcinoma, *FFPE* formalin-fixed paraffin-embedded, *FF* Freshly frozen tissue, *FISH* fluorescence in situ hybridization, *LSCC* laryngeal squamous cell carcinoma, *ND* not detected, *PCR* polymerase chain reaction, *qPCR* quantitative polymerase chain reaction, *rt-PCR* real-time polymerase chain reaction, *RISH* RNA fluorescence in situ hybridization, *Seq* Sanger sequencing, *TC* tonsillar carcinoma, *TETs* thymic epithelial tumors, *TSCC* tonsillar squamous cell carcinoma

In body fluids specimens, Chan et al. investigated the presence of HPyVs in the bile fluid of patients with a variety of hepatobiliary malignancies ⁶⁴. HPyV6 but not HPyV7 was detected in 27.6, 10.7, 16.7, and 20% of bile specimens from cholangiocarcinoma (CCA), hepatocellular carcinoma, pancreatic carcinoma, and gallbladder carcinoma patients, respectively ⁶⁴. We recently identified the presence of HPyV6- and 7-DNA in cholangiocarcinoma cases at the single-cell level by diverse molecular biology techniques for the detection of viral DNA, mRNA, and protein expression ⁷⁷. We found that HPyV7 was more prevalent than HPyV6 and indeed the presence of these viruses was not restricted to CCA tissues but also found in adjacent microscopically non-neoplastic hepatocytes ⁷⁷.

Overall, among patients with different non-cutaneous cancers, HPyV7-DNA was more frequently detected than HPyV6-DNA in tissues. Yet, it remains unclear if and how HPyV7 might contribute to the tumorigenesis of these thymic epithelial tumors or cholangiocarcinomas, or that it rather is present in the context of viral latency.

Latency and tropism

The frequent prevalence of HPyV6- and 7-DNA in non-neoplastic tissues along with their high seropositivity indicates that these viruses remain in long-term latency in humans. Therefore, it is important to unmask the host cells facilitating this long-term latency in

healthy individuals to understand the host cell-type specific encoded proteins needed for viral entrance, replication, and transcription.

So far, the underlying mechanisms of tissue tropism or the possible latency of HPyV6 and 7 are still unknown. Other HPyVs have been reported to remain in latency within the epithelium of the proximal tubule of the kidney (BKPyV) while JCPyV is found in the brain³. In other studies, BKPyV and JCPyV were suggested to be found latent in lymphoid tissues and lymphocytes⁷⁸. Since the seroprevalence of HPyV6 and 7 is high in the general population, it is difficult to ultimately determine the latency compartment of these two HPyVs based on the currently available literature. However, keratinocytes have been identified to be the potential primary target of HPyV7 infection and replication⁵⁰. In addition, since HPyV 6 and 7 have been detected in BCC, further studies are needed to investigate whether they act similar to HPV by infecting the basal epithelial cells through lesions in the epidermis and undergo episomal replication in the parabasal layers^{25, 27, 29, 68, 79}.

Direct versus indirect HPyVs-related carcinogenesis

The potential contribution of oncogenic viruses is either direct and/or indirect in the carcinogenesis of virus-associated tumors^{80, 81}. MCPyV is the only HPyV found to be clonally integrated into the genome of human cancer, namely MCC. Next to its clonal integration, the MCPyV genome carries tumor-specific mutations within the large tumor antigen (LTag) introducing stop codons and thus abrogating the helicase expression and rendering MCPyV replication-deficient^{15, 17}. In direct carcinogenesis, one would expect a high expression of viral proteins in every tumor cell, which indeed is seen for the MCPyV-LTag protein in MCPyV positive MCC^{15, 17, 82}. In particular, the binding of truncated LTag to the retinoblastoma protein-binding (RB) maintains the replication of MCPyV-positive MCC cells^{3, 26, 83}. During the preparation of this review it occurred, that only a few studies report the protein expression of HPyV6 and 7 in neoplastic cells, possibly indicating such a direct tumorigenic role as shown for MCPyV and MCC. However, HPyV6- and 7-LTag expression are not found in all tumor cells in the above mentioned possibly HPyV6 and 7-associated human cancers. Thus, it seems unlikely that both HPyVs play an important direct contribution to human tumorigenesis^{26, 31, 77}.

Human tumor viruses also can induce neoplasms by indirect mechanisms via the interaction between viral antigens, environmental factors, and other modifications to the host genome⁸⁰. Inflammatory reactions due to the immune response to viral infection exposure can become eradicated and induce acute inflammation which may also result in chronic inflammation^{84, 85}. Reactive oxygen radicals can be produced years later following chronic inflammation and trigger modifications in the host nucleic acids and then inducing transformation as it is known for hepatitis B and C viruses^{84, 86}.

For instance, the hepatitis C virus is not detected in the cancer cells that may develop after many years of infection but are found in the adjacent lesions to the hepatocellular carcinoma^{87,88,89,90}. The infection by the virus alone is not sufficient to develop a tumor, and persistent viral infection precedes possibly many years before a cancer develops by an indirect mechanism such as chronic inflammation. The recent finding of HPyV6 and 7 in hepato- and cholangiocellular tissues possibly indicates such a physiopathologic mechanism of chronic inflammation-related indirect carcinogenesis also for HPyV6 and 7⁷⁷. However, more studies are needed to further elucidate and establish chronic inflammation-related indirect carcinogenesis for HPyV6 and 7.

Besides the direct and indirect HPyVs-related carcinogenesis, the “hit and run” hypothesis has been proposed to explain a possible role of viral agents to mediate cellular transformation⁹¹. The “hit and run” concept was proposed by Skinner in 1976 as a model where the virus is required to initiate the tumorigenesis process by leading to mutations, which maintain the tumorigenesis resulting in tumor with loss of the viral genes in the later stages⁹¹. Few studies have suggested the “hit and run” mechanism to mediate transformation by BKPyV, JCPyV, and MCPyV^{92,93,94,95,96}. However, this hypothesis remains speculative until further evidence for “hit and run” for HPyV associated diseases is established.

In addition, HPyVs may contribute to human tumorigenesis together with other oncogenic viruses assuming, that two or maybe more different viruses potentially infect the same cells and interact with each other in the etiopathogenesis of human cancers^{97,98,99,100}. Primary evidence for interplay and helper functions of viruses had been established since long, especially for adenoviruses and herpesviruses^{101, 102}. Of interest, Heilbronn et al. reported the finding that cytomegalovirus (CMV) infection can trigger JCPyV-DNA replication in cultured human fibroblasts¹⁰³. Indirect potential evidence for possible interplay and helper functions is currently restricted to observational findings: BKPyV has been found to be associated with the induction of cervical intraepithelial neoplasias, particularly in immunosuppressed patients^{104, 105}. Although these findings might point to an interaction of certain high-risk HPV types with BKPyV, they are by far no causal proof that BKPyV contributes to the HPV-related etiopathogenesis of cervical cancer. Coinfection with different human viruses has been also reported in oropharyngeal and oral cavity cancers¹⁰⁰. Although there are yet no functional data available concerning possible interplay and helper functions of HPyVs with other viruses in human cancers, it might be interesting to study this for HPyV6 and 7.

Further studies to elucidate the role of HPyV6 and 7 in etiopathogenesis of human cancers

The HPyV6 and 7 contributions to human tumorigenesis are not yet elucidated. Therefore, several important questions regarding the pathogenesis of both viruses need to be addressed. For example, how to confirm the interaction of HPyV6 and HPyV7 in the inactivation of p53 and retinoblastoma protein (pRb), or in the transformation function. Rozenblatt-Rosen et al. namely proposed this mechanism in which HPyV6 and 7 both LTA_g and sTA_g proteins to bind p53 and inactivate it leading to tumorigenesis³². MCPyV is the only known HPyV that is found to be clonally integrated into MCC cases, in particular, the binding of truncated LTA_g to the pRb was found to maintain the growth of MCC in MCPyV-positive cell lines^{3, 83, 106}. Furthermore, SV40, which is a non-HPyV, was reported to bind p53 and Rb and inactivate their function^{107, 108, 109}. In addition, one study has shown that HPyV6 sTA_g is capable to inactivate the tumor suppressor protein phosphatase 2A (PP2A) that results in hyperphosphorylation of the MEK-ERK pathway and leads to abnormal proliferation^{110, 111}.

The presence of HPyV6- and 7-DNA and their association with human malignancies have commonly been tested by PCR and qPCR. However, the conclusions that can be drawn from these studies restricting their level of evidence to the proof of viral nucleic acids in homogenized tissue extracts are unfortunately very limited. The meaning of the presence of viral nucleic acids must remain inconclusive because these findings cannot be interpreted on the single-cell level in the histomorphological context of the specific disease. In addition, only limited reliable data are available on the viral protein expression, because the detection of antibodies by IHC in FFPE tissues are lacking. A possible screening approach might start with the use of the PAb416 antibody followed by specific HPyV6 and 7 PCR. In addition, studies definitely are needed in order to investigate next to the presence of HPyV6-DNA and 7-DNA, if these viruses are integrated into the tumor genome and possibly carry tumor-specific mutations within the viral helicase of LTA_g in analogy to MCPyV^{15, 17}. This will e.g. also help to understand the role of HPyV6 and 7 in skin carcinogenesis as a possible co-factor of UV-radiation as it is discussed for HPV5 and 8 association skin carcinogenesis^{49, 112}.

Conclusion

In this review, we aimed to comprehensively analyze the literature for a possible role of the two closely related HPyV6 and 7 in human diseases and cancers. The seropositivity of HPyV6 was found to be higher than HPyV7 and both increased with age. The etiopathogenetical contribution of these viruses to human cancers remains unclear. Studies revealed that low HPyV6 and HPyV7 prevalence in non-neoplastic tumors resembled those in malignancies. Interestingly, HPyV6 prevalence was higher in skin malignancies than that of HPyV7. In contrast, HPyV7 was more frequently detected in non-cutaneous malignancies compared to HPyV6.

Based on sero- and tissue-prevalence HPyV6 and 7 remain important putative candidates for contributing to the etiopathogenesis of human disease, including skin cancers. There is an urgent need to further define their presence within cutaneous lesions e.g., keratoacanthoma, squamous cell carcinoma, and basal cell carcinoma, and to apply additional molecular techniques to unravel the molecular basis of virus-host interactions and assess a possible role of viral integration or mutation of the LTA_g in skin cancer development. The same applies for HPyV7-associated tumors e.g., cholangiocellular carcinomas or thymomas. Further studies are needed to understand the contribution of HPyV6 and HPyV7 to human cancers.

Abbreviations:

HPyVs: Human polyomaviruses; HPyV6: Human polyomavirus 6; HPyV7: Human polyomavirus 7; PyV's: Polyomaviruses; MCPyV: Merkel cell polyomavirus; MCC: Merkel cell carcinoma; VP1: Viral protein 1; VLP: Virus-like particles; ELISA: Enzyme-linked immunosorbent assays; GST: glutathione S-transferase; HPV: human papillomaviruses; ALHE: Angiolymphoid hyperplasia with eosinophilia ; FISH: Fluorescence in situ hybridization; FFPE: Formalin-fixed-paraffin-embedded; MM: Malignant melanomas; BCC: Basal cell carcinoma; SCC: Squamous cell carcinoma; IHC: Immunohistochemistry; CTCL: Cutaneous T-cell lymphomas; CCA: cholangiocarcinoma; LTA_g: Large tumor antigen.

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Author Contributions

FK and AzH conceived and designed the study. FK, GM, and DL searched the literature, extract and analyzed the data. FK and AzH wrote the manuscript. AK, VW, RA, EJS were contributed to writing specific parts of the manuscript. AzH is the principal investigator and set up and supervised this review. All the authors read and approved this manuscript for publication.

Author details

¹ Department of Pathology, GROW-School for Oncology & Developmental Biology, Maastricht University, Medical Centre+, Maastricht, the Netherlands. ² Department of Laboratory Medicine, Faculty of Applied Medical Sciences, Albaha University, Albaha, Saudi Arabia. ³ Department of Medical Laboratories Technology, Faculty of Applied Medical Sciences, Jazan University, Jazan, Saudi Arabia. ⁴ Department of Hematology, The Affiliated Hospital of Southwest Medical University, Luzhou, China. ⁵ Department of Internal Medicine IV, RWTH Aachen University Hospital, Aachen, Germany

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CHAPTER 3

High Prevalence of Human Polyomavirus 7 in Cholangiocarcinomas and Adjacent Peritumoral Hepatocytes: Preliminary Findings

Faisal Klufah, Ghalib Mobaraki, Emil Chteinberg, Raed A. Alharbi, Véronique Winnepenninckx, Ernst Jan M. Speel, Dorit Rennspiess, Steven W. Olde Damink, Ulf P. Neumann, Anna Kordelia Kurz, Iryna Samarska, and Axel zur Hausen

Graphical abstract:

ABSTRACT

Cholangiocarcinoma (CCA) is a rare biliary-duct malignancy with poor prognosis. Recently, the presence of the human polyomavirus 6 (HPyV6) has been reported in the bile of diverse hepatobiliary diseases, particularly in the bile of CCA patients. Here, we investigated the presence of novel HPyVs in CCA tissues using diverse molecular techniques to assess a possible role of HPyVs in CCA. Formalin-Fixed Paraffin-Embedded (FFPE) tissues of 42 CCA patients were included in this study. PCR-based screening for HPyVs was conducted using degenerated and HPyV-specific primers. Following that, we performed FISH, RNA in situ hybridization (RNA-ISH), and immunohistochemistry (IHC) to assess the presence of HPyVs in selected tissues. Of all 42 CCAs, 25 (59%) were positive for one HPyV, while 10 (24%) CCAs were positive for 2 HPyVs simultaneously, and 7 (17%) were negative for HPyVs. Of the total 35 positive CCAs, 19 (45%) were positive for HPyV7, 4 (9%) for HPyV6, 2 (5%) for Merkel cell polyomavirus (MCPyV), 8 (19%) for both HPyV7/MCPyV, and 2 (5%) for both HPyV6/HPyV7 as confirmed by sequencing. The presence of viral nucleic acids was confirmed by specific FISH, while the RNA-ISH confirmed the presence of HPyV6 on the single-cell level. In addition, expression of HPyV7, HPyV6, and MCPyV proteins were confirmed by IHC. Our results strongly indicate that HPyV7, HPyV6, and MCPyV infect bile duct epithelium, hepatocytes, and CCA cells, which possibly suggest an indirect role of these viruses in the etiopathogenesis of CCA. Furthermore, the observed hepatotropism of these novel HPyV, in particular HPyV7, might implicate a role of these viruses in other hepatobiliary diseases.

KEYWORDS

HPyV7; HPyV6; small DNA viruses; non-neoplastic hepatocytes; cancer; bile duct; Merkel cell polyomavirus; tumorigenesis

1. INTRODUCTION

Cholangiocarcinoma (CCA) is a rare but very aggressive biliary duct neoplasia with a very poor prognosis ¹. The etiology of CCA is still largely obscure. However, several risk factors have been identified to be associated with the etiopathogenesis of CCA, e.g., primary sclerosing cholangitis, hepatolithiasis, inflammatory bowel disease, cirrhosis, diabetes, and biliary-duct cysts. Additionally, the role of environmental factors, e.g., toxins and smoking and microbiological infections, including hepatic parasites (flukes), and Hepatitis B and C viruses has been established in the etiopathogenesis of CCA ¹⁻³. Although CCA is rather uncommon in Western countries, the incidence of CCA has been steadily—yet inexplicably—rising during the last few decades ¹. Chan et al. recently reported the presence of the novel human polyomavirus 6 (HPyV6) in the bile of Chinese patients with diverse hepatobiliary diseases ⁴.

Human polyomaviruses (HPyVs) are small DNA viruses in which e.g., BK polyomavirus (BKPv) and JC polyomavirus (JCPv) have been identified since the early 1970s ^{5,6}. The current number of members of HPyVs has recently been expanded to fourteen, most of which have been identified within the past twelve years ^{7,8}. However, the only HPyV that has been identified as a human tumor virus is the Merkel cell polyomavirus (MCPyV) ⁹. Approximately 80% of Merkel cell carcinoma (MCC) cases are caused by clonal integration of MCPyV in the tumor genome of this highly malignant MCC. In addition, MCPyV-positive MCCs harbor tumor-specific oncogenic mutations in the viral oncogene encoding large tumor antigen (LTAg) ^{7,10-13}.

Here, we assessed the prevalence of human polyomavirus 7 (HPyV7), HPyV6, and the oncogenic MCPyV in paraffin-embedded tissue sections (FFPE) of patients diagnosed with CCA, using diverse molecular techniques.

2. MATERIALS AND METHODS

2.1. Patients and Specimens

Forty-two formalin fixed and paraffin embedded (FFPE) tissue specimens of CCA patients collected at the Department of Pathology, Maastricht University Medical Centre+ (Maastricht Pathology Tissue Collection number: 2015-13) were included in this study. The study was approved by the Medical Ethics Review Committee of the Maastricht UMC+, the Netherlands (2019-0977), all tissue resections were collected and studied in accordance with the protocol of the Dutch Code of Conduct for Observational Research with Personal Data (2004) and Tissue ¹⁴.

Of these 42 CCA patients, 25 (60%) were male and 17 (40%) female. The mean age was 65.2 years (range: 29–85 yrs.). Three experienced pathologists (I.S., V.W., and A.z.H.) reviewed the histopathological and cytopathological features, and a consensus diagnosis of CCA subtypes was established. The recognition of the tubular non-neoplastic bile duct epithelium and neoplastic CCA cells has been performed by the above-mentioned pathologists.

DNA was isolated from all 42 FFPE tissues using techniques as previously described¹⁵. In brief, tissue sections were deparaffinized with xylene and the DNA was then extracted using the protocol of Genomic DNA from a tissue kit by Macherey-Nagel. The DNA concentration was assessed using a spectrophotometer (NanoDrop 2000, Thermo Scientific., Wilmington, DE, USA). Per sample, 250 ng was added in a PCR reaction. All isolated DNAs were assessed for quality and integrity using multiplex primers (SCS: specimen control size) as described previously^{15,16}.

2.2. Degenerated PCR

In order to screen for the presence of HPyVs DNA, we modified the degenerated primers as recently published by Chan et al.⁴. The primer modification was performed according to the multiple alignment of the conservative region which is located within the conserved region in LTag of HPyVs genome (Supplementary Figure S4a,b). To validate these degenerate primer sets, diverse PyVs-carrying plasmids were used, in addition to the MCPyV-positive MCC cell line (MKL-1) as positive controls in the PCR analysis. Bio-performance certified water was used as a non-template negative control.

The first nineteen isolated DNAs were screened for the presence of HPyVs DNA using degenerate primers, followed by sequencing of the obtained PCR products. DNA sequences were compared with the reference sequences of the National Center for Biotechnology Information (NCBI) Entrez Nucleotide database using the NCBI Blast program.

2.3. Specific HPyVs DNA PCR

In addition to the degenerated PCR technique, the specific PCRs targeting different gene regions of HPyV7, HPyV6, and MCPyV were performed using the protocols as previously described¹⁵. We used PCR to test all 42 CCA DNAs to amplify small tumor antigen (sTag) and large tumor antigen (LTag) of HPyV7, while sTag genes of HPyV6 were amplified, as well as for three MCPyV genes, i.e., LT3, VP1, and M1/M2. In addition, multiple sequence alignment was performed for each primer result using the Clustal Omega algorithm (by The European Bioinformatics Institute) to compare with the positive control sequencing in the reference sequences of NCBI. All HPyV specific primers used in this study are summarized in Table S2.

2.4. Fluorescence In Situ Hybridization (FISH)

All FISH procedures were carried out as described previously^{15,17,18,19}. Additionally, to check whether or not the FISH protocol earlier described by Hopman et al. was optimal for our CCA FFPE tissues cohort, we hybridized selected tissues with the alpha satellite (centromeric) DNA probe (Vysis-CEP 12 by Abbott Co., Abbott Park, IL, USA) to ensure the quality of the pretreatment for both parenchymal and non-parenchymal liver cells. The majority of FFPE specimens that were tested positive by DNA PCR for one of the HPyVs were tested by DNA FISH for the presence of HPyV6, or 7 or MCPyV to confirm the presence of the DNA in single cell level with an alternative technique. In addition, we evaluated and validated the viral DNA signals to confirm if the signals were specific. We treated the positive controls and selected CCA tissues from our cohort with a FISH protocol in combination with either DNase I or RNase prior to hybridization. By adding DNase then retesting, we ensured that the signal captured was DNA-specific; and by adding RNase, we ensured that the signal was not RNA-specific. Positive controls revealed specific signals that diminished slightly when the tissue was treated with RNase prior to hybridization. However, all specific viral DNA signals vanished completely in the positive controls when treated with DNase I (Supplementary Figure S3a) prior to hybridization. No cross-reactivity for the probes of HPyV 6 or 7 or MCPyV was observed. In addition, tissues previously tested negative for viral DNA served as negative controls for FISH, and no signals were seen. Furthermore, no specific nuclear signals were generated by omission of the probe during the hybridization. Therefore, HPyV6, HPyV7, and MCPyV biotin probes appeared to be sufficiently specific to detect the specific virus DNA (Supplementary Figure S3a).

The FISH slides were analyzed by at least three members (F.K., E.J.S., and A.z.H.) and the results of all cases were discussed together with all authors according to the criteria as previously described by Hafkamp et al.²⁰. In addition, the specificity of the FISH probes was assessed on the liver tissues, by both DNase I and RNase treatment, as described above (Supplementary Figure S3b).

2.5. RNA In Situ Hybridization (RISH)

The RNAscope assay using complementary RNA probes for the localization of specific expression of HPyVs mRNA was applied to selected cases: A specific probe for both HPyV6 and 7 LTag genes was designed by Advanced Cell Diagnostics (ACD). A total of 20 pairs of 50 bp pooled probe designed for the HPyV7 target region between 3688 and 4876 bp (V-Polyomavirus-HPyV7, Accession No: HM011566.1). Another 20 pairs of RNA probes for the HPyV6 target region 3786–4898 bp (V-Polyomavirus-HPyV6, Accession No: HM011563.1). The sections were pretreated using the RNAscope® HD Red 2.5 Kit (Advanced Cell Diagnostics, Cat No. 322350, Newark, CA, USA) according to the manufacturer's instructions.

A probe for human peptidylprolyl isomerase B (Hs-PPIB) mRNA expression was used as a positive control, while a probe for bacterial dihydrodipicolinate reductase gene (DapB) expression was used as a negative control. First, Hs-PPIB and DapB probes were applied to FFPE HeLa cells to validate the pretreatment and hybridization conditions according to ACD protocols ²¹. In addition, the two control probes were applied to each tested CCA case to confirm the expression of Hs-PPIB RNA and no expression of DapB. All RISH slides were evaluated and graded according to the ACD protocols and photos were taken using the VENTANA iScan-HT slide scanner ²¹.

2.6. Immunohistochemistry (IHC)

The following antibodies and dilutions were used: the monoclonal antibodies 2T10 directed against HPyV7-sTAG and 1T1 directed against HPyV6-sTAG were provided by C.Buck, NCI, Bethesda, MD, USA, and used at a 1:100 dilution in combination with the EnVision-FLEX™ visualization Kit (K8008, DAKO, Carpinteria, CA, USA) according to standard protocols ¹⁵. 2T10-Ab is known to weakly cross-react with HPyV6, but not with MCPyV. 1T1-Ab is known to cross-react with HPyV7 and MCPyV while 2T10 Ab is known to weakly cross-react with HPyV6 ²².

In addition, the CM2B4 monoclonal antibody was used to detect LT-antigen expression of MCPyV, which is more sensitive and specific than 2T10 and 1T1 Ab as described by Moshiri et al. (clone: CM2B4, dilution 1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) ²³. The protocol used for HPyV6 and HPyV7 IHC was described previously, as was the protocol used for CM2B4 MCPyV IHC ^{15,24}.

HPyV7-positive thymoma tissues were used as previously published ¹⁵. Keratoacanthoma tissue positive for HPyV6 was used as a control for 1T1 IHC ¹⁸. MCC cell line MKL-1 positive for MCPyV was used as a positive control for the CM2B4 antibody. All slides were evaluated by three pathologists (I.S., V.W., and A.z.H.) and photos were taken from the scans obtained by the VENTANA iScan-HT slide scanner. IHC was performed in selected PCR virus-positive cases and randomly for selected PCR virus-negative cases.

The immunohistochemistry stainings were scored either microscopically or using digital slides. The whole tissue sections were evaluated for the presence of positive nuclei and cytoplasm in hepatocytes, large bile duct, portal tracts, and neoplastic CCA cells. The percentage of positive cells was assessed. Inflammatory infiltrate was also evaluated if it was present. The intensity of HPyVs staining was evaluated and scored as strong (+++) if the positive nuclei were seen at 100× magnification, moderate (++) if the positive nuclei were seen at 200× magnification and weak (+) if positive nuclei were seen at 400× magnification.

In all subanatomic histological compartments (hepatocytes, large bile duct, and tumor cells), the quantitative analysis of HPyV7 IHC positive cells was evaluated using morphometry by (I.S., F.K.) on digital slides. The slides were scanned with the VENTANA iScan-HT slide scanner (Roche Diagnostics Inc., Tucson, AZ, USA) at 400× magnification. The morphometry was performed by calculating positive nuclei in the epithelial lining of the portal bile ducts, hepatocytes, and tumor cells in the hotspots in 5 photos at 400× magnification (HPF, high power field). The average of positive nuclei in the five images was calculated, and the results were present as a number of positive cells per HPF.

2.7. Statistical Analysis

The overall survival (OS) curve was estimated using the Kaplan–Meier method, and differences between HPyV-positive and -negative CCA cases were evaluated using a log rank test. OS was defined as the time between the date of tumor biopsy and date of death. Follow-up data for 39 (93%) patients were available, of which two patients were excluded due to the lack of survival data, only iCCA and pCCA were selected. Statistical analysis was performed with R-studio, and a p-value of <0.05 was considered to be statistically significant. The cohort was used to build the survival model. For feature selection, we first removed correlated features (Pearson's $r > 0.90$), using the publicly available Caret package²⁵. The remaining features were used to build a Cox proportional hazards model, using the publicly available rms package²⁶. The hazards ratio was calculated from the regression coefficients.

3. RESULTS

3.1. Histopathology and Specimen Quality

All isolated DNAs of the FFPE blocks revealed sufficient DNA quality with PCR products > 300 bp based on the results of the specimen control size (SCS) ladder PCR (Supplementary Figure S1a). A consensus histopathological diagnosis by three pathologists revealed that thirty (71.4%) patients had intrahepatic-CCA (iCCA), eleven (26.2%) had perihilar-CCA (pCCA), and one (2.4%) had distal-CCA (dCCA). No virus-like cytopathic effects or changes were observed in the cholangiocytes or hepatocytes during the review of HPyVs positive patient tissue specimens. Overall, 55% of patients were diagnosed at stage I, 26% at stage II, 14% at stage III, and 5% at stage IV.

3.2. Screening for Human Polyomaviruses Using Degenerate and HPyVs-Specific PCR

We used the MCPyV-positive MKL-1 cell line and diverse polyomaviruses (PyVs) plasmids as positive controls for PCR with the modified degenerated primers. Accordingly,

the modified degenerated primers were able to amplify PyVs sequences from MCPyV, HPyV6, 7, 9, NJPyV, WUV, CaPyV, and BPyV plasmids as well as the MKL-1 cell line (Supplementary Figure S1b), as confirmed by subsequent sequencing of PCR products. Screening of the first 19 CCA cases with this PCR technique revealed that fifteen (79%) cases were positive for one of the novel HPyVs as confirmed by sequencing. HPyV7 DNA was detected in twelve CCA cases (63%), followed by MCPyV DNA in three (16%) cases. Of interest, no HPyV6 DNA was detected in the CCAs using degenerated PCR (Figure 1a).

Using HPyV7-specific PCR for all CCA cases of the cohort, 62% (26/42) of CCA tissues were tested positive for the LTA_g in comparison to 26% (11/42) CCAs which were tested positive for the sTA_g DNA-PCR (Figure 1b) (Supplementary Figure S2a,b). In contrast, only 14% (6/42) of cases were positive by HPyV6-specific PCR, which were negative in the degenerated primer PCR. MCPyV-specific PCR revealed seven cases (17%) positive for the tumor antigen common region (M1/M2), two (5%) positive for the viral protein gene 1 (VP1), and three (7%) for the LTA_g gene (LT3) (Supplementary Figure S2c,d). All PCR products were confirmed by sequence analysis, with few nucleotide differences between the cases (Table 1).

In summary, the results of all PCR approaches revealed that 59% (25/42) of CCA tissues were positive for one of the HPyVs, while 10 (24%) CCA tissues were positive for 2 HPyVs, and 7 (17%) CCA tissues were negative for any HPyVs. Of the total 35 positive CCA tissues, 19 (45%) were positive for HPyV7, 4 (9%) for HPyV6, 2 (5%) for Merkel cell polyomavirus (MCPyV), 8 (19%) for both HPyV7/MCPyV, and 2 (5%) for both HPyV6/HPyV7 (Figures 1c, d). Combined, the results of the HPyV7-specific and modified degenerate primer PCR showed that 29 (69%) CCA tissues were positive for HPyV7 as confirmed by sequencing. Degenerated primer PCR showed 3 (16%) CCA tissues positive for MCPyV after sequencing. Both degenerated and MCPyV-specific PCR combined detected 10 (24%) CCA tissues positive for MCPyV (Table 1). In this CCA cohort, we did not observe an association of a specific HPyV with a specific CCA subtype. Only one HPyV type was detected in the samples of each CCA subtype, except for iCCA in which 10 (24%) CCAs were simultaneously harboring two different HPyVs. Eight cases were coinfecting with HPyV7 and MCPyV, and two cases were coinfecting with HPyV6 and HPyV7 (Figure 1d).

3.3. Fluorescence In Situ Hybridization (FISH)

Selected FFPE tissues chosen for assessing the FISH pretreatment with the alpha satellite (centromeric) DNA probe quality revealed intense FISH signals in all parenchymal and non-parenchymal liver cells. Thus, the protocol was optimal and was used for subsequent HPyV FISH analyses without modifications (Supplementary Figure S3a).

Figure 1. Degenerated and specific HPyV7 DNA-PCR. **(a)** The results of screening for HPyVs using FFPE tissues of Cholangiocarcinoma (CCA) by performing degenerated PCR-DNA. **(b)** Specific DNA-PCR amplifying HPyV7 sTag (181 bp). **(c)** A chart summarizing the total HPyV results for both degenerated and specific DNA-PCR. The numbers on the gels refer to the number of CCA tissues of the respective CCA patients. **(d)** Venn diagram showing cases with two different HPyVs simultaneously. Abbreviations: Numbers on gels: represents the patient ID; H₂O: water non-template negative control; MKL-1: MCPyV-positive Merkel cell carcinoma cell line used as a positive control; empty slot (no mastermix added to the gel); MW: molecular weight marker

Twelve CCA tissues were subjected to HPyV7-specific FISH analysis and four of them revealed specific nuclear signals in CCA tumor cells as well as non-neoplastic bile duct epithelium and hepatocytes (Table 2). HPyV7 DNA was detected as weak to moderate nuclear punctate signals in neoplastic CCA cells, and as moderate to strong punctate nuclear signals in non-neoplastic hepatocytes (Figure 2a). To assess if the FISH signals are DNA-specific, FISH analysis using the full length HPyV7 probe was performed on three tissue slides of case CCA1 (CCA patient with ID number 1). The first slide was hybridized with the HPyV7 probe resulting in HPyV7-specific green signals in

Table I. Clinical data and DNA PCR results.

Patient ID.	Gender	Age	Diagnosis	Clinical Stage	Degenerated PCR	HPyV7		HPyV6	MCPyV		Result
						LTAg	sTAg		sTAg	M1/M2	
CCA1	F	67	iCCA	II	HPyV7	+	+	-	+	+	HPyV7/MCPyV
CCA2	M	59	iCCA	II	-	-	-	+	-	-	HPyV6
CCA3	F	68	iCCA	I	-	-	-	-	-	-	-
CCA4	F	29	iCCA	III	-	-	-	+	-	-	HPyV6
CCA6	M	74	iCCA	I	MCPyV	+	+	-	-	-	HPyV7/MCPyV
CCA7	M	64	pCCA	I	MCPyV	-	-	-	-	+	MCPyV
CCA8	F	64	pCCA	III	HPyV7	+	-	-	-	-	HPyV7
CCA9	M	45	pCCA	I	MCPyV	-	-	-	+	-	MCPyV
CCA11	F	50	dCCA	II	HPyV7	+	-	-	-	-	HPyV7
CCA12	M	70	iCCA	II	HPyV7	+	+	-	-	-	HPyV7
CCA13	M	69	iCCA	I	HPyV7	+	-	-	-	-	HPyV7
CCA14	M	70	iCCA	I	HPyV7	+	+	-	-	-	HPyV7
CCA15	F	71	iCCA	I	HPyV7	+	-	+	-	-	HPyV6/HPyV7
CCA16	F	64	iCCA	II	-	-	-	-	-	-	-
CCA17	M	59	pCCA	III	HPyV7	+	-	-	-	-	HPyV7
CCA19	M	69	iCCA	I	HPyV7	+	+	-	+	-	HPyV7/MCPyV
CCA20	M	71	iCCA	II	HPyV7	+	+	-	-	-	HPyV7
CCA21	F	63	iCCA	III	HPyV7	+	-	-	-	-	HPyV7
CCA22	M	61	iCCA	I	HPyV7	+	-	-	-	-	HPyV7
CCA23	M	63	iCCA	I	NA	+	-	-	-	-	HPyV7
CCA24	M	73	iCCA	I	NA	+	-	-	+	-	MCPyV/HPyV7
CCA25	F	74	iCCA	II	NA	-	-	+	-	-	HPyV6
CCA26	F	60	iCCA	II	NA	+	+	-	-	-	HPyV7
CCA27	M	75	iCCA	I	NA	+	-	-	+	-	MCPyV/HPyV7
CCA28	M	50	pCCA	I	NA	+	-	-	-	-	HPyV7
CCA29	F	45	iCCA	I	NA	-	-	-	-	-	-
CCA30	F	66	pCCA	I	NA	-	+	-	-	-	HPyV7

Patient ID.	Gender	Age	Diagnosis	Clinical Stage	Degenerated PCR	HPyV7		HPyV6		MCPyV		Result
						LTAg	sTAg	LTAg	sTAg	M1/M2	VP1	LT3
CCA31	F	74	iCCA	III	NA	+	+	+	-	-	-	HPyV7
CCA32	F	77	iCCA	I	NA	+	-	-	-	+	-	MCPyV/HPyV7
CCA33	M	77	iCCA	IV	NA	+	-	-	-	-	+	MCPyV/HPyV7
CCA34	M	77	pCCA	I	NA	-	-	-	+	-	-	HPyV6
CCA35	M	59	pCCA	IV	NA	-	+	-	-	-	-	HPyV7
CCA36	M	72	pCCA	II	NA	+	-	-	-	-	-	HPyV7
CCA37	M	60	iCCA	I	NA	-	+	-	+	-	-	HPyV7/HPyV6
CCA38	F	85	iCCA	I	NA	-	-	-	-	-	-	-
CCA39	M	52	iCCA	II	NA	+	-	-	-	-	-	HPyV7
CCA40	M	63	iCCA	II	NA	-	-	-	-	-	-	-
CCA41	M	78	pCCA	I	NA	-	-	-	-	-	-	-
CCA42	F	72	iCCA	I	NA	+	-	-	-	-	-	HPyV7
CCA43	F	74	iCCA	I	NA	+	-	-	-	+	-	MCPyV/HPyV7
CCA44	M	60	iCCA	III	NA	-	-	-	-	-	-	-
CCA45	M	66	pCCA	I	NA	+	-	-	-	-	-	HPyV7
Results	F:17	iCCA:30	pCCA:11 dCCA:1		HPyV7:12	26/42	11/42	6/42	7/42	2/42	3/42	HPyV7:29
	M:25				MCPyV:3							HPyV6: 6
												MCPyV:10

Abbreviations: Patient ID, lab identification number; CCA, Cholangiocarcinoma; iCCA, intrahepatic; pCCA, perihilar; dCCA, distal; PCR, Polymerase chain reaction; +, positive; -, negative; NA, not applicable; HPyV6, human polyomavirus 6; HPyV7, human polyomavirus 7; MCPyV, Merkel cell polyomavirus; sTAg, small tumor antigen; LTAg, large tumor antigen; M1/M2, common region between sTAg and LTAg; VP, viral protein.

Figure 2. Detection of HPyV6, HPyV7, and MCPyV on the DNA in FFPE of CCA and non-neoplastic hepatocytes. Merged green (FITC) and blue (nuclei were counterstained with DAPI) show specific green signals. (a) Whole HPyV7 genome FISH probe performed on CCA1 tissue as a representative example of FISH for HPyV7 in CCA and non-neoplastic liver parenchyma cell while CCA2 tissue represents a negative case for HPyV7 DNA. (b) Whole HPyV6 genome FISH probe showed positive signals in both CCA and hepatocytes of CCA15, while the PCR-negative CCA8 case tissue revealed no signals. (c) Example of results of FISH specific nuclear of MCPyV in the nuclei of CCA epithelial, and the non-neoplastic hepatocytes. MKL-1 cell lines served as a positive control for the MCPyV probe. The images were taken at 630x magnification, a white square area was magnified 6x in the top right corner of each figure.

the bile duct tumor tissues as well as non-neoplastic hepatocytes. Pretreatment of the second slide with RNase reduced the FISH signal intensity, while DNase pretreatment diminished the specific signals almost completely as have been seen with the positive control (Supplementary Figure S3b).

All three HPyV6 DNA PCR positive cases showed specific nuclear HPyV6 DNA signals after FISH, although the dots were observed more often in the hepatocytes with normal morphology than in the CCA cells (Figure 2b). Additionally, the presence of MCPyV

Figure 3. Detection of HPyV6 on the transcriptional level in FFPE CCA tissue by RNA-ISH, the CCA2 patient tissue section was hybridized with 20 sets labeled probes to detect HPyV6 LTag mRNA using RNAscope RNA in situ hybridization assay. Additionally, FFPE hybridized with both the bacterial gene (DapB) as a negative control and the housekeeping gene (PPIB) as a positive control. Positive red signals were detected using fast red chromogen. HPyV6 LTA transcript seen as red signals in CCA and hepatocytes. No HPyV6 RNA was seen when the tissue was treated with RNase, nuclei were counterstained with hematoxylin. The images were taken at 200x magnification, a black square area was magnified 6x in the top right corner of each figure.

was confirmed by FISH in four out of five MCPyV DNA positive PCR cases (Figure 2c) (Table 2).

3.4. RNA In Situ Hybridization (RISH)

The RNAscope technique was applied on 5 CCA tissues (2 HPyV6-positive CCA tissues and 3 HPyV6-negative CCA tissues, Table 2). The selection of these was based on the DNA PCR results and the availability of tissue specimens. HPyV6 mRNA was detected as specific red punctate spots in the nucleus and/or cytoplasm in two cases in both non-neoplastic hepatocytes and CCA cells, which had also tested positive by specific sTag-PCR, FISH, and immunohistochemistry (IHC) (Figure 3). Unfortunately, RNAscope for HPyV7 mRNA detection did not work in our hands. The background ratio in the selected tissues made an interpretation of specific HPyV7 mRNA signals impossible.

Figure 4. Detection of HPyV6, HPyV7, and MCPyV on the translational level in FFPE of CCA and non-neoplastic hepatocytes. (a) Representative examples of IHC using 2t10 antibodies showed the specific nuclear expression in the nucleus (brown) of both CCA and liver parenchyma. CCA2 tissue is an example of negative HPyV7. (b) In a representative IHC for CCA15 tissue, 1t1 antibodies show positivity in both CCA and hepatocytes, while no protein expression was seen in CCA3. (c) Example of results of IHC for MCPyV, specific nuclear expression (brown) of MCPyV (CM2B4 antibody) in the nuclei of CCA epithelial, and the non-neoplastic hepatocytes. MKL1 cell line served as a positive for MCPyV antibodies. The images were taken at 200x magnification, a black square area was magnified 6x in the top right corner of each figure.

3.5. Detection of HPyV7-sTag, HPyV6-sTag, and MCPyV-LTag Expression by Immunohistochemistry

Twelve of sixteen CCA tissues were tested positive by HPyV7-IHC. Nine CCA tissues expressed HPyV7-sTag in tumor cells, three of which showed strong nuclear expression in at least 20% of the tumor cells (3/9; 33%) and six showed moderate to weak nuclear expression in five–ten percent of the tumor cells (Figure 4a). The expression of HPyV7-sTag within the histomorphological non-neoplastic adjacent large and small bile duct epithelium was also assessed and revealed nuclear positivity in eleven cases (11/16; 69%) (Table 2). No strong expression was observed, and seven cases showed weak expression in

Table 2. Fluorescence in situ hybridization, immunohistochemistry, and RNA in situ hybridization results.

Patient ID	HPyV7				HPyV6			MCPyV		Result
	FISH	IHC			FISH	RNA-Ish	IHC	FISH	IHC	
		Hepatocyte	Bile Duct Epithelium	CCA						
CCA1	++	+++	++	++	–	NA	+++	+	+	HPyV7 MCPyV
CCA2	–	–	–	–	+	+	++	–	NA	HPyV6
CCA4	–	NA	NA	NA	+	NA	+++	NA	NA	HPyV6
CCA6	–	+	++	–	–	NA	++	+	++	HPyV7 MCPyV
CCA7	NA	–	–	–	–	–	+++	++	+++	MCPyV
CCA8	–	+	+	+++	–	–	++	NA	NA	HPyV7
CCA9	–	–	–	–	–	NA	+++	–	++	MCPyV
CCA11	–	++	++	+++	–	NA	NA	NA	–	HPyV7
CCA12	–	+	++	+	–	NA	NA	NA	NA	HPyV7
CCA13	NA	–	–	+	NA	NA	NA	NA	NA	HPyV7
CCA14	+	+++	+	–	NA	–	+++	NA	NA	HPyV7
CCA15	+	+++	+	+++	++	+	+++	NA	NA	HPyV6 HPyV7
CCA16	NA	–	–	–	NA	NA	–	–	NA	–
CCA17	NA	+	+	–	NA	NA	+	NA	NA	HPyV7
CCA19	NA	+	+	+	NA	NA	–	+	+	HPyV7 MCPyV
CCA20	NA	+	+	+	NA	NA	+	NA	NA	HPyV7
CCA21	+	+	+	+	–	NA	+	NA	NA	HPyV7
Total	4/11	11/16	11/16	9/16	3/11	2/5	12/14	4/7	5/6	

Abbreviations: Patient ID, lab identification number; CCA, Cholangiocarcinoma; +, weak positive; ++, moderate positive; +++, strong positive; –, negative; NA, not applicable; HPyV6, human polyomavirus 6; HPyV7, human polyomavirus 7; MCPyV, Merkel cell polyomavirus; sTA_g, small tumor antigen; LT_{Ag}, large tumor antigen; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; RNA-ISH, RNA in situ hybridization.

less than 5% of bile ducts. Of interest, adjacent histomorphological non-neoplastic liver parenchyma also revealed specific nuclear positivity for HPyV7 in 11 cases (11/6; 65%) (Figure 4a). Strong hepatocellular expression was seen in three cases, moderate intensity in one case, and weak staining in seven cases (Table 2). The percentage of positive nuclei varied from less than 5% up to approximately 80%. No cytoplasmic expression was observed in neoplastic or non-neoplastic bile duct epithelium and hepatocytes, and no differences in zonal distribution were observed. In cases with a prominent inflammatory infiltrate, scattered lymphocytes also showed nuclear positivity for HPyV7. Furthermore, endothelium of the blood vessels was completely negative for HPyV7-sTA_g expression.

Moreover, the number of HPyV7-positive nuclei in hepatocytes, bile duct epithelium, and tumor cells was counted in the hotspots in 5 high power fields (HPF; 400X) using morphometric analysis (Table S1). Thus, these findings confirm at least a low HPyV7 protein expression in at least a few positive cells per HPF in all positive IHC cases of CCA regardless of the CCA subtype and/or clinical stage. Moreover, periportal hepatocytes

showed at least a focal positivity in all of these cases. In cases with reactive ductular reaction within the limiting plate of the portal tract, the newly formed small ducts also showed at least focal immunoreactivity.

Twelve of the selected fourteen CCA tissues were tested positive for HPyV6-sTag IHC expression. However, more CCA tissues revealed HPyV6-IHC positivity than PCR due to the fact that 1t1 antibodies are well known to have cross-reactivity with HPyV7 and MCPyV. The intensity of the HPyV6-IHC staining was weak to moderate with a maximal 10% of positive cells (Figure 4b). No sTag expression within the cytoplasm was observed, and no expression was found in endothelial cells of blood/lymphatic vessels. Of interest, twelve CCA tissues revealed specific nuclear HPyV6-sTag expression in non-neoplastic hepatocytes (12/14; 86%). Six cases revealed strong nuclear reactivity with percentages of positive nuclei between 20% and 80% (Figure 4b). No differences in zonal distribution were observed. Normal epithelial lining of bile duct epithelium was only weakly positive in 5 to 40% of the nuclei of cholangiocytes.

Six CCA tissues were tested for MCPyV-LTag expression by IHC, five of which showed nuclear staining (5/6; 83.3%) (Figure 4c). Three CCA tissues revealed both weak nuclear positivity in maximal 30% of tumor cells. All of the hepatocytes showed a weak to moderate staining pattern (Figure 4c). One CCA tissue revealed cytoplasmic staining, which was interpreted as unspecific and thus negative. No differences in zonal distribution were seen. Normal epithelial lining of bile duct epithelium was only positive in three cases up to 30% of nuclei of bile duct epithelium (ranging between weak and strong intensity). No cytoplasmic staining was observed (Table 2).

3.6. Overall Survival Analysis

Statistics data analysis revealed no significant association between overall survival (OS) and HPyV7 ($p = 0.726$), HPyV6 ($p = 0.366$), and MCPyV ($p = 0.953$) in iCCA and pCCA. The hazards ratio and the p-value are reported in Cox regression data (Table 3). The Kaplan–Meier curve showed no significant risk stratification for CCA patients (Figure 5).

Table 3. Cox regression model for multivariable analysis of iCCA and pCCA cohort.

Variable	Hazards Ratio	p-value
Sex	0.617888	0.521
Age	1.004683	0.893
Subtype	2.639623	0.174
Stage	0.761135	0.551
HPyV7	0.741641	0.726
HPyV6	2.293245	0.366
MCPyV	1.057001	0.953

Figure 5. Kaplan–Meier method survival curve for overall survival.

4. DISCUSSION

The etiology of CCA is largely unknown. Here, we investigated the presence of HPyV7, 6, and MCPyV in CCA tissue specimens, including adjacent histomorphological non-neoplastic bile duct epithelium and non-neoplastic hepatocytes, using diverse molecular detection techniques. We used a combined approach of a degenerated primer set for HPyVs and diverse HPyVs-specific PCRs, all PCRs were followed by sequence analysis. Degenerated PCR revealed to be a reliable detection of HPyVs in FFPE tissues demonstrating that the use of degenerated HPyV primers is applicable for screening purposes of FFPE tissues. (Figure 1a). To the best of our knowledge, this is the first study to perform FISH and IHC to confirm the presence of HPyV6, HPyV7, and MCPyV in the bile duct epithelium and hepatocytes, and also to show HPyV6 transcript by RNA-ISH in human tissues.

Compared to the prevalence of HPyV6 DNA in CCA tissues of our cohort 6 (14%), HPyV7 DNA was detected much more frequently in 29 (69%) of the CCA tissues. Of interest, some of the cases that tested HPyV7 DNA positive by degenerate LTag PCR were negative by specific HPyV7 DNA-PCR targeting sTAG. However, assessing the whole cohort with a specific LTag HPyV7 DNA-PCR within the conserved region which is targeted by the degenerated primers revealed a good agreement (Figure S2). This apparent incongruency of HPyVs DNA (LTag vs sTAG) detection by PCR in FFPE

tissue using different primer pairs is a well-known phenomenon. It is most likely due to the abundance of the diverse targeted sequences of the viral genome and is in line with prior observations of us and others in the detection of HPyVs such as MCPyV, HPyV6, and HPyV7^{4,11,27,28}. We have proven this discrepant phenomenon by using two specific HPyV7-DNA PCR targeting different regions (sTAg and LTA_g). Our results showed that the rate of detection of specific HPyV7 LTA_g is higher than the detection of sTAg, matching the results of the degenerated PCR.

An explanation for the discrepancy between high HPyV7 prevalence found by us and high HPyV6 prevalence found by Chan et al. might be the primary source of the DNA, i.e., primary CCA tissues versus bile fluids of CCA patients, respectively, or due to variation in the ethnical and geographical distribution of both cohorts. Recent studies have assessed HPyVs serum antibody levels across a range of age groups including one from the Netherlands. HPyV7 and HPyV6 were found to be highly seroprevalent in all age groups^{29,30,31,32,33}. HPyV6 seroprevalence was slightly higher than HPyV7 and MCPyV. However, the mean age in our patient cohort was 65.2 years compared to 77 years in the study of Chan et al., largely ruling out age related effects to explain these diverse findings.

In addition to HPyV7 specific DNA PCR, we were able to visualize the presence of HPyV7 by FISH and IHC. The FISH results were in good agreement with the PCR results and allowed the identification of the virus at the single cell level within the histomorphological context. FISH revealed the presence of HPyV7 within non-neoplastic hepatocytes and bile duct epithelium as well as CCA tumor tissue (Figure 2a). However, not all tumor cells revealed the presence of HPyV7 DNA by FISH. On the protein level using the 2t10 IHC antibody, nine CCA cases revealed an accumulation of the HPyV7 sTAg in tumor cells. Compared to non-neoplastic bile duct epithelium as well as adjacent non-neoplastic hepatocytes, eleven cases showed expression of HPyV7 sTAg. Finding less expression in CCA tumor cases might be explained by the fact that CCA can be derived from different cellular origins, including mature hepatocytes^{34,35}. Due to the different origins of cholangiocytes, we have seen some CCAs which were positive and some which were negative. Therefore, one can speculate that HPyV7 might infect hepatocytes at an early stage, considering that hepatocytes have the potential to differentiate between bile ducts or CCA. CCA cells with negative HPyV7 expression might differentiate from the non-hepatocyte origin.

Unfortunately, we were unable to detect specific mRNA signals for HPyV7 RISH due to technical reasons, which could be explained by two possibilities: the background ratio in the selected tissues made an interpretation of specific HPyV7 mRNA signals impossible; or the selected target sequences of the HPyV7 probe need to be adapted.

The above findings indicate that HPyV7 is most probably not directly related to the etiopathogenesis of CCA. However, the presence of HPyV7 in only a part of the tumor cells may also have been caused by technical restrictions of the FISH and IHC assays. The detection sensitivity of these assays is dependent on optimal pre-analytic tissue processing and preservation of nucleic acid quality and protein epitopes, which sometimes can be limited in FFPE tissues.

In the underlying study, a relatively low 14% prevalence of HPyV6 was detected in CCA tissues by HPyV6 specific PCR, which is lower than the 27% reported by Chan et al. in the bile fluid of CCA patients. By using the RNAscope technique, we were able to demonstrate HPyV6 transcripts on the single cell level in both non-neoplastic hepatocytes and CCA cells. This is in line with the DNA FISH and sTag IHC results. The IHC results, however, need to be interpreted with care, since we also detected the HPyV6 sTag expression in HPyV6 PCR-negative but HPyV7- and MCPyV- PCR positive cases, which is most likely due to the known cross-reactivity of this antibody with HPyV7 and MCPyV ²².

MCPyV was found in 24% of CCAs by both degenerated and specific DNA PCR, which is consistent with previous reports ^{36,37}. Loyo and colleagues reported the presence of MCPyV DNA by qPCR in five (33%) out of fifteen normal liver tissues, ten (63%) out of sixteen liver cancers, and two (100%) out of two biliary cirrhosis cases ³⁶. Additionally, the prevalence of MCPyV has been reported in three (24%) of 124 nonmalignant liver tissues by generic polyomavirus PCR ³⁷. MCPyV FISH analysis was able to detect the virus DNA in CCA tumors as well as non-neoplastic hepatocytes and bile duct epithelium (Figure 2c), showing relatively weak signals similar to the general weak to faint amplicons detected by PCR. On the protein level, weak immunostaining of MCPyV LTag viral proteins within both the CCA neoplastic cell and the normal looking bile duct epithelium was also observed. Thus, all three detection methods provided evidence for the presence of MCPyV in a subset of CCAs and normal liver and bile duct tissue. It is well known that malignant tumors associated with DNA viruses have a better survival as reported previously ^{38,39}. Our study revealed that iCCA and pCCA carrying HPyV7, HPyV6, or MCPyV DNA did not predict an association with overall survival and further larger studies are needed to confirm our findings.

The contribution of HPyV 7 to CCA carcinogenesis cannot be definitely elucidated by the present data. However, it remains speculation in as much as HPyV7 possibly contributes to CCA carcinogenesis. This might be based on a direct HPyV7-interaction and subsequent deactivation of p53, as recently reported by Rozenblatt-Rosen et al. ⁴⁰, or alternatively, by clonal integration of the HPyV7 genome with or without tumor-specific viral mutations as has been shown for MCPyV-positive MCCs ^{11,41}. The latter seems to

be rather unlikely for HPyV7-positive CCAs as one would then expect a high expression of viral proteins in every tumor cell. The most likely contribution of HPyV7 and maybe other HPyVs is an indirect mechanism via the induction of (chronic) inflammation as has been convincingly shown for Hepatitis B and C viruses ¹.

There are some limitations to our study. These limitations are mainly due to the rarity of CCA which also explains the sample size of our patient cohort (n = 42). Of course, it would be of clinical interest to further elucidate possible other correlations between these HPyVs and clinical endpoints of CCA.

5. CONCLUSIONS

Our results strongly indicate that HPyV7, HPyV6, and MCPyV infect non-neoplastic hepatocytes, bile duct epithelium, and CCA lesions. Although all three HPyVs are hepatotropic, HPyV7 was more frequently detected in both neoplastic and non-neoplastic cells in our CCA subset compared to HPyV6 and MCPyV. To the best of our knowledge, this is the first study to report the presence of HPyV7, HPyV6, and MCPyV on the single cell level in liver tissues of non-neoplastic bile duct epithelium and hepatocytes and CCA. We used diverse molecular techniques to assess the presence of these viruses. Although our results might point to indirect role of these HPyVs to CCA carcinogenesis, possibly by inflammation, the role of these HPyVs in the etiopathogenesis of CCA needs to be further elucidated. Furthermore, the frequent finding of HPyVs, especially HPyV7 in liver tissues might also point to a possible role of HPyV7 in other hepatocellular diseases.

Supplementary Materials

The following are available online at <https://www.mdpi.com/2076-2607/8/8/1125/s1>. Figure S1: (a) Agarose gel showing the specimen control size (SCS) ladder protocol to validate the DNA quality to be eligible for PCR testing. (b) Representative figure for validating the modified degenerated primer set by testing diverse PyVs positive control plasmids. Figure S2: (a,b) representative gel figures of DNA-PCR for HPyV7 targeting LTA_g (98 bp) for the whole cohort. (c,d) A representative gel figure of DNA-PCR for MCPyV targeting the common region of LTA_g and sTA_g (M1/M2—178 bp). Figure S3: validating the pretreatment of FFPE and labelled probes for FISH. Figure S4: (a,b) genome organization of HPyV6 627a (HM011563.1) showing the degenerated primers location in the LTA_g (designed by genome compiler software). Table S1: morphometric analysis for HPyV7 IHC immunohistochemistry. Table S2: PCR-primers used in this study.

Author Contributions

Conceptualization, F.K. and A.z.H.; methodology, F.K., G.M., E.C., E.J.M.S., and A.z.H.; investigation, F.K., G.M., D.R., and R.A.A.; data curation, F.K., G.M., and I.S.; writing—original draft preparation, F.K., G.M., and R.A.A.; writing—review and editing, A.z.H., V.W., and E.J.M.S.; visualization, F.K., I.S., R.A.A., A.K.K., V.W., E.J.M.S., and E.C.; resources, D.R., S.W.O.D., U.P.N., and A.K.K.; principal investigator, supervision, and project administration, A.z.H. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary:

Figure S1. (a) Agarose gel showing the specimen control size (SCS) ladder protocol to validate the DNA quality to be eligible for PCR testing. (b) Representative images of DNA agarose gels for validating the modified degenerated primer set by testing diverse PyVs positive control plasmids and MKL1 cell line (142 to 158bp PCR products). **Abbreviations:** H₂O; water non-template negative control, PF; free paraffin without tissue, MW; molecular weight marker, MKL1; Merkel cell carcinoma cell line positive for MCPyV, Free; no master mix added to the gel.

Figure S2. (a & b) A representative gel figures of DNA-PCR for HPV7 targeting LTA9 (98bp). (c & d) A representative gel figures of DNA-PCR for MCPyV targeting the common region of LTA9 and sTA9 (M1/M2-178bp). **Abbreviations:** H₂O; water non-template negative control, MW; molecular weight marker, HPV7; Human polyoma virus, MKL1; Merkel cell carcinoma cell line positive for MCPyV as a positive control,-; no master mix added to the gel slot.

Figure S3. Validating the pretreatment of FFPE and labelled probes for FISH. (a) Validating the DNA quality and integrity for subsequent HPyV FISH analyses. Using the CEP probe directed against alpha satellite DNA located at the centromere of chromosome 12 (orange). Two copies of each signal were observed in the nuclei all parenchymal and non-parenchymal liver cells. Validating the whole HPyV7 genome probe labeling, specific green punctate dot FISH signals in thymic cells which used as control for HPyV7. HPyV7 Probe with RNase for thymoma tissues section demonstrate a slightly diminished of the fluorescence signal dots. Treatment the thymoma tissue with DNase I in combination with the HPyV7 probe erased the signals completely. Merkel cell carcinoma tissue with positive MCPyV tested with HPyV7 probe to exclude the cross reactivity of the labeled probe with MCPyV. Testing the labeled pFunny backbone probe which used as backbone for HPyV7 plasmid and revealed no signals in the CCA and non-neoplastic hepatocyte cells which were positive for HPyV7 probe FISH. (b) Representative images for validating FISH for the HPyV7 probe using CCA tissue positive for HPyV7 by PCR, specific green punctate dot FISH signals in CCA and hepatic cells representing HPyV7 DNA. The signals were slightly decreased after RNase A treatment, however using DNase I erased the signals completely.

Figure S4. (a) Genome organization of HPyV6 627a (HM011563.1) showing the degenerated primers location in the LTag (Designed by genome compiler software). (b) Sequence alignment of LTag conservative region of all 14 HPyVs as well as SV40 which can be detected with the degenerated primers (designed by the Clustal Omega algorithm by The European Bioinformatics Institute). **Abbreviations:** H₂O; water non-template negative control, MW; molecular weight marker.

Supplementary Table 1: Morphometric analysis for HPyV7 IHC immunohistochemistry

Patient ID	HPyV7 IHC		
	Average of positive cells by HPF		
	Hepatocyte	Bile duct epithelium	CCA
CCA1	23	18	11
CCA6	3	11	-
CCA8	6	10	26
CCA11	13	13	21
CCA12	4	12	2
CCA13	-	-	8
CCA14	52	8	-
CCA15	40	2	40
CCA17	1	1	-
CCA19	2	2	2
CCA20	2	4	3
CCA21	7	3	3

Abbreviations: Patient ID, lab identification number; CCA, Cholangiocarcinoma; HPyV7, human polyomavirus 7; IHC, immunohistochemistry; HPF, high power field

Supplementary Table 2: PCR-primers used in this study

Primer	Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Products size	Reference
Modified Degenerated	LTAg	CTGYCCYTTYACATCCTCAA	GCCCYATHAAYAGrTGGVAAAAC	142 to 158 bp	Our group
HPyV7	sTAg	AGATTTAGCTGTCCCCAAAG (3946 – 3965)	AAGAAGGCCAAAGAGTATGC (4108 – 4127)	181 bp	(1)
HPyV7	LTAg	GTTCAAAGTTTATCTTGTCAC TGC (3119 – 3142)	CAACAGTGGGAAAACAACCTTAGCA (3192 – 3216)	98 bp	Our group
HPyV6	sTAg	ATCAGCTTCCACAGGTAGGC (3438 – 3457)	TTGCCTTCTCAAAAAGGAGC (3542 – 3561)	123 bp	(1)
MCPyV	LT3	TTGTCTCGCCAGCATTTGTAG (571 – 590)	ATAATAGGGGCCCTCGTCAAACC (860 – 879)	308 bp	(2)
MCPyV	VP1	TGGATCTAGGCCCTGATTTT (3786 – 3806)	TTTGCCAGCTTACAGTGTGG (4118 – 4137)	351 bp	(2)
MCPyV	M1/ M2	GGCATGCCCTGTGAATTAGGA (1711 – 1730)	TTGCAGTAATT'TGTAAGGGGGCT (1867 – 1889)	178 bp	(3)

Abbreviations: HPyV6, human polyomavirus 6; HPyV7, human polyomavirus 7; MCPyV, Merkel cell polyomavirus; sTAg, small tumor antigen; LTAg, large tumor antigen; LT, large antigen; VP, viral protein; M1/M2, the common region between sTAg and LTAg; bp, base pair.

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CHAPTER 4

Reactivation of BK Polyomavirus in Urine Cytology is Not Associated with Urothelial Cell Carcinoma

Faisal Klufah, Ghalib Mobaraki, Axel zur Hausen, and Iryna V. Samarska

ABSTRACT

BK polyomavirus (BKPyV) has been associated with some high-grade and special urothelial cell carcinoma (UCC) subtypes in immunosuppressed patients. Here, we evaluated the relationship of BKPyV-positive urine cytology specimens (UCS) with UCC. A large single-institution database was retrospectively searched for UCS positive for decoy cells, suggesting BKPyV infection. These were tested for the presence of BKPyV by PCR and immunohistochemistry (IHC) in urine sediments and formalin-fixed paraffin-embedded (FFPE) tissue samples of UCC. Decoy cells were reported in 30 patients out of the database with 22,867 UCS. Of these 30 patients, 16 (53.3%) had no history of UCC. Six patients out of these 16 had a history of transplantation, 4 had a history of severe chronic medical conditions, and 6 had no chronic disease. The other fourteen patients were diagnosed with either in situ or invasive UCC of the urinary bladder (14/30; 46.6%) prior to the detection of decoy cells in the urine. Nine of these UCC patients received intravesical treatment (BCG or mitomycin) after the first presentation with UCC. However, the clinical data on the treatment of the other five UCC patients was lacking. IHC identified BKPyV-positivity in the urine samples of non-UCC and UCC patients, while no BKPyV positivity was found in FFPE tissues of primary UCCs and metastases. In addition, BKPyV-PCR results revealed the presence of BKPyV DNA in the urine of the UCC cases, yet none in the UCC tissues itself. These data strongly indicate that BKPyV reactivation is not restricted to immunosuppression. It can be found in UCS of the immunocompetent patients and may be related to the intravesical BCG or mitomycin treatment of the UCC patients.

Keywords: BKPyV; small DNA viruses; bladder cancer; cancer; polyomavirus; tumorigenesis; decoy cells

1. INTRODUCTION

BK polyomavirus (BKPyV) is a human polyomavirus that has been suspected as a putative oncogenic virus in the development of urothelial cell carcinomas (UCC) in immunocompromised patients ^{1,2}. Approximately 90% of the human population is infected during early life with BKPyV. The virus remains latent in the epithelium of the proximal tubule of the kidney, within the urothelial cell layer of the bladder, ureters, renal pelvis, and other tissue types ¹. BKPyV reactivation frequently occurs following immunosuppression, especially in the context of kidney and solid organ transplantations, with the risk of developing BKPyV-associated nephropathy (PyVAN) ^{3,4}. BKPyV replication has been described to be a significant risk factor for bladder cancer development following kidney transplantation ^{2,5}. Thus, BKPyV-positivity has been reported previously in the urinary bladder and kidney tumors occurring after PyVAN ^{2,5,6,7,8,9,10}. The spectrum of the BKPyV positive urinary bladder lesions includes high-grade urothelial carcinoma (UCC; both in situ and invasive), micropapillary urothelial carcinoma, pleomorphic giant cell carcinoma, bladder adenocarcinoma, and nephrogenic adenoma ^{3,5,6,7,8,9}. Moreover, BKPyV-DNA was detected in many other tumors, including pancreas, liver, oral, oropharyngeal, laryngeal squamous cell carcinomas, rhabdomyosarcoma, Kaposi's sarcoma, prostate adenocarcinoma, and brain tumors ^{2,10,11}. In 2012, the International Agency for Research on Cancer (IARC) classified BKPyV as a group 2B possibly carcinogenic candidate to humans ².

BKPyV-infected cells from renal tubules and urothelium can be detected by cytology as decoy cells that have enlarged and altered nuclei with large homogeneous basophilic nuclear inclusions, which mimic cellular changes observed in UCC in situ ^{3,12}. The identification of BKPyV-IHC positive decoy cells in voided urine has thus been interpreted as a strong indicator of BKPyV reactivation in urothelial cells following immunosuppressive treatment ^{3,4}. These cells have been shown to express BKPyV large T antigen (LTag) using immunohistochemistry (IHC) ⁷. Due to its known cross-reactivity with the LTag of BKPyV, an anti-Simian virus 40 (SV40) LTag antibody is used in clinical practice as a surrogate marker of BKPyV infection ^{1,13}.

The aim of this study was to assess the relation of BKPyV-positive urine cytology specimens (UCS) to the detection of UCC in a large UCS database and the following evaluation of BKPyV in the UCC of the urinary bladder. In addition, we aimed to evaluate the relation of BKPyV to intravesicular BCG or mitomycin treatment of UCC patients.

2. MATERIALS AND METHODS

2.1. Study Population

The starting point of this study was a single-institution database that included 22,867 UCS. This database was searched for UCS containing decoy cells as detected by cytology over a 15 year period (January 2004–December 2019) using the laboratory information system of the department of pathology, Maastricht University Medical Center+ (MUMC+), the Netherlands. Thirty patients were identified and included in this study for further workup and analyses. All cytology slides, including immunocytochemistry for SV40, were retrieved and independently reviewed by two pathologists (IVS and AzH). Cases that also had bladder biopsies or resections obtained following and preceding the urinary cytology specimen were collected from the archive and independently reviewed. Clinical information regarding medical history, transplantation, chronic medical condition (autoimmune disease, cancer, diabetes, immunosuppressive treatment, chemotherapy, radiotherapy, and connective tissue disease), as well as the history of renal and bladder tumors were obtained from the medical records of the MUMC+. This study was approved by the Medical Ethics Review Committee of the Maastricht University Medical Center in the Netherlands (2019-0977). All specimens were collected and studied in accordance with the protocol of the Dutch Code of Conduct for Observational Research with Personal Data (2004) and Tissue ¹⁴.

2.2. Cytology

Unfixed urine samples were prepared by the cytopsin method, according to the previously described protocol ¹⁵. One cytopsin was stained with Papanicolaou stain, screened by a cytotechnologist, and subsequently reviewed and reported by a pathologist. The other cytopsin was stained with the immunoperoxidase staining for SV40 LTA_g immunocytochemistry (ICC). The ICC was performed using SV40 LTA_g antibody (clone: PAb416, dilution 1:500, Calbiochem Inc., San Diego, CA, USA) with a Dako's autostainer Link 48 using the EnVision FLEX visualization kit (K8008, DAKO, Carpinteria, CA, USA) according to the diagnostic standard routine and manufacturer's protocol. Only nuclear staining was regarded as a positive reaction for the SV40 antibody.

2.3. Histology

Urinary bladder biopsy and resection specimens were fixed in 4% buffered formalin and embedded in paraffin, according to the routine pathology diagnostic procedures. For light microscopy, hematoxylin and eosin (H&E)–stained sections were performed on 3–5 μ m-thick tissue sections.

2.4. Immunohistochemistry

Representative formalin-fixed paraffin-embedded tissues (FFPE) were chosen from bladder biopsy and resection cases, and IHC was performed using the SV40 LTag antibody (clone: PAb416, dilution 1:500, Calbiochem Inc. San Diego, CA, USA). The IHC staining was performed on 3–5 µm-thick FFPE sections using Dako's autostainer as described above in the cytology section. Only nuclear protein expression was interpreted as a positive reaction for the SV40 antibody. All slides were scanned by the VENTANA iScan-HT slide scanner (Roche Diagnostics Inc., Tucson, AZ, USA).

2.5. BKPyV PCR

Nine FFPE specimens and five urine sediments of UCC-patients were retrieved from the archive at the Department of Pathology, Maastricht University Medical Center+. DNA was extracted from all specimens using the protocol of genomic DNA isolation by (NucleoSpin® Tissue, Macherey–Nagel GmbH & Co., Düren, Germany). The DNA concentration was assessed using a spectrophotometer (NanoDrop 2000, Thermo Scientific., Wilmington, DE, USA). Per sample, 125 ng DNA were added to the PCR reaction. All isolated DNAs were assessed for quality and integrity using multiplex primers (SCS: specimen control size) as described previously^{16,17}.

Two PCR primer sets were used to screen for the presence of BKPyV DNA. One primer set targeted the viral LTag, while the other targeted the viral protein 1 (VP1), all primer sequences listed in Supplementary Table S3. Sequencing of all obtained PCR products was compared and analyzed with the reference sequences of the National Center for Biotechnology Information (NCBI), Entrez nucleotide database using the NCBI Blast program.

3. RESULTS

3.1. Study Population and Urine Cytology

Of the 22,867 retrospectively evaluated UCS, 46 urine specimens (0.2%), obtained from 30 patients, were positive for decoy cells, indicating BKPyV-infection (Figure 1A). Six of these patients had two or more urine specimens with these viral changes. UCS were obtained from 25 males with a median age of 68.6 years (range 25–90) and 5 females with a median age of 55 years (range 38–80). All clinicopathological data of the patients are summarized in Table 1. The follow-up period ranged from 7 months to 170 months, with a median of 70.5 months following or preceding the urinary cytology. All 30 patients showed typical decoy cells in their urine specimens by cytology. These cells were characterized by an enlarged nucleus with an intranuclear inclusion, which has an amorphous basophilic ground-glass appearance (Figure 1).

Figure 1. (A) Flow chart depicting the study design and immunohistochemistry results. (B) Decoy cells (arrows) in urine cytology (Papanicolaou staining) and (C) immunohistochemistry for BKPyV (arrows), and an enlarged image in the lower right corner showing the expression of BKPyV. The images were taken at 200× magnification.

Table 1. Patients clinicopathological data, demographics, and results.

Clinicopathological Characteristic	Patients (<i>n</i> = 30)
Age range (years)	25–90
Male	25–90
Female	38–80
BKPyV-IHC+ve in urine cytology	27/30
Non-UCC patients	13/16
UCC-patients	14/14
Non-UCC patients	16
Immunosuppressed	10
Transplantation	6
Renal	5
Stem cell	1
Chronic disease (IBD, AIH, CTD, other malignancy)	4
No chronic disease	6
UCC patients	14
LGUCC	6
HGUCC	1
INUCC	1
CIS	2
LGUCC and HGUCC	2
CIS and HGUCC	1
CIS and INUCC	1
UCC with treatment	9
Intravesical treatment	7
BCG	4
Mitomycin	2
Both BCG and mitomycin	1
Radiotherapy	1
Intravesical treatment and radiotherapy	1
UCC with unknown treatment	5

UCC, urothelial cell carcinoma; IHC, immunohistochemistry; +ve: positive; IBD, inflammatory bowel disease; AIH, autoimmune hepatitis; CTD, connective tissue disease; LGUCC, low-grade non-invasive urothelial cell carcinoma; HGUCC, high-grade non-invasive urothelial cell carcinoma; CIS, carcinoma in situ; INUCC, invasive urothelial cell carcinoma; BCG, Bacillus Calmette–Guerin.

3.2. BKPyV in Patients without a History of Urothelial Cell Carcinoma

Of the thirty patients, sixteen had no history of UCC (53.3%). Out of the sixteen patients without a history of UCC, five patients (5/16; 31.25%) had a history of kidney transplantation, and one patient (1/16; 6.25%) had a history of stem cell transplantation. Other four patients (4/16; 25%) from the non-UCC history group had a history of chronic diseases that required immunosuppression and systemic therapy/radiotherapy,

such as ulcerative colitis, diabetic nephropathy, autoimmune thyroiditis, autoimmune hepatitis, and not-urolithelial cancer (prostate adenocarcinoma, colon adenocarcinoma, high-grade sarcoma of the soft tissue). The other six patients (6/16; 37.5%), who had follow-up history in the database for at least one year after the first presentation with BKPyV positive urine cytology, did not have any history of chronic diseases, nor were diagnosed with UCC during the follow-up period (Table 1 and Supplementary Table S1). Those six patients could be considered as immunocompetent for the period of their follow-up in our institution. Of the sixteen non-UCC patients, thirteen (81.25%) were positive for BKPyV IHC in urinary samples (Figure 1C). IHC could not be performed in three patients because no material was left due to multiple usages for additional analyses (Table S1).

3.3. Presence of BKPyV in Urothelial Cell Carcinoma

All fourteen patients with a history of UCC developed cancer prior to the detection of BKPyV-IHC positive decoy cells in their urine. Decoy cells in their urine were detected in the follow-up after the first presentation with UCC with a median period of 3.5 years (range 1–13 years) and were positive for the SV40 LTA α by immunohistochemistry, indicating the BKPyV-reactivation in urothelial cells (Figure 1C; Table 2; Supplementary Table S2). Additionally, BKPyV-DNA PCR of the five available urine sediments revealed BKPyV-positivity as confirmed by sequencing (Table 2). Interestingly, none of these patients had decoy cells in urine by preoperative cytology prior to the diagnosis of UCC.

Nine of these patients (9/14; 64.3%) were known to have a history of intravesical treatment, either BCG, mitomycin, or/and radiotherapy (Table 1). The treatment history of the other five UCC patients is lacking. Two patients had recurrent UCC after intravesical treatment, and the diagnosis of BKPyV infection was confirmed by cytology. Twelve patients (12/14; 85.7%) presented with a conventional type UCC (either low-grade, high-grade UCC, or CIS) and two patients had invasive UCC with sarcomatoid differentiation (Supplementary Table S2). No micropapillary urothelial carcinoma, pleomorphic giant cell carcinoma, or bladder adenocarcinomas were found in the patients' cohort. The H&E-stained slides of all 14 UCC patients were examined histologically and revealed no virus-related cytopathic changes (Figure 2A). BKPyV-IHC was performed on the available UCC FFPE specimens (with either in situ or invasive UCC of the urinary bladder) of 12 patients (Table 2, Supplementary Table S2). BKPyV-IHC was negative in all tested UCC samples (Figure 2B, Table 2), including metastatic and recurrent UCC. In addition, BKPyV DNA PCR was performed on the available UCC FFPE tissues of the nine patients, and all nine samples were negative for BKPyV-DNA (Table 2). Due to multiple previous histopathological and immunochemical diagnostic procedures, no FFPE tissues of the remaining five UCCs were available to perform BKPyV-IHC or PCR (Table S2).

Table 2. Clinical data, BKPyV IHC, and DNA PCR results.

Patient ID.	Diagnosis	(Intravesical) Treatment	BKPyV IHC		BKPyV PCR			
			Urine Cytology	FFPE Tissue	Urine Sediments		FFPE Tissue	
					LTA _g	VP1	LTA _g	VP1
I.7	LGUCC	Mitomycin	+	n.a.	n.a.	n.a.	n.a.	n.a.
I.8	LGUCC	Unknown	+	-	n.a.	n.a.	n.a.	n.a.
I.9	LGUCC	Unknown	+	-	n.a.	n.a.	n.a.	n.a.
I.10	LGUCC, HGUCC	BCG/Mitomycin	+	-	n.a.	n.a.	-	-
I.12	INUCC	Unknown	+	n.a.	n.a.	n.a.	n.a.	n.a.
I.13	CIS, INUCC	BCG/radiotherapy	+	-	n.a.	n.a.	-	-
I.15	LGUCC	Unknown	+	-	n.a.	n.a.	-	-
I.17	CIS	BCG	+	-	n.a.	n.a.	-	-
I.21	HGUCC	Radiotherapy	+	-	n.a.	n.a.	-	-
I.22	CIS	BCG	+	-	+	-	-	-
I.23	LGUCC, HGUCC	BCG	+	-	+	-	-	-
I.26	CIS and HGUCC	BCG	+	-	+	+	-	-
I.29	LGUCC	Mitomycin	+	-	+	-	n.a.	n.a.
I.30	LGUCC	Unknown	+	-	+	-	-	-

Patient ID, lab identification number; FFPE, formalin-fixed paraffin-embedded tissues; UCC, urothelial cell carcinomas; PCR, polymerase chain reaction; +, positive; -, negative; LTA_g, large tumor antigen; VP1, viral protein; UCC, urothelial cell carcinoma; n.a., not applicable; IHC, immunohistochemistry; LGUCC, low-grade non-invasive urothelial cell carcinoma; HGUCC, high-grade non-invasive urothelial cell carcinoma; INUCC, invasive urothelial cell carcinoma; CIS, carcinoma in situ; BCG, Bacillus Calmette–Guerin.

Figure 2. High-grade urothelial cell carcinoma and immunohistochemistry for BKPyV. (A) H&E-stained section shows neoplastic UCC cells with no visible viral changes in the nuclei. (B) a representative BKPyV-negative UCC as tested by IHC (200× magnification).

4. DISCUSSION

In this study, we evaluated the association of BKPyV-positive urine cytology with UCC and the presence of BKPyV in UCS and FFPE samples of the UCC cohort. The overall cytology results revealed a very low percentage (0.2%) of BKPyV-positive urine cytology in the database of our institution over a 15 year period (January 2004–December 2019). Several studies reported that decoy cells were detected in 18 to 28% of the urine sediments of patients with kidney transplantation^{18,19,20,21,22,23}. In contrast to those studies, our study was not restricted to immunocompromised patients but also included patients with no previous history of organ transplantation, chemotherapy, radiotherapy, malignancy, or chronic disease, requiring immunosuppressive therapy (such as colitis ulcerosa, autoimmune disease, diabetes, connective tissue disease). Previous studies reported similar low detection rates of BKPyV in UCS when all patients (immunocompromised and immunocompetent) were included in the search^{12,24}.

The main finding of our work is that BKPyV was found in the UCS of the patients diagnosed with UCC and was detected in the follow-up period after the initial diagnosis of the UCC was made. Moreover, this BKPyV-infection may be related to the intravesical treatment of their UCC. In our study, UCC developed before the diagnosis of BKPyV-infection in urine cytology and were negative for BKPyV-IHC or PCR in FFPE tissue samples.

BKPyV has been associated with some types of UCC in the context of immunosuppression, polyomavirus-associated nephropathy (PVAN), and hemorrhagic cystitis^{1,2,25,26}. The asymptomatic urinary shedding of BKPyV has been reported in healthy immunocompetent individuals as well²⁷. It is known that BKPyV remains latent following the infection of the urinary tract in the proximal tubular epithelium of the kidney, the urothelial cell layer of the bladder, ureters, or renal pelvis¹. Immunosuppression due to any clinical reason leads to the reactivation of BKPyV from viral latency^{28,29}. The detection of decoy cells in voided urine is a widely used diagnostic tool as a cytopathological surrogate marker of a BKPyV-infection³⁰ and has several clinical implications. These cells mimic atypical neoplastic cells and can be misdiagnosed as carcinoma in situ¹². The reactivation of BKPyV can lead to BKPyV-associated nephropathy. BKPyV-associated nephropathy develops in 1–10% of kidney transplant recipients and can ultimately result in renal graft loss in 30% up to 80% of the cases^{25,28}.

The prevalence of BKPyV varies from one study to another, which probably depends on the number of transplantations performed in the corresponding institution. Decoy cells are more often found in patients with a history of kidney or stem cell transplantation^{25,28}. Our results show that BKPyV-positive decoy cells in voided urine samples were

also detected in patients with chronic diseases, such as autoimmune diseases, diabetes, ulcerative colitis, diabetic nephropathy, and non-urological cancer, which could be explained by immunosuppressive or systemic therapy. Our findings are in line with previously reported results by Reploeg et al.³¹.

Interestingly, 20% of the patients in our cohort did not have any history of immunosuppression, transplantation, or cancer, which could explain the reactivation of BKPyV. Thus, our data show that BKPyV-infection can occur in immunocompetent patients who did not have any history of UCC, organ transplantation, autoimmune or immunomodulated disease, or immunosuppressive medication. The reactivation of BKPyV was reported previously in immunocompetent patients with hemorrhagic and non-hemorrhagic cystitis, ulcerative painful bladder syndrome, and interstitial cystitis^{31,32}, possibly suggesting a pathophysiological role for BKPyV in the development of these diseases.

BKPyV was associated with several types of cancer, including high-grade urothelial carcinoma (both in situ and invasive), micropapillary urothelial carcinoma, pleomorphic giant cell carcinoma, bladder adenocarcinoma, and nephrogenic adenoma^{3,5,6,7,8,9}. Our study did not find any of these rarely seen types of urothelial cell carcinoma. Our cohort included conventional type urothelial cell carcinoma, and none of the samples were positive for BKPyV. Similar data were published previously. Csoma et al. reported no evidence of BKPyV in 76 bladder cancer samples in their cohort²⁹. Kumari et al. reported no association of BKPyV with urothelial carcinoma in immunocompetent patients³³. Lu et al. showed no association of BKPyV cytopathic effects in urine cytology with the high risk of developing a high-grade urothelial carcinoma²⁴. In our study, the BKPyV in urine was not related to the urothelial cell carcinomas resected prior to the virus detection in urine, making its role in the oncogenesis of UCC very unlikely in our cohort.

Nevertheless, based on our results, BKPyV may be associated with intravesical therapy and/or radiotherapy since nine patients with UCC (9/14:64%) in our cohort were treated with either BCG, mitomycin, or radiotherapy before the detection of the virus in urine. To the best of our knowledge, this is the first study reporting such findings. None of the patients had a decoy cell in the urine cytology prior to the diagnosis of UCC. Further research is necessary to clarify the exact mechanism of its occurrence or the nature of the association. It is known that intravesical treatment can lead to BCG cystitis and even disseminated systemic BCG infection^{34,35}. Eosinophilic cystitis is a relatively common complication of intravesical mitomycin therapy^{36,37}. Potentially, therapy-induced cystitis could explain the reactivation of the latent BKPyV. Still, this question was not the aim of our present study, and further research is needed to elucidate this issue. Moreover, the standard treatment of all patients diagnosed with UCC usually includes Bacillus

Calmette–Guerin (BCG) or mitomycin C. Therefore, we cannot exclude that the other five patients with unknown UCC-treatment have also received the treatment, but we could not confirm this information in the database. However, it is known that reactivation of BKPyV can occur following various reasons, and further research is needed to elucidate its cause and mechanism in the case of intravesical treatment.

5. CONCLUSIONS

In our study, the BKPyV detected in urine was not associated with the prior resected urothelial cell carcinomas, potentially excluding the role of this virus in the oncogenesis of the conventional type UCC in our cohort. BKPyV-reactivation is not restricted to immunosuppression but also found in the urine of patients diagnosed with urothelial cell carcinoma and patients without any history of transplantation, malignancy, or chronic diseases. The intravesical treatment could be associated with the reactivation of the latent BKPyV. BKPyV-positivity in the immunocompetent patients may be explained by cystitis, possibly causing viral reactivation. BKPyV testing in post-UCC patients is of potential clinical relevance for the risk assessment of BKPyV-nephropathy, and further research is needed to unravel this complex relationship.

Supplementary Materials:

The following are available online at <https://www.mdpi.com/1999-4915/12/12/1412/s1>. Table S1: Clinicopathological data, demographic and relevant follow-up information of the patients without a history of urothelial cell carcinoma. Table S2: Clinicopathological, histopathological and immunohistochemistry data in the patients diagnosed with urothelial cell carcinoma (either in situ or invasive). Table S3: PCR-primers used in this study.

Author Contributions

Conceptualization, F.K., A.z.H., and I.V.S.; methodology, F.K., G.M., and I.V.S.; investigation, F.K., G.M., and I.V.S.; data curation, F.K., G.M., and I.V.S.; writing—original draft preparation, F.K., G.M., and I.V.S.; writing—review and editing, A.z.H., and I.V.S.; visualization, F.K., and I.V.S.; resources, I.V.S.; principal investigator, supervision, and project administration, I.V.S. and A.z.H. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary:

Table S1. Clinicopathological data, demographic and relevant follow up information of the patients without a history of urothelial cell carcinoma.

Patient Lab-ID	Age	Gender	Follow up period (months)	First presentation with BKPyV urine cytology	BKPyV IHC on cytology	Patient history including transplantation and immunosuppression
I.1	25	M	12	2006	+	Kidney transplantation
I.2	57	F	135	2007	+	Kidney transplantation
I.3	50	M	134	2007	n.a.	Colitis ulcerosa
I.4	60	M	7	2008	+	Kidney transplantation
I.5	74	M	12	2008	+	Kidney transplantation
I.6	63	M	85	2009	+	Autoimmune thyroiditis, diabetes, prostate adenocarcinoma Grade group 1
I.11	67	M	12	2011	n.a.	No chronic disease
I.14	72	M	75	2012	+	Kidney transplantation 2011
I.16	55	F	82	2012	+	Autoimmune hepatitis
I.18	66	M	27	2014	+	2014 colon adenocarcinoma. 2015 High grade sarcoma soft tissue leg. No information about treatment.
I.19	78	M	12	2014	+	No chronic disease
I.20	38	F	22	2014	+	No chronic disease
I.24	77	M	51	2015	+	No chronic disease
I.25	69	M	12	2016	+	Stem cell transplantation, GVHD liver.
I.27	76	M	16	2016	n.a.	No chronic disease
I.28	45	F	48	2017	+	No chronic disease

Abbreviations: M, Male; F, Female; BKPyV, BK polyomavirus; n.a. no material available for immunohistochemistry.

Table S2: Clinicopathological, histopathological and immunohistochemistry data in the patients diagnosed with urothelial cell carcinoma (either in situ or invasive).

Patient Lab-ID	Age	Gender	Follow up period (months)	Presentation UCC (Year)	First presentation with BKPyV in urine cytology (Year)	Number of years between presenting UCC and detecting BKPyV urine cytology	pT stage	Patient history
I.7	83	M	66	2006	2010	4	pT _{1a}	- 2004: LGUCC of the distal ureter and nephrectomy. - 2006: First presentation with LGUCC urinary bladder. - 2008: Last presentation LGUCC urinary bladder. - No tissue blocks available for IHC.
I.8	55	M	56	2009	2010	1	pT _{1a}	- 2009: LGUCC urinary bladder. Negative BKPyV-IHC of the UCC TURB.
I.9	71	M	169	2005	2010	5	pT _{1a}	- 2010 and 2018: BKPyV positive in urine. - 2005: First presentation LGUCC urinary bladder. - 2013: Negative BKPyV-IHC of the UCC TURB. - History of prostate adenocarcinoma.
I.10	89	M	132	2008	2010	2	pT ₁ and later pT _{1a}	- 2008: HGUCC. Negative BKPyV-IHC of the UCC TURB.
I.12	90	M	85	2004	2011	7	pT ₂	- 2009 LGUCC. Negative BKPyV-IHC of the UCC TURB. - pT2G3 detrusor invasive UCC with sarcomatoid features. - No tissue blocks available for IHC.
I.13	55	M	88	2008	2011	3	pT _{1s} progressed to pT ₃ and multiple metastases	- 2008: CIS with BCG treatment. Negative BKPyV-IHC of CIS specimen. - 2010: INUCC with radiotherapy and chemotherapy. Negative BKPyV-IHC. - 2012: lymph node metastasis. Negative BKPyV-IHC. - 2014: bladder resection from pT ₃ (INUCC); on two samples of metastatic UCC in rectum. Negative BKPyV-IHC. - 2015: small intestine resection with metastases UCC and lymph node metastasis. Negative BKPyV-IHC.
I.15	76	M	10	2011	2012	1	pT _{1a}	- 2011: LGUCC. Negative BKPyV-IHC in the TURB sample
I.17	74	M	94	2011	2013	2	pT _{1s}	- 2011: CIS urinary bladder. Negative BKPyV-IHC in the TURB samples.
I.21	66	M	75	2013	2014	1	pT _{1a}	- 2013: HGUCC in the diverticulum of the urinary bladder, with partial squamous differentiation. Negative BKPyV-IHC in the samples of high grade UCC from the diverticulum of the urinary bladder, with partial squamous differentiation; - 2018: Negative BKPyV-IHC in the urinary bladder biopsy with chronic inflammation

Patient Lab-ID	Age	Gender	Follow up period (months)	Presentation UCC (Year)	First presentation with BKPyV in urine cytology (Year)	Number of years between presenting UCC and detecting BKPyV urine cytology	pT stage	Patient history
I.22	70	M	115	2008	2015	7	pTis	- 2008 and 2014: Multiple CIS with multiple BCG courses. Negative BKPyV-IHC.
I.23	72	M	134	2007	2015	8	pT1	- 2007: INUCC. Negative BKPyV-IHC in high grade stromal invasive UCC. - 2015 and 2018: Negative BKPyV-IHC of non-invasive LGUCC.
I.26	80	F	78	2011	2016	5	pT1, pTis	- 2011 and 2014: Multiple INUCC and CIS in the period. Negative BKPyV-IHC. - 2018: Liver metastasis UCC. Negative BKPyV-IHC.
I.29	59	M	24	2017	2018	1	pTa	- 2017: LGUCC. Negative BKPyV-IHC in the TURB.
I.30	78	M	170	2005	2018	13	pTa	- 2005 and 2018: LGUCC. Negative BKPyV-IHC.

Abbreviations: UCC, Urothelial cell carcinoma; M, Male; F, Female; n.a, not applicable; IHC, Immunohistochemistry; LGUCC, Low-grade non-invasive urothelial cell carcinoma; HGUCC, high-grade non-invasive urothelial cell carcinoma; INUCC, invasive urothelial cell carcinoma; CIS, Carcinoma in Situ; BCG, Bacillus Calmette-Guerin ; FFPE, Formalin-Fixed Paraffin-Embedded ; BKPyV, BK polyomavirus; pTa, non -invasive stage; pT1, invasive stage in the stroma of urinary bladder; pT2- invasive stage in the detrusor muscle of urinary bladder; pT3 – invasive stage in the fibro-adipose tissue beyond urinary bladder.

Table S3: PCR-primers used in this study

#	Primer	Gene	Region	Forward primer (5' to 3')	Reverse primer (5' to 3')	Products Size (bp)	Reference
1	BKPyV-TA	LTA _g	(4322-4567)	ACAGCAAAAGCAGGCAAG	GGTGCCAACCTATGGAACAG	246	(1)
2	BKPyV-VP	VP1	(1600-1726)	GCAGCTCCCAAAAAGCCAAA	CTGGGTTAGGAAGCATTCTA	127	(2)

Abbreviations: LTA_g, large tumor antigen; TA, tumor antigen; VP, viral protein; bp, base pair.

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CHAPTER 5

5

Low Prevalence of Merkel Cell Polyomavirus in Human Epithelial Thymic Tumors

Emil Chteinberg, **Faisal Klufah**, Dorit Rennspiess, Mick F. Mannheims, Myrurgia A. Abdul-Hamid, Mario Losen, Marlies Keijzers, Marc H. De Baets, Anna Kordelia Kurz, Axel zur Hausen

ABSTRACT

Background

The etiology of thymic epithelial tumors is unknown. Murine polyomavirus strain PTA has been shown to induce thymomas in mice. Recently, using diverse molecular techniques, we reported the presence of human polyomavirus 7 (HPyV7) in thymic epithelial tumors. In the present study, we investigated the prevalence of Merkel cell polyomavirus (MCPyV) in thymic epithelial tumors.

Methods

Thirty-six thymomas were screened for MCPyV by PCR and subsequently tested by DNA and RNA in situ hybridization and immunohistochemistry. Twenty-six thymomas were diagnosed with myasthenia gravis (MG).

Results

MCPyV DNA was detected by PCR in 7 (19.4%) of the 36 thymic epithelial tumors and in six of these, the presence of MCPyV was confirmed by fluorescence situ hybridization. Of these, 3 (28.6%) revealed weak MCPyV LT-antigen protein expression. In addition, one of the MCPyV positive thymomas tested positive for MCPyV LT RNA with RNAscope. Of interest, two out of the three thymomas that previously tested positive for MCPyV by immunohistochemistry also tested positive for HPyV7. One of the 11 MG-negative and 2 of the 25 MG-positive were positive for MCPyV.

Conclusions

MCPyV DNA and MCPyV protein expression can be detected in human epithelial thymoma; however, to a far lesser extent than HPyV7. Our data strongly indicate that because of its infrequent detection and weak expression, MCPyV is unlikely to play an important role in the etiopathogenesis of human thymomas.

Keywords: Fluorescence in situ hybridization; immunohistochemistry; Merkel cell polyomavirus; thymoma; tumorigenesis.

INTRODUCTION

Thymomas are rare thymic epithelial tumors that are frequently associated with autoimmune diseases, such as myasthenia gravis (MG, 24–40%).¹ The underlying etiology of thymomas is unknown. Several studies have investigated the role of viruses in thymomagenesis.^{1–3} We previously assessed the presence of the novel human polyomaviruses 6 and 7 (HPyV6 and 7) in human thymic epithelial tumors, based on the reported induction of thymomas in mouse strains C3H/BiDa and AKR by the polyomavirus strain PTA, and frequently detected HPyV7 in these tumors.^{4–6} The number of human polyomaviruses detected has continually increased in recent years.^{7, 8} To date, only Merkel cell polyomavirus (MCPyV) has been established as a novel human tumor virus in the majority of Merkel cell carcinomas (MCC).^{8, 9} In MCC, MCPyV is clonally integrated in the tumor DNA and harbors tumor specific truncating oncogenic mutations within the large T (LT) antigen.^{9–11} Truncated LT antigen and small T antigen (sTAg) of MCPyV have been shown to inhibit the tumor suppressor protein retinoblastoma (RB) and p53.^{12–14} Nicol et al. recently reported the seroprevalence of five novel human polyomaviruses (MCPyV, HPyV6, HPyV7, HPyV9, TSPyV [trichodysplasia spinulosa-associated polyomavirus]) is increased in an age-dependent manner.¹⁵ In other studies, 86.4% and 56.8–63.6% of adult blood sera (≥ 20 years) were positive for MCPyV- and HPyV7-DNA, respectively.^{16, 17} In two independent studies using clinicopathologically not further classified thymomas ($n = 10$), no MCPyV DNA could be detected by PCR.^{18, 19} Because of these reports and our recent finding that 62.2% of thymomas are HPyV7 positive,⁶ we aimed to comprehensively assess the prevalence of MCPyV in this clinicopathologically well-classified cohort of human thymic epithelial tumors by diverse molecular techniques.

Testing the prevalence of MCPyV in formalin-fixed and paraffin-embedded (FFPE) tissues of MCC has generated conflicting results in previous studies.²⁰ According to Moshiri et al., the use of a PCR-based approach in combination with the CM2B4 anti LT-antigen antibody has the highest specificity and sensitivity.²⁰ Herein, we used a PCR based approach in combination with MCPyV fluorescence situ hybridization (FISH) and immunohistochemistry (IHC) to elucidate the presence of MCPyV in thymic epithelial tumors.

METHODS

Patients and tissues

Thirty-six FFPE thymoma resection specimens were included in this study (from 19 women and 17 men; mean age 58.3 years, range 34–82 years). Thirty-five of these

had previously been tested for the presence of HPyV7.6 Twenty-six thymoma patients were known to have a history of MG, of which 24 were anti-acetylcholine receptor (AChR) antibody positive and two were negative. Twelve of the 26 MG-positive thymoma patients were immunosuppressed. All clinical pathological data are summarized in Table 1. In addition, non-neoplastic thymic hyperplasia (n = 20) and fetal thymic (n = 20) tissues were included in this study (summarized in Table S1). All use of this tissue and patient data was in agreement with the Dutch Code of Conduct for Observational Research with Personal Data (2004) and Tissue (2011, www.federa.org/sites/default/files/digital_version_first_part_code_of_conduct_in_uk_2011_12092012.pdf). Diagnoses were previously defined by histology in routine diagnostics and were reviewed by two experienced pathologists.

Merkel cell polyomavirus (MCPyV) detection

PCR was performed with 250 ng of genomic DNA using the AmpliTaq Gold DNA Polymerase (Thermo Fisher Scientific, Naarden, The Netherlands) in a final volume of 50 μ L, as recently described.¹⁰ DNA quality and integrity were assessed by specimen control size ladder (SCS) as previously described and published for the DNA used.^{6, 21} All gained PCR fragments were confirmed by sequencing. In addition, Merkel cell carcinoma cell lines MS-1 and MCC26 were used as positive and negative controls, respectively, to detect MCPyV.

MCPyV detection by fluorescence in situ hybridization (FISH)

MCPyV FISH was performed as previously described.^{22, 23} In brief, deparaffinized 3 μ m thick sections were pretreated with 0.2 M hydrochloric acid, incubated with 1 M NaSCN and digested with 1 mg/mL pepsin (2500–3500 U/mg, Sigma Chemical, St. Louis, MO, USA). The biotin labeled “specific” MCPyV DNA probe was added to the samples at a concentration of 5 ng/ μ L, followed by denaturation of DNA (five minutes, 80°C) and hybridization overnight (37°C, humid chamber; ThermoBrite System, Abbott Molecular, Abbot Park, IL, USA). Unbound MCPyV DNA probe was stringently washed away. Bound probe was detected by sequential incubation in a combination of secondary antibodies: fluorescein isothiocyanate (FITC) avidin secondary antibody (1:500) and biotin conjugated goat anti-avidin (1:100; Vector, Brunswick Chemie, Amsterdam, The Netherlands). Prior to incubation, aspecific binding sites were blocked with Boehringer Blocking reagent (Roche, Molecular Diagnostics Inc., South Branchburg, NJ, USA). Cell nuclei were counterstained, and cover slipped with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; 0.2 μ g/mL; Vectashield, Vector Laboratories, Burlingame, CA, USA). Samples were visualized using a DM 5000B fluorescence microscope (Leica, Wetzlar, Germany) coupled to an online digital camera (Leica DC 300 Fx) for independent evaluation of FISH signals by two investigators.

RNA-in situ hybridization

RNAscope 2.5 HD RED assay (cat. no. 322350; Advanced Cell Diagnostics, Newark, CA, USA) was performed using target probes to MCPyV on tissue sections according to the manufacturer's instructions and methods followed by Wang et al.²⁴ Human peptidylprolyl isomerase B (Hs-PPiB) and bacterial dihydrodipicolinate reductase (DapB) were used as positive and negative controls, respectively. FFPE HeLa cells (Advanced Cell Diagnostics) were also used as negative and positive controls, as suggested by Advanced Cell Diagnostics. In addition, we used a FFPE MCC tissue block that previously tested MCPyV-positive by DNA-PCR and immunohistochemistry as a positive control. Four investigators independently evaluated the slides.

Immunohistochemistry (IHC) and double staining procedure

We used an anti LT-antigen MCPyV antibody (clone: CM2B4, dilution 1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Immunohistochemical staining was conducted on a Dako Autostainer Link 48 using the EnVision FLEX Visualization Kit K8008 (Dako, Carpinteria, CA, USA) according to standard diagnostic routine protocols and manufacturer's instructions.

RESULTS

MCPyV detection by DNA PCR and FISH

A total of 13 of 36 (36.1%) thymomas tested positive using M1/2, 8 (22.2%) tested positive using VP1, and 4 tested positives using LT3 primers (Table 1). Sequence analyses of the PCR amplicons identified all PCR products as MCPyV DNA sequences. In total, 7 (19.4%) of 36 thymomas tested positive for two out of three different MCPyV DNA PCRs (Table 1, Fig 1) and thus were considered MCPyV-positive. Of these seven MCPyV-positive thymomas, five had a history of MG. In these five, the presence of MCPyV was confirmed by MCPyV FISH (Fig 2a). FISH analysis revealed relative weak punctuate hybridization signals, which were mostly heterogeneous patterns in all cases tested. None of the follicular hyperplasia or fetal thymus tissues were positive for MCPyV-DNA.

MCPyV LT IHC and MCPyV LT RNA-ISH

All seven positive MCPyV thymomas were assessed for MCPyV LT-antigen expression by IHC. Three out of seven (42.9%) revealed the presence of LT-antigen in thymoma tissues (Fig 2e, Table 1). The expression of MCPyV LT in these tissues was weak. In addition, three thymoma tissues, two MCPyV-negative (ID 1–26 and ID 1–32) and one MCPyV-positive (ID 1–8); and one MCPyV-positive MCC tissue were analyzed for MCPyV LT-antigen RNA (Fig 2b,d, Table 1). The MCC cells revealed strong MCPyV

Table 1. Clinicopathological data and MCPyV DNA PCR, FISH, IHC, and RISH results with HPyV7 positivity of the tissues

Clinicopathological data										MCPyV					HPyV7		
ID	G	Age	MG	Thymtype	Masaoka-Koga	Anti AChR	IS/ster.	M1/M2 (178bp)	VP1 (351bp)	LT3 (308bp)	MCPyV FISH	MCPyV RISH	MCPyV IHC	sTAg-PCR	LTAg-PCR	FISH	IHC 2+10
1-1	F	73	+	B1/B2	I	+	+	-	-	NA				+	+	+	-
1-2	F	75	+	B1/B2	I	+	+	-	-	NA				+	+	+	-
1-11	F	34	+	B2	I	+	-	-	-	-				-	-	++	-
1-12	F	36	+	A	I	-	+	+	(+)	-	+	n.d.	-	-	-	-	-
1-16	F	34	+	A	I	+	+	+	(+)	-	+	n.d.	-	-	-	-	-
1-17	M	69	+	AB	I	+	+	-	-	-				-	+	++	-
1-18	F	47	+	AB	I	+	+	-	-	-				+	+	-	-
1-19	M	68	-	AB	I	NA	NA	-	-	-				+	+	++	+
1-21	F	38	+	AB	I	+	-	-	-	-				-	+	-	-
1-22	M	65	+	AB/B2	I	+	-	+	-	(+)	NA	n.d.	-	-	-	-	-
1-28	M	38	-	B1	I	NA	NA	-	-	-				-	-	-	-
1-31	F	82	+	A	I	+	+	+	-	-				+	+	+	+
1-32	M	47	+	B2	I	+	-	+	-	-	n.d.	-	n.d.	-	-	-	-
1-34	M	59	-	AB	I	NA	NA	-	-	-				+	-	++	+
1-36	F	82	-	AB	I	NA	NA	-	-	-				-	(+)	++	+
1-39	F	78	-	B1	I	NA	NA	-	-	-				-	-	-	-
1-43	F	78	-	AB	I	NA	NA	+	+	-	+	n.d.	+	-	-	++	(+)
1-23	M	37	+	AB	IIA	+	-	+	-	-				+	-	+	+
1-24	M	68	+	B2	IIA	+	-	-	-	-				+	+	++	+
1-33	F	43	+	B2	IIA	+	-	-	-	-				-	-	++	+
1-35	M	45	+	AB	IIA	+	-	-	-	-				+	+	++	+
1-37	M	77	+	AB	IIA	+	-	-	-	-				-	-	+++	(+)
1-38	F	80	+	AB	IIA	+	+	-	-	+				-	-	-	-
1-3	F	57	+	B2/B3	IIB	+	-	+	-	-				+	+	+	+
1-5	M	37	+	B3	IIB	+	+	+	-	-				+	+	++	+
1-7	F	79	+	A	IIB	-	-	+	-	-				+	+	++	(+)

Clinicopathological data																	
ID	G	Age	MG	Thymtype	Masaoka-Koga	Anti AChR	IS/ster.	M1/M2 (178bp)	VP1 (351bp)	LT3 (308bp)	MCPyV			HPyV7			
											MCPyV FISH	MCPyV RISH	MCPyV IHC CM2B4	sTAg-PCR	LTAg-PCR	FISH IHC 2t10	
1-8	M	58	+	A/B2	IIB	+	-	(+)	(+)	+	++	(+)	(+)	+	+	+++ (+)	
1-9	M	64	-	B2	IIB	NA	NA	-	-	-				+	+	++ (+)	
1-15	F	53	+	B3	IIB	+	+	-	-	-				-	-	+	
1-26	M	65	-	AB	IIB	NA	NA	-	-	(+)	-	-	-	-	-	-	
1-42	F	57	+	AB	IIB	+	-	-	-	-				-	-	-	
1-10	F	73	+	B3	III	+	+	-	-	-				-	-	-	
1-13	F	54	-	B2	III	NA	NA	-	-	-				-	+	(+)	
1-20	M	64	-	B3	III	NA	NA	++	++	++	+	n.d.	-	-	-	-	
1-41	M	65	-	B3	III	NA	NA	-	-	-				+	(+)	+	
1-25	M	37	+	B2	IVA	+	+	+	+	NA	+	n.d.	+	n.d.	n.d.	n.d.	
		25/36				24/25	12/25	13/36	8/36	4/33	6/7	1/3	3/7	15/35	17/35	22/35	15/35
		69.4%				96.0%	48.0%	36.1%	22.2%	12.1%	85.7%	33.3%	37.5%	42.9%	48.6%	62.9%	42.9%

Clinicopathological data and the results of Merkel cell polyomavirus (MCPyV) DNA PCR, fluorescence in situ hybridization (FISH), immunohistochemistry (IHC) and RNA in situ hybridization (RISH) in relation to the recently reported HPyV7 positivity in these thymomas as published by Rennspies et al., 2017. MCPyV-negative ID-32 and ID-26 were used as negative controls for IHC and RISH. Anti-AChR, anti-acetylcholine receptor antibodies; bp, base pairs; F, female; G, gender; HPyV7, human polyomavirus 7; ID, laboratory identification; IS/Ster., immunosuppression/steroids; LTAg, large T-antigen; M, male; MG, myasthenia gravis; sTAg, small T-antigen; Thym. type, thymoma type; ++, strong positive; +, positive; (+), weak positive; -, negative; NA, not applicable; n.d., not done.

Figure 1: Representative PCR of 11 thymic tissues. Representative results of Merkel cell polyomavirus (MCPyV) M1/2 (178 base pairs [bp]), VP1 (351 bp), and LT3 (308 bp) PCR primer set of 11 human epithelial thymic DNA and two Merkel cell carcinoma cell lines. MS-1 was used as a positive control and MCC26 as a negative control. Amplification could be observed for VP1 and M1/2 primer sets for ID 1-8, ID 1-9 and ID 1-20. For the LT3 primer set, ID 1-8, ID 1-20, and ID-26, a PCR product could be detected. free, free space; H₂O, water control; MW, molecular weight marker.

RNA detection in the cytoplasm (Fig 2c). ID 1–8 showed weak detection of MCPyV RNA. ID 1–26 and ID 1–32 showed no RNA amplification (Fig 2c, Table 1).

Of interest, thymoma tissue ID 1–8 was positive for both polyomaviruses: HPyV7 and MCPyV (Fig 2f, Table 1), whereas the MCPyV expression was weaker compared to HPyV7.

DISCUSSION

We recently reported the presence of HPyV7 in 46.0% (protein level) to 62.2% (DNA level) of thymomas.⁶ Herein, we assessed the presence of MCPyV on DNA level by PCR and FISH, on RNA level by RNA-ISH (RISH), and on protein level by IHC. In order to reliably detect MCPyV in FFPE tissues, Moshiri et al. recently reported that the use of a PCR-based approach in combination with the CM2B4 anti LT-antigen antibody yielded the highest specificity and sensitivity.²⁰ In the present study, we used a PCR based approach in combination with MCPyV FISH, RISH, and IHC in order to comprehensively elucidate the presence of MCPyV in the same clinicopathologically well-classified thymoma cohort in which we recently assessed the presence of HPyV7. None of thymic hyperplasia (n = 20) or fetal thymic tissues were positive for MCPyV assessed by PCR. Overall 19.4% (n = 7) thymomas tested positive for the presence of MCPyV DNA (VP1, M1/2, and LT3). The presence of MCPyV DNA was confirmed in six of seven PCR positive thymomas by FISH analysis. The bands and signals obtained by PCR and FISH were weak. Correspondingly, weak MCPyV LT-antigen expression on protein level was found in three of seven thymomas. The prevalence of MCPyV was far lower than the prevalence of HPyV7 in these thymomas.

Of all MG-positive thymomas tested for MCPyV, five were positive on DNA level and three on protein level. Based on this small number, no conclusion can be drawn concerning an association between MCPyV and MG in thymoma. In addition, the weak DNA and protein detection correlates with the weak prevalence of MCPyV RNA compared to the RNA detection of the MCPyV-positive MCC tissue assessed by RISH in ID 1–8. In contrast to Torrachio et al. and Hashida et al. we used a larger number of MCPyV detection techniques in a larger thymoma cohort, which very likely explains why we were able to identify some MCPyV positive thymoma cases.^{18,19} However, given the weak DNA amplification and the low protein expression in these few, it can be concluded that MCPyV is weakly present in three thymomas, thus MCPyV is very unlikely to play a role in human thymomagenesis. In the context of our previously reported frequent finding of HPyV7 within the same thymoma cohort, the low prevalence of MCPyV indirectly supports a role of HPyV7 in human thymomagenesis.

Figure 2: Detection of Merkel cell polyomavirus (MCPyV) and human polyomavirus 7 (HPyV7) in thymoma ID 1-8. (a) Magnification (63x) an overlay of nuclei 4,6-diamidino-2-phenylindole dihydrochloride staining and MCPyV fluorescence in situ hybridization of thymoma ID 1-8 shows that MCPyV DNA is present in thymoma nuclei. Single dots per nuclei were detected. (b) RNAscope RNA in situ hybridization (10x) was performed on the thymoma ID 1-8. (c) MCPyV-positive Merkel cell carcinoma (MCC) tissue, and (d) and thymoma ID 1-32. MCPyV LT RNA are detected in (b) thymoma cells, which is comparable to the dot-like signals in (c) MCPyV-positive MCC cells. (d) In thymoma ID-32, no MCPyV RNA is present. Immunohistochemistry (10x) for (e) MCPyV with the CM2B4 antibody and (f) HPyV7 with the 2t10 antibody reveal MCPyV and HPyV7 expressing thymoma cells. MCPyV expression is weaker than HPyV7.

Remarkably, most thymoma cases that tested positive for HPyV7 or MCPyV in this cohort were of stage II or III according to the Masaoka classification (Table 1). Eleven of ¹⁴ stage II thymomas were positive for one of these human polyomaviruses. In addition, one of the three MCPyV positive tissues was classified as stage IIIB (Table 1). The higher thymoma stages are considered to be more invasive.^{25, 26} The sTag of MCPyV was recently observed to provide cells the ability to migrate;²⁷ however, in as much this applies to HPyV7 and thymic epithelial cells currently remains speculation.

Of interest, to the best of our knowledge, we show for the first time the concomitant detection of HPyV7 and MCPyV in the same tumor tissue using diverse molecular techniques. Interestingly, MCPyV and HPyV7 are also present with a high seroprevalence in the same blood.^{16, 17} Detection of the MCPyV genome by PCR and FISH reveals that MCPyV can infect thymic epithelial cells. In the present study we show that thymic epithelial tumors can harbor both HPyV7 and MCPyV, although the prevalence of MCPyV in human epithelial tumors is far lower than reported for HPyV7. Our data strongly indicate that MCPyV is very unlikely to play an important role in the etiopathogenesis of human thymomas.

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Disclosure

No authors report any conflict of interest.

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Supplementary data:

Table S1. Clinicopathological Data of follicular hyperplasia tissue results with human polyomavirus 7 (HPyV7) positivity, as published by Rennspieß et al., 2017.

Clinicopathological data												
ID	Gender	Age	MG	Anti-AChR	IS/Ster	MCPyV			HPyV7			
						M1/M2 (178bp)	VP1 (351bp)	LT3 (308bp)	sTAg-PCR	LTAg-PCR	FISH	IHC 2t10
1-1	M	16	+	+	-	-	-	-	-	-	+	-
1-2	F	15	+	+	-	-	-	-	-	-	+	-
1-3	F	20	+	+	-	-	-	-	-	+	+	-
1-4	F	25	+	+	-	-	-	-	-	+	-	(+)
1-5	F	24	+	+	-	-	-	-	+	-	+	(+)
1-6	F	26	+	+	-	-	-	-	+	-	+	+
1-7	F	30	+	+	-	-	-	-	-	-	+	-
1-8	F	32	+	+	-	-	-	-	-	-	+	-
1-9	F	40	+	+	-	-	-	-	-	-	-	-
1-10	F	13	+	+	-	-	-	-	+	-	+	-
1-11	M	40	+	+	-	-	-	-	-	+	+	(+)
1-12	F	34	+	+-	-	-	-	-	-	-	-	-
1-13	F	26	+	+	+	-	-	-	-	-	-	(+)
1-14	F	24	+	+	-	-	-	-	-	-	+	-
1-15	F	31	+	+	-	-	-	-	-	-	+	-
1-16	F	22	+	-	+	-	-	-	-	-	-	-
1-17	F	48	+	+	-	-	-	-	-	-	+	(+)
1-18	M	39	+	-	+	-	-	-	-	-	+	-
1-19	M	31	-	NA	NA	-	-	-	-	+	-	(+)
1-20	M	12	+	+	-	-	-	-	+	-	+	+

Anti-AChR, anti-acetylcholine receptor antibodies; F, female; G., gender; ID, laboratory identification; IS/Ster., immunosuppression/ steroids; M, male; MG, myasthenia gravis; NA, not applicable; Thym. type, thymoma type.

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CHAPTER 6

Presence of Human Papillomavirus and Epstein–Barr Virus, but Absence of Merkel Cell Polyomavirus, in Head and Neck Cancer of Non-Smokers and Non-Drinkers

Frans J. Mulder, **Faisal Klufah**, Famke M. E. Janssen, Farzaneh Farshadpour, Stefan M. Willems, Remco de Bree, Axel zur Hausen, Mari F. C. M. van den Hout, Bernd Kremer and Ernst-Jan M. Speel

ABSTRACT

Objective:

Determine the presence and prognostic value of human papillomavirus (HPV), Epstein-Barr virus (EBV), Merkel cell polyomavirus (MCPyV), and cell cycle proteins in head and neck squamous cell carcinoma (HNSCC) of non-smokers and non-drinkers (NSND).

Methods:

Clinical characteristics and tumors of 119 NSND with HNSCC were retrospectively collected and analyzed on tissue microarrays. RNAscope in situ hybridization (ISH) was used to screen for the presence of HPV and MCPyV mRNA. Immunohistochemistry was performed for expression of p16 as surrogate marker for HPV, Large T-antigen for MCPyV, and cell cycle proteins p53 and pRb. Positive virus results were confirmed with polymerase chain reaction. For EBV, EBV encoded RNA ISH was performed. Differences in 5-year survival between virus positive and negative tumors were determined by log rank analysis.

Results:

All oropharyngeal tumors (OPSCC) (n = 10) were HPV-positive, in addition to one oral (OSCC) and one nasopharyngeal tumor (NPSCC). The other three NPSCC were EBV-positive. MCPyV was not detected. Patients with HPV or EBV positive tumors did not have a significantly better 5-year disease free or overall survival. Over 70% of virus negative OSCC showed mutant-type p53 expression.

Conclusion:

In this cohort, all OPSCC and NPSCC showed HPV or EBV presence. Besides one OSCC, all other oral (n = 94), hypopharyngeal (n = 1), and laryngeal (n = 9) tumors were HPV, EBV, and MCPyV negative. This argues against a central role of these viruses in the etiopathogenesis of tumors outside the oro- and nasopharynx in NSND. So, for the majority of NSND with virus negative OSCC, more research is needed to understand the carcinogenic mechanisms in order to consider targeted therapeutic options.

Keywords: head and neck cancer, human papillomavirus, Epstein-Barr virus, polyomavirus, non-smokers, nondrinkers, cell cycle protein, in situ hybridization

INTRODUCTION

Viruses play an increasing role in head and neck squamous cell carcinoma (HNSCC). High-risk human papillomavirus (HPV)-positive oropharyngeal squamous cell carcinoma (OPSCC) has been identified as an entity with a different carcinogenesis than traditional HNSCC resulting from excessive tobacco and alcohol consumption. HPV is also an independent prognostic factor for a better disease free survival (DFS) and overall survival (OS), which has led to a down staging of these tumors in the eighth edition of the American Joint Committee on Cancer (AJCC) and union for International Cancer Control tumor-node-metastasis (TNM) classification¹⁻³. Because of the better prognosis, de-escalation strategies are proposed for HPV-positive OPSCC patients⁴. The prevalence of HPV-positive OPSCC is rising in the Western World. A HPV prevalence above 50% has already been reported in America, Europe, and Australia, based on HPV DNA in combination with either E6*I mRNA or p16 immunohistochemistry (IHC) detection^{5,6}. Combining these HPV detection methods has been recommended because only OPSCC with transcriptionally active HPV is related to a better survival compared to biologically inactive infections^{7,8}.

Another virus known for its carcinogenic potential in the head and neck region is the Epstein-Barr virus (EBV). EBV has a strong association with nasopharyngeal squamous cell carcinoma (NPSCC), approaching a prevalence of 100% in these tumors, and is endemic in Southern China, Southeast Asia, Northern Africa, and the Mediterranean basin^{9,10}. It is suggested to cause an immunosuppressive microenvironment in these tumors, among others via PD-L1 overexpression, making these patients interesting candidates for checkpoint blockade therapy¹⁰. Detection of EBV presence can be performed reliably with EBV encoded RNA (EBER) in situ hybridization (ISH)^{9,11}.

Lately, besides these acknowledged oncogenic viruses, there is attention for polyomaviruses in HNSCC. Merkel cell polyomavirus (MCPyV) has not only been detected by digital transcriptome subtraction and polymerase chain reaction (PCR) in up to 80% of Merkel cell carcinoma of the skin, but also in non-malignant tonsillar tissue, oral squamous cell carcinoma (OSCC), and pharyngeal cancer, with a reported prevalence of 23, 6.6–29, and 50% respectively¹²⁻¹⁷. Although it was thought not to play a role in oral carcinogenesis because of low viral loads detected with quantitative real-time PCR, the presence of MCPyV appears to be predictive for a better DFS¹⁶.

Cell cycle deregulation plays a central role in head and neck carcinogenesis, with frequent inactivation of TP53 and CDKN2A, leading to cell proliferation and prevention of apoptosis, among others. In HPV-related OPSCC, HPV integration in the host cell DNA genome leads to deregulation of oncoproteins E6 and E7, resulting in inactivity of

p53 and retinoblastoma tumor suppressor gene product pRb, respectively. The negative feedback of pRb inactivation leads to p16 overexpression¹⁸. In EBV infected NPSCC, it has been suggested that the cell cycle pathway is the most deregulated pathway, promoting the progression of the G1/S phase via inhibition of p16 expression and pRb overexpression¹⁹. For MCPyV, oncogenetic transformation requires both integration of the viral genome into the host genome and truncation of the Large T-antigen (LTAg) to render the viral genome replication deficient²⁰. LTAg mutations disrupt the DNA binding domain and the helicase domain distal to the pRb-binding motif, thereby promoting cell cycle progression by retaining its ability to bind to pRb^{20, 21}.

There is a small group of HNSCC patients without any exposure to the traditional risk factors. The mechanisms underlying carcinogenesis in these non-smokers and non-drinkers (NSND) remain largely unclear, but a significant role of oncogenic viruses would be expected. Indeed, a higher prevalence of HPV in these tumors has been reported in several studies, though in small numbers of patients^{22–25}. Therefore, the goal of this study was to determine the presence of HPV, EBV, and MCPyV in a series of 119 well-characterized NSND with HNSCC. Secondary analyses evaluate differences in tumor suppressor proteins p16, p53, and pRb expression regarding viral presence and whether the presence of these viruses is predictive for a better DFS and OS.

MATERIALS AND METHODS

Patients

Consecutive patients with HNSCC were selected at the University Medical Center Utrecht (UMCU) and Maastricht University Medical Center (MUMC). In the UMCU, patients were prospectively selected between 1980 and 2004, as described previously²⁶. In the MUMC, HNSCC patients have been selected retrospectively between 2011 and 2016, in addition to all patients with OPSCC between 2003 and 2010. Inclusion criteria were: ≥18-years-old NSND patients with HNSCC, available formalin fixated and paraffin embedded (FFPE) tumor tissue, and >2 years follow up. Patient characteristics, risk factors, World Health Organization tumor classification, AJCC seventh edition staging, and information concerning recurrent disease or death were collected from the medical records. Non-smoking was defined as having no history of smoking, non-drinking as having no history of alcohol consumption (not even ‘sporadic’ alcohol consumption), as reported in the patients’ medical records during both their first presentation at the Head and Neck outpatient clinic, as well as during the anesthesiological screening before panendoscopy or surgical resection. Patients with a second primary tumor in the head and neck region, tumors outside the upper aerodigestive tract, a cervical metastasis of

unknown origin, or a histopathologic diagnosis other than squamous cell carcinoma were excluded.

The Medical Ethics Review Committee of the MUMC (2018-0567) has approved this study and the principles outlined in the Declaration of Helsinki were followed. All data and tissues were handled according to General Data Protection Regulation.

Tissue Microarrays

FFPE blocks of either the diagnostic biopsy or tumor resection were retrieved and hematoxylin and eosin sections were digitally evaluated with a senior head and neck pathologist (SW or MH), using Panoramic viewer (3DHISTEC, Budapest, Hungary). Per patient, three 0.6mm tumor tissue cores and one normal epithelium core were selected, placed in a tissue microarray (TMAs), and cut into 5 μ m sections.

RNA *In Situ* Hybridization

To screen for the presence of HPV and MCPyV mRNA, the RNAscope 2.5 RED assay kit and HPV-16/18 or V-MCPyV-LT-ST-Ag probe cocktails (Advanced Cell Diagnostics, Newark, California) were used according to the manufacturer's instructions. In short, TMA sections were deparaffinized and pretreated with RNAscope Hydrogen Peroxide for 10 min. Antigen retrieval comprised of boiling the slide sections in the provided Target Retrieval Reagents solution at 100°C for 15 min. After washing, the TMAs were dried over night at room temperature and treated for 30 min with RNAscope Protease Plus. In situ hybridization was performed applying four droplets of the provided probes prior to each of the six amplification steps (30 min at 40°C, 15 min at 40°C, 30 min at 40°C, 15 min at 40°C, 30 min at room temperature, and 15 min at room temperature, respectively). After each hybridization step, the slides were washed in the RNAscope wash buffer for 2 min at room temperature. Subsequent to alkaline phosphatase Fast Red chromogenic visualization of hybridized probes, the slides were counterstained with hematoxylin and assessed under a bright field microscope at 200x magnification. Tissue with at least 1 red punctate signal dot in the cytoplasm and/or nucleus of malignant cells was considered to be positive, as suggested by the manufacturer for genes with an expression level varying between 1 to >10 copies per cell. Probes for housekeeping gene transcript human peptidylprolyl isomerase B (Hs-PPIB) and bacterial dihydrodipicolinate reductase gene (dapB) transcript were used as positive and negative controls, respectively. As virus specific positive controls, virus positive tumor tissue was used, in addition to the HPV-18 positive cell line HeLa, HPV-16 positive cell lines SiHa and Caski, and MCPyV positive cell lines MKL-1, MKL-2, and WaGa (Figure 1). Negative controls were tumor tissue of a virus negative patient, cell lines MKL-1 and MKL-2 for HPV, and cell lines MCC13 and MCC26 for MCPyV²⁷.

EBER-ISH was performed using the Dako fluorescein-labeled EBV peptide nucleic acid (PNA) probe mixture and PNA ISH Detection kit (Agilent Technologies, Santa Clara, California) according to the manufacturer's instructions. Briefly, following TMA section deparaffinization, target retrieval was performed with the Dako Omnis ISH Pre-Treatment solution for 5 min. Subsequently, enzyme pre-treatment was carried out using two steps of 3 min ethanol 96% application and one step of Dako ISH Pepsin for 15 min. Once the provided EBER RNA CISH probe was applied, denaturation at 66°C for 10 min, and hybridization at 45°C for 90 min followed. The slides were washed with the ISH Stringent Wash Buffer for 3 min, and the provided reagents were applied for staining: CISH Endogenous Enzyme Block for 3 min, Anti-FITC-AP for 30 min, and BCIP-NBT Substrate for 15 min. Sections were counterstained with Nuclear Fast Red and analyzed under a bright field microscope at 200x magnification. Strong blue staining of more than 50% of tumor nuclei was considered to be positive. In parallel, a probe for housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to ensure the presence of mRNA in the TMA and a case of EBV-positive infectious mononucleosis served as a positive control (Figure 1).

Immunohistochemistry

Three-µm FFPE TMA sections were subjected to IHC, using primary monoclonal antibodies directed against p16, p53, pRb, and the LTA_g of MCPyV (Table 1). Immunostainings were performed on a Dako Omnis autostainer (Agilent Technologies) using the EnVision FLEX+ Mouse (LINKER) kit. In short, antigen retrieval was performed on the TMA with sodium citrate-solution (pH 6.0) for p16, or a high pH-buffer (pH 9.0) for p53, pRb, and MCPyV. Endogenous peroxidase was blocked with Dako REAL Peroxidase-Blocking Solution prior to 20 minutes of incubation with the primary antibody. Binding of the antibodies was visualized by an enzymatic reaction with horseradish peroxidase and 3,3'-Diaminobenzidine as substrate, producing a brown precipitate (Figure 1). Slides were counterstained with hematoxylin and evaluated under a bright field microscope by two independent assessors, blinded for patients' clinical characteristics. In case of dissonance between the assessors, a third assessor evaluated the staining and agreement was reached by discussion. For p16, strong homogenous staining in the cytoplasm and nuclei of >70% of the tumor cells was considered to be overexpression²⁸. p53 staining was assessed as 0-mutant type (0% nuclear staining), mutant-type overexpression (>70% strong nuclear staining in the non-keratinizing tumor cells), or wild-type (heterogeneous nuclear staining)²⁹. For pRb, nuclear staining in <25% of tumors cells was evaluated as loss of pRb³⁰. MCPyV LTA_g was considered positive if >10% of nuclei were stained³¹. For p16, a case of HPV-positive OPSCC was used as a control, and for p53 and pRb normal tonsil tissue was used. MCPyV positive cell lines MKL-1, MKL-2, and WaGa served as positive controls for the LTA_g of MCPyV.

Figure 1 Representative images of RNAscope in situ hybridization on cell lines Caski (A), HeLa (B), and WaGa (C), positive for HPV-16, HPV-18, and MCPyV, respectively. A positive and negative control with housekeeping gene transcript PPIB (D) and bacterial transcript dapB (G) on the TMA, and a control patient positive for HPV-16 and MCPyV (E, F). Positive internal control for housekeeping gene GAPDH (H) and a control case of EBV-positive infectious mononucleosis (I) following Epstein-Barr virus encoded RNA in situ hybridization. Study TMA cores of patients positive for HPV-16 mRNA (at least 1 red punctate dot per tumor cell), p16 immunohistochemistry (>70% strong brown staining of tumor nuclei and cytoplasm), and EBV mRNA (>50% strong blue staining of tumor nuclei) are presented in (J–L), respectively. The images were taken at 200x magnification, an area of 100 μm^2 is marked in each image and 3x magnified in its top right corner.

Table 1 Immunohistochemistry primary antibodies and evaluation criteria.

Antibody characteristics		Source	Clone	Dilution	Retrieval	Localization	Evaluation criteria	
Antibody	Company						Cut off	References
p16	Immunologic	Monoclonal, Mouse	MX007	1:200	Citrate (pH 6.0)	Nuclear and cytoplasmic	>70%	²⁷
p53	Dako Omnis	Monoclonal, Mouse	DO-7	Ready-to-use	High pH buffer (pH 9.0)	Nuclear	≥25%	²⁸
pRb	Leica Biosystems	Monoclonal, Mouse	13a10	1:100	High pH buffer (pH 9.0)	Nuclear	≥25%	²⁹
Large T-antigen	Santa Cruz Biotechnology	Monoclonal, Mouse	CM2B4	1:50	High pH buffer (pH 9.0)	Nuclear	>10%	³⁰

Human Papillomavirus-Specific Polymerase Chain Reaction

Of patients with a positive result for HPV RNA-ISH and/or p16 IHC, DNA was isolated from eight 5-μm FFPE whole tissue sections with the Maxwell RSC DNA FFPE kit (Promega, Madison, Wisconsin). DNA concentrations were determined using the Quantus Fluorometer and the QuantiFluor ONE dsDNA system (Promega). Next, 250 ng DNA was added to 1 ml SurePath preservative fluid (VWR International, Amsterdam, Netherlands) and used for HPV-DNA analysis utilizing the COBAS 4800 platform (Roche, Basel, Switzerland), according to the manufacturer's instructions. The COBAS 4800 tests specifically for HPV-16, HPV-18, and a combination of 12 other HR-HPV types^{31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68}. Results were considered reliable when the controls were labeled "valid". Housekeeping gene β-globin was used as a control for the human DNA, in addition to DNA samples of an HPV-positive and HPV-negative tumor.

Statistical Analysis

Patients were considered to be HPV or MCPyV positive when the virus was detected with at least two techniques (ISH, IHC and/or PCR). EBV presence was based on the EBER-ISH result. Differences in clinical parameters between virus positive and virus negative patients were evaluated by Mann-Whitney U test for age because of a non-normal distribution, Fisher's exact test for binominal variables (sex, M-stage, recurrence, p16, pRb), and the Fisher-Freeman-Halton exact test for tumor location, T-stage, N-stage, and p53 because of expected counts of less than five. The 5-year DFS and OS were estimated with Kaplan-Meier curves and differences between virus positive and negative tumors were determined by log rank test. DFS was defined as the last date of treatment until the biopsy date of a histologically proven recurrence or second primary tumor in the head and neck region. OS was defined as the time between the primary tumor biopsy date and death. Censoring took place when patients were lost to follow-up, deceased without recurrent disease for DFS, or at the cut-off point of 60 months. All clinical and pathological parameters were assessed in bivariate analysis regarding survival. Variables with significant ($p < 0.05$) or near significant ($p < 0.1$) relationships were evaluated in multinomial logistic regression to assess predictors for DFS and OS. Analyses were

performed using IBM SPSS Statistics 25.0 (IMB corp., Armonk, NY) and a p-value of <0.05 was considered to be statistically significant.

RESULTS

A total of 119 patients were included in this study. These patients had a median age of 74.9 years (inter quartile range = 14.6 years) and were mainly women (78%) with a tumor of the oral cavity (80%) and no regional or distant metastases (66 and 95%, respectively). Thirty-one patients (26%) had recurrent disease within 5 years (Table 2).

Human Papillomavirus

ISH on the TMAs showed HPV-16/18 mRNA expression in tumors of ten patients. All of these tumors showed p16 overexpression by IHC as well, in addition to five tumors with no HPV-16/18 mRNA expression. COBAS analysis on HR-HPV DNA in these 15 patients detected the presence of HPV-16 in ten tumors and another HR-HPV type in one other case. For patient 61, the quality of the DNA was insufficient for COBAS analysis. This resulted in a total of 12 tumors being HPV-positive based on at least two detection techniques (Table 3).

Compared to HPV-negative tumors, HPV-positive tumors were associated with lower age (67.3 versus 76.2 years old, $p = 0.003$), oropharyngeal origin (83% versus 0%, $p < 0.001$), and N2-stage (58% versus 13%, $p = 0.004$) (Table 2). All oropharyngeal tumors ($n = 10$) were HPV-positive, in addition to one OSCC of the alveolar process and a NPSCC. Although the OSCC case showed HPV-16 DNA and p16 overexpression, no mRNA was detected with ISH, neither on the TMA nor on a whole section.

Four of the twelve patients with HPV-positive tumors (33%) died within five years, two of which had recurrent disease. For patient 1 there were no details recorded on the cause of death (OS = 59.6 months), patient 5 died of the complications of an aortic valve prosthesis endocarditis (OS = 23.7 months), patient 21 received palliative treatment because of distant metastasis (DFS = 1.6 months), and patient 61 developed liver metastases 20.1 months after initial therapy (Table 3). The presence of HPV was no predictor for a better DFS or OS ($p = 0.33$ and $p = 0.27$, respectively), compared to HPV-negative HNSCC in NSND (Figures 2A, B). A younger age at cancer diagnosis ($p = 0.008$), T1 stage ($p = 0.0047$), and N0 or N1 stage (both $p < 0.001$) were retained in the best multivariable model as predictors for OS in HNSCC of NSND (Supplementary table 1, Table 4). The model explained 35% of the variation (model fit: omnibus test of model coefficients: $X^2 = 36.0$ and $p < 0.001$; M-stage was omitted because of low case numbers).

Table 2 Comparison of clinical characteristics between human papillomavirus (HPV) and Epstein–Barr virus (EBV) positive and negative head and neck squamous cell carcinoma in non-smokers and non-drinkers.

Clinical characteristics		Total (n=119)		HPV-positive (n=12)		HPV-negative (n=107)		p-value	EBV-positive (n=3)		EBV-negative (n=116)		p-value
Age (years)	Median (interquartile range)	74.9	(14.6)	67.3	(13.2)	76.2	(14.7)	0.003	48.0	(NA*)	75.3	(14.1)	0.062
Sex	Female	93	(78)	10	(83)	83	(78)	1.0	2	(67)	91	(78)	0.53
	Male	26	(22)	2	(17)	24	(22)		1	(33)	25	(22)	
Location	Hypopharynx	1	(0.8)	0	(0)	1	(0.9)	<0.001	0	(0)	1	(0.9)	<0.001
	Larynx	9	(7.6)	0	(0)	9	(8.4)		0	(0)	9	(7.8)	
	Nasopharynx	4	(3.4)	1	(8.3)	3	(2.8)		3	(100)	1	(0.9)	
	Oral cavity	95	(80)	1	(8.3)	94	(88)		0	(0)	95	(82)	
	Oropharynx	10	(8.4)	10	(83)	0	(0)		0	(0)	10	(8.6)	
		33	(28)	3	(25)	30	(28)	0.35	1	(33)	32	(28)	1.0
T-stage	2	36	(30)	2	(17)	34	(32)		1	(33)	35	(30)	
	3	13	(11)	3	(25)	10	(9.3)		0	(0)	13	(11)	
	4	37	(31)	4	(33)	33	(31)		1	(33)	1	(31)	
		78	(66)	5	(42)	73	(68)	0.004	0	(0)	78	(67)	0.061
N-stage	1	18	(15)	0	(0)	18	(17)		1	(33)	17	(15)	
	2	21	(18)	7	(58)	14	(13)		2	(67)	19	(16)	
	3	2	(1.7)	0	(0)	2	(1.9)		0	(0)	2	(1.7)	
M-stage	0	113	(95)	11	(92)	102	(95)	0.42	3	(100)	111	(96)	1.0
	1	6	(5.0)	1	(8.3)	5	(4.7)		0	(0)	5	(4.3)	
Recurrence	Yes	31	(26)	2	(17)	29	(27)	0.73	2	(67)	29	(25)	0.17
	No	88	(74)	10	(83)	78	(73)		1	(33)	87	(75)	

*Not applicable because of small number of patients

Table 3 Demographics and viral analysis results of 15 virus positive tumors in non-smokers and non-drinkers.

Virus	Study ID	Age (years)	Sex	Tumor location	T	N	M	Recurrence	ISH	p16 IHC	COBAS PCR
HPV	1	56.4	Male	Oropharynx	3	2	0	No	+	+	HPV-16
	3	53.9	Female	Oropharynx	1	2	0	No	+	+	HPV-16
	4	71.5	Male	Oropharynx	4	2	0	No	+	+	HPV-16
	5	76.6	Female	Oropharynx	2	2	0	No	+	+	HPV-16
	9	69.5	Female	Oropharynx	3	0	0	No	-	+	HR-HPV
	16	68.3	Female	Oropharynx	1	0	0	No	+	+	HPV-16
	21	71.8	Female	Oropharynx	4	2	1	Yes	+	+	HPV-16
	29	57.8	Female	Nasopharynx	2	0	0	No	+	+	HPV-16
	32	63.5	Female	Oral cavity	4	0	0	No	-	+	HPV-16
	40	67.9	Female	Oropharynx	4	2	0	No	+	+	HPV-16
	61	66.6	Female	Oropharynx	3	0	0	Yes	+	+	Invalid
	122	57.9	Female	Oropharynx	1	2	0	No	+	+	HPV-16
EBV	6	48.0	Male	Nasopharynx	4	2	0	Yes	+	NA	NA
	13	47.3	Female	Nasopharynx	2	1	0	No	+	NA	NA
	18	74.6	Female	Nasopharynx	1	2	0	Yes	+	NA	NA

ISH: in situ hybridization; IHC: immunohistochemistry; PCR: polymerase chain reaction; +: positive; -: negative; †: using RNAscope, ‡: using EBER-ISH, HR-HPV: high-risk human papilloma virus; EBV: Epstein-Barr virus; NA: not applicable

Table 4 Multivariable analysis of predictors for 5-year overall survival in non-smokers and non-drinkers with head and neck squamous cell carcinoma.

Parameter	Coefficient (β)	Standard error	Wald X ²	OR	95% CI		p-value
					Lower	Upper	
Age	0.053	0.020	7.0	1.05	1.0	1.1	0.008
<i>N-stage (reference T4)</i>							
T1	-1.2	0.86	3.9	0.31	0.099	0.99	0.047
T2	-0.67	0.54	1.5	0.51	0.18	1.5	0.22
T3	0.79	0.74	1.1	2.2	0.52	9.4	0.29
<i>N-stage (reference N3)</i>							
N0	-19	0.59	1081	<0.001	<0.001	<0.001	<0.001
N1	-17	0.78	506	<0.001	<0.001	<0.001	<0.001
N2	-18	0.00	NA	<0.001	<0.001	<0.001	NA

NA: not available because of small number of cases

Nagelkerke $R^2 = 0.35$

HPV-positive tumors showed significantly more often p16 overexpression, p53 wild-type expression, and loss of pRb than HPV-negative tumors (p16: 100 versus 2.8%, $p < 0.001$; p53: 83 versus 27%, $p < 0.001$; pRb: 83 versus 19%, $p < 0.001$) (Table 5).

Epstein–Barr Virus

Three tumors were EBV positive as detected by EBER-ISH. These patients all had a tumor of the nasopharynx (100 versus 0.9% in EBV-negative tumors, $p < 0.001$), resulting in virus positivity of all four nasopharyngeal tumors in this cohort (three containing EBV and one HPV). Two of the three patients (67%) with EBV-positive tumors were below 50 years of age and non-Caucasian (Northern African and East Asian) and they all had regional metastases (Tables 2, 3).

Figure 2 Kaplan-Meier curves estimating the survival of patients with HPV (A, B) and EBV (C, D) positive and negative tumors. HPV and EBV were no significant predictors for a better disease free ($p = 0.33$ and $p = 0.11$, respectively) or overall survival ($p = 0.27$ and $p = 0.62$, respectively).

Patient 6 and 18 were both diagnosed with recurrent disease, the former with distant metastases 8.3 months after chemoradiotherapy and the latter with regional metastases 5.5 months after locoregional radiotherapy, which eventually led to their death. Although this resulted in a 33% 5-year survival for patients with EBV-positive tumors, EBV was no significant predictor for DFS or OS in this cohort, compared to EBV-negative HNSCC ($p = 0.11$ and $p = 0.62$, respectively) (Figures 2C, D).

None of the EBV-positive tumors showed p16 overexpression, all were positive for pRb and two of the three tumors (66%) showed p53 mutant-type overexpression. This did not differ significantly from cell cycle protein expression in EBV-negative tumors, most probably because of the small number of EBV-positive tumors (Table 5).

Merkel Cell Polyomavirus

MCPyV was not detected in any of the samples with RNA-ISH or IHC against the LTA_g of MCPyV.

Table 5 Expression of cell cycle proteins p16, p53, and pRb in virus positive and negative tumors.

Cell cycle protein		Virus positive		Virus negative		p-value	HPV-positive		HPV-negative		p-value	EBV-positive		EBV-negative		p-value
		n	(%)	n	(%)		n	(%)	n	(%)		n	(%)	n	(%)	
p16	Over-expression	12	(80)	3	(2.9)	<0.001	12	(100)	3	(2.8)	<0.001	0	(0)	15	(13)	1.0
	No over-expression	3	(20)	101	(97)		0	(0)	104	(97)		3	(100)	101	(87)	
p53	Mutant-type over-expression	2	(13)	51	(49)	0.002	0	(0)	53	(50)	<0.001	2	(67)	51	(44)	0.80
	Wild-type	11	(73)	28	(27)		10	(83)	29	(27)		1	(33)	38	(33)	
	0-mutant type	2	(13)	25	(24)		2	(17)	25	(23)		0	(0)	27	(23)	
pRb	Positive (preserved expression)	5	(33)	84	(81)	<0.001	2	(17)	87	(81)	<0.001	3	(100)	86	(74)	0.57
	Loss	10	(67)	20	(19)		10	(83)	20	(19)		0	(0)	30	(26)	

HPV, human papillomavirus; EBV, Epstein-Barr virus

Virus Negative Tumors

None of the squamous cell carcinomas of the oral tongue (OTSCC) (n = 39), larynx (LSCC) (n = 9), or hypopharynx (HPSCC) (n = 1) showed involvement of HPV, EBV, or MCPyV. Except for one tumor of the alveolar process, all other OSCC (n = 54) were virus negative as well (Table 2). Although the patients with virus negative tumors were relatively old (mean age of 75 years) when being diagnosed with HNSCC, the 5-year OS of these patients was still 50% (Figure 2).

The three patients with virus negative OSCC containing p16 overexpression all had recurrent disease within 6 months after surgical resection [a T4N1M0 floor of mouth tumor, after an irradiated resection the patient wished no further treatment (DFS = 0 months), OS = 27.4 months; a T4N0M0 oral cavity tumor (not otherwise specified), DFS = 5.4 months after resection, OS = 8.2 months; a T2N1M0 retromolar triangle tumor, DFS 4.8 months after resection, OS = 11.8 months]. Two of these tumors showed loss of pRb expression. A poor DFS (2.3, 8.8, and 20.3 months) was also found in three other tumors in this cohort with some p16 expression (>50%): all OTSCC with a pRb expression above 50%. Apart from one LSCC, all tumors with loss of pRb were OSCC (19/20) without any expression of p16. A younger age at cancer diagnosis (p = 0.021) and N0 or N1 stage (both p < 0.001) were retained in the best multivariable model as predictors for OS in NSND with virus negative OSCC (Supplementary Table 2, Table 6). The model explained 31% of the variation (model fit: omnibus test of model coefficients: X² = 24.4 and p < 0.001; M-stage and p16 were omitted because of low case numbers).

Over 70% (76/104) of virus negative tumors showed mutant-type p53 expression, with 0-type mutant expression in 20% (19/94) of OSCC, 56% (5/9) of LSCC, and 100%

Table 6 Multivariable analysis of predictors for 5-year overall survival in non-smokers and non-drinkers with virus negative oral squamous cell carcinoma.

Parameter	Coefficient (β)	Standard error	Wald X^2	OR	95% CI		p-value
					Lower	Upper	
Age	0.051	0.022	5.3	1.05	1.0	1.1	0.021
N-stage (reference N3)							
N0	-20	0.72	803	<0.001	<0.001	<0.001	<0.001
N1	-18	0.89	414	<0.001	<0.001	<0.001	<0.001
N2	-19	0.00	NA	<0.001	<0.001	<0.001	NA

NA: not available because of small number of cases

Nagelkerke $R^2 = 0.31$

(1/1) of HPSCC, and mutant-type overexpression in 52% (49/94) of OSCC and 22% (2/9) of LSCC (Table 5). Cell cycle protein expression was no predictor for a better DFS or OS in patients with virus negative tumors (data not displayed).

DISCUSSION

The objective of this study was to determine if HPV, EBV, and MCPyV play a role in head and neck carcinogenesis of NSND, the role of cell cycle proteins p16, p53, and pRb regarding viral presence, and the influence of these viruses and proteins on patient survival. In this cohort of 119 NSND, the ten oropharyngeal (100%) and four nasopharyngeal (100%) tumors contained either HPV or EBV. Besides one oral cavity tumor, all other specimens of the oral cavity, hypopharynx, and larynx were HPV, EBV, and MCPyV-negative. Virus positivity did not predict better disease free or overall survival. Regarding cell cycle protein expression, HPV-positive tumors showed more p16 overexpression, wild-type p53 expression, and loss of pRb compared to HPV-negative tumors. OSCC with >70% p16 expression had a poor DFS and OS, with loss of pRb in two of the three cases. The other pRb negative tumors were mainly OSCC as well and did not show p16 expression. Mutant type p53 expression was observed in over 70% of virus negative HNSCC.

As the worldwide HPV prevalence is rising, a wide range has been reported in the literature, ranging from 20% in OPSCC patients from Eastern Asia or Central America, to over 50% in Europe and Australia, based on HPV DNA combined with E6*I mRNA or p16 IHC^{5,6}. A recent systematic review specifically analyzing patients without tobacco or alcohol consumption reported an OPSCC HPV prevalence of over 60% in non-smokers and over 40% in non-drinkers, compared to 20% in smokers and drinkers, based on at least two detection techniques (combining PCR, ISH, IHC, or sequencing)²⁵. This is a lower prevalence than the 100% (10/10) HPV infections of OPSCC in the current study. Possibly, the low number of OPSCC in the current study explains the 100%

prevalence, as it could be a coincidence that they were all HPV-positive. Nonetheless, it is acknowledged that HPV plays an increasingly substantial role in OPSCC carcinogenesis of patients without the traditional risk factors. The HPV prevalence of 1.8% (2/109) in non-OPSCC as found in the current study is comparable to the low prevalence in other studies^{32–35}.

In this study, 4.2% (4/95) of OSCC showed p16 overexpression with IHC, and one of those contained HPV-16 DNA following COBAS analysis resulting in HPV-positivity according to detection by two methods. p16 overexpression could result from loss of pRb function via structural alterations, or maybe as a result of other oncogenic viruses affecting pRb expression that we are not aware of³⁶. Lechner and colleagues speculated that high levels of protein p16 could also occur in cells irrespective of pRb expression or HPV-positivity, as a result of enrichment for NSD1 mutations in CDKN2A wild-type tumors^{36, 37}. NSD1 is coding for Histone H3K36 methyltransferase, which is associated with DNA hypomethylation, resulting in p16 overexpression when mutated by not being able to regulate its expression via methylation anymore³⁶. Indeed, p16 overexpression has been reported in non-OPSCC, without a correlation to HPV infection nor as a predictor for survival^{34, 38}. Therefore, The College of American Pathologists does not endorse routine p16 screening for non-OPSCC³⁹. As the one HPV-positive OSCC in this cohort had no loss of pRb and lacked HPV mRNA in a whole section following RNAscope ISH analysis, the p16 overexpression could be a result of CDKN2A mutation, and HPV a commensal with the HPV DNA not located in the tumor cells but in the adjacent mucosal epithelial cells⁴⁰. The three HPV-negative OSCC with p16 overexpression in the current study had a poor DFS. This could be a result of pRb loss, although for the whole study group p16 overexpression was no significant predictor for survival. Additionally, HNSCC could be the result of a genetic predisposition. OSCC has been associated with specific CDKN2A germline mutations, accompanied with loss of heterogeneity of the wild-type allele, in a small fraction of young NSND⁴¹.

The 5-year OS of HNSCC patients in general is 40–50%, whereas it is 70–80% for patients with HPV-positive OPSCC^{2, 42}. In this study, there was no significant difference in OS or DFS between virus positive and virus negative patients. However, it is not certain if the survival comparison of HPV-positive versus HPV-negative HNSCC was one of HPV-positivity or of tumor location (OPSCC versus non-OPSCC), as in this cohort HPV-negative tumors were exclusively non-OPSCC. The same applies to EBV-positive tumors, which were all NPSCC. Therefore, these survival analyses should be interpreted with caution. The 5-year OS of 67% for patients with HPV-positive tumors was lower than expected, but these patients were relatively old with a median age of 67.3 years. Patients with HPV-negative HNSCC (53%) did not differ significantly in 5-year OS from patients with HPV-positive OPSCC, even though they had a median age of almost

9 years older than the patients with HPV-positive tumors (76.2 versus 67.3 years). So, considering their age at the time of HNSCC diagnosis, the HPV-negative NSND, mainly with OSCC, had a relatively good 5-year OS. Nevertheless, a young age at the time of cancer diagnosis was predictive of a better OS in both the virus negative OSCC group and the whole NSND cohort, besides a T1 stage and a N0 or N1 stage.

Viral association in all four NPSCC patients of this cohort was as expected. Although the worldwide incidence of EBV related NPSCC has been decreasing over the past decade, the prevalence is still high with almost 100% in endemic regions (southern China, Southeast Asia, Northern Africa, and the Mediterranean basin), and 60–85% in non-endemic regions^{9, 43–46}. NPSCC infections with HPV are less common, and have been reported in studies from non-endemic regions like West Africa and Europe (outside the Mediterranean basin), with a prevalence between 1.6–16%^{43, 45, 47, 48}. Based on 517 U.S. NPSCC patients with known HPV testing (34.8% HPV-positive) in the Surveillance, Epidemiology and End Results database, predictors for HPV-positivity in NPSCC have been established: being younger than 25-years-old, Caucasian (rather than East-Asian or other ethnicities), an AJCC-7 stage other than stage 1, and no distant metastases (M0)⁴⁹. Indeed, the one patient in our cohort with HPV-positive NPSCC was a Caucasian patient with stage 2 disease.

Expression of cell cycle proteins p16 and pRb was as expected in the HPV-positive OPSCC of this cohort, with overexpression of the former and loss of the latter. Although p53 is usually degraded by HPV's viral oncoprotein E6, only two HPV-positive tumors showed 0-mutant type p53 expression, whereas the other 10 showed wild-type p53 expression. This is in agreement with earlier observations, where there was p53 expression in 7/10 HPV-positive tumors of non-smokers, despite absence of TP53 mutations⁵⁰. Possible explanations for this finding are virally induced processes, such as hypoxia, oxidative stress, or impaired repair of double strand DNA breaks^{50, 51}. On the other hand, up to 55–75% of HPV-negative OSCC contain TP53 mutations, which is comparable to the 72% of HPV-negative OSCC showing mutant-type p53 expression (either 0-type or overexpression) in the current study^{36, 52}. The EBV-positive tumors were all without p16 overexpression and with preserved pRb expression. Zhang and colleagues reported that the cell cycle pathway is the most deregulated pathway in NPSCC in comparison to non-tumor nasopharyngeal epithelium, with down-regulation of p16 and up-regulation of pRb¹⁹. Although the precise mechanism of p16 inactivation by EBV in NPSCC remains unclear, it has been suggested that the Late Membrane Protein 1 of EBV could inhibit p16 expression and induce pRb phosphorylation, promoting the progression of the G1/S phase¹⁹. This is in accordance with the cell cycle protein expressions found in the current study.

No MCPyV was detected in the current study, which contrasts earlier findings. Hamiter and colleagues performed PCR using specific primers for the regulatory and LTA_g of MCPyV, followed by DNA sequence analysis to confirm viral presence in 6/21 (29%, three were NSND) patients with OTSCC¹⁶. Although the OTSCC group in the current study was almost twice the size ($n = 39$), no MCPyV presence was detected. Other studies report MCPyV DNA in 4–50% of HNSCC based on quantitative real-time PCR, though with low viral loads^{14, 15, 17}. This discrepancy could be the result of differences in sensitivity between the used detection methods (RNA-ISH and LTA_g IHC versus DNA PCR and sequencing). However, the MCPyV presence in the literature was solely based on PCR and was not confirmed with another detection method. Nevertheless, the significance of MCPyV presence in HNSCC has yet to be determined, but our data strengthen the premise that MCPyV is not likely to play an important role in head and neck carcinogenesis.

HPV has its clinical relevance in routine practice as a prognostic marker in OPSCC, with a better DFS and OS, in addition to an improved radiosensitivity compared to HPV-negative OPSCC because of altered DNA repair, reduced hypoxic regions, and an increased cellular immune response^{3, 53}. With the conduction of multiple phase III de-escalation trials for HPV-positive OPSCC, and the high HPV prevalence in OPSCC of NSND, the treatment of NSND may be affected in the near future⁴. For EBV-positive NPSCC, treatment usually consists of radiotherapy, with or without chemotherapy. Besides the anti-tumor effects of radiotherapy based on direct and indirect DNA damage, it also induces an immune response comprising of a network of immune-stimulatory and –inhibitory signals like up-regulation of immune checkpoint proteins such as PD-1/PD-L1⁵⁴. Consequently, there are a number of clinical trials evaluating the incorporation of immune checkpoint inhibitors in the treatment of EBV-positive NPSCC^{10, 54}. Mutant p53 has been associated with resistance to chemoradiation in OSCC and an increased risk of locoregional recurrence and metastases⁵⁵. Since NSND mainly have OSCC and frequent mutant-type p53 expression (as presented in this study), p53 could be used to predict therapy failure in case of recurrent disease.

One of the limitations of this study was that the definition of when a patient was a non-smoker and non-drinker was collected retrospectively from their medical records. Nevertheless, a strict definition was used (for example with exclusion of patients with “sporadic” alcohol consumption), based on a standard history taking template including specific questions on any current or previous tobacco and alcohol consumption, during two separate hospital visits (Head and Neck outpatient clinic and Anesthesiology screening). Secondly, there was a small group of patients with tumors at anatomical sites other than the oral cavity (OSCC). In combination with the strict NSND definition, this resulted in no virus negative OPSCC and NPSCC. However, it has been reported that

NSND are mainly patients with OSCC, so a small number of tumors outside the oral cavity was expected^{25, 56}. Thirdly, there might be a higher percentage of HNSCC positive for HPV DNA in this cohort, because COBAS PCR analysis was not performed on all samples. Conversely, HPV is only considered to be predictive for survival when being transcriptionally active, and since all tumors were tested on HPV-16/18 mRNA and p16 IHC, we expect to have detected the tumors with biologically active HPV infection^{7, 8}. Finally, some of the FFPE material was rather old (>25 years of storage), which is known to often result in breakdown of the nucleic acids. Indeed, DNA quality of DNA extracted from one tumor was insufficient for COBAS analysis. Nevertheless, the TMA blocks were freshly sectioned before ISH, IHC, or PCR, and all the positive controls were adequate.

CONCLUSION

A high prevalence of HPV and EBV was observed in OPSCC and NPSCC of NSND respectively, but not in HNSCC outside these locations. Although a significant role of oncogenic viruses would be assumed in this specific patient group lacking the traditional risk factors for developing HNSCC, HPV, EBV, and MCPyV were not detected in this relatively large cohort of 95 OSCC apart from one case, using clinically relevant cut-off values. This argues against a central role of these viruses in the etiopathogenesis of oral, hypopharyngeal, and laryngeal squamous cell carcinoma in this specific patient group. With ongoing de-escalation trials for HPV-positive OPSCC and trials for immune checkpoint inhibitors in the treatment of EBV-positive NPSCC, the treatment of NSND with tumors at those locations may change in the near future. However, for the majority of NSND with virus negative OSCC, more research is needed to understand the carcinogenic mechanisms in order to consider targeted therapeutic options.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics Statement

The studies involving human participants were reviewed and approved by Maastricht University Medical Center (METc 2018-0567). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author Contributions

All authors have contributed substantially to the conception and design (FM, SW, BK, and ES), acquisition of data (FM, FK, FJ, and FF), analysis and interpretation of data

(FM, SW, RB, AH, MH, BK, and ES); drafting the article (FM, FJ, and ES) or revising it critically for important intellectual content (FK, FF, SW, RB, AH, MH, and BK); final approval of the version to be published (FM, FK, FJ, FF, SW, RB, AH, MH, BK, and ES); and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

Conflict of Interest

SW receives a research grant from Roche, MSD, AstraZeneca, Pfizer, Nextcure, Amgen, and Bayer. BK and ES receive a research grant from Pfizer and Novartis. The funders had no role in the design of the study, in the collection, analyses, or interpretation of the data, in the writing of the manuscript, or in the decision to publish the results.

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Supplementary data:

Supplementary table 1. Predictors for 5-year DFS and OS in non-smokers and non-drinkers with HNSCC

Clinical characteristics		Total (n=119)		5-year DFS (n=88)		Recurrence (n=31)		p-value		5-year OS (n=65)		Death (n=54)		p-value	
Age (years)	Median (IQR)	74.9	(14.6)	75.5	(16.0)	74.2	(12.9)	0.51		70.8	(18.2)	77.4	(11.2)	0.007	
		n	(%)	n	(%)	n	(%)			n	(%)	n	(%)		
Sex	Female	93	(78)	70	(80)	23	(74)	0.61		12	(19)	14	(26)	0.38	
	Male	26	(22)	18	(21)	8	(26)			53	(82)	40	(74)		
Location	Hypopharynx	1	(0.8)	1	(1.1)	0	(0)	0.72		0	(0)	1	(1.9)	0.53	
	Larynx	9	(7.6)	6	(6.8)	3	(9.7)			3	(4.6)	6	(11)		
	Nasopharynx	4	(3.4)	2	(2.3)	2	(6.5)			2	(3.1)	2	(3.7)		
	Oral cavity	95	(80)	71	(81)	24	(77)			54	(83)	41	(76)		
	Oropharynx	10	(8.4)	8	(9.1)	2	(6.5)			6	(9.2)	4	(7.4)		
T-stage	1	33	(28)	25	(28)	8	(26)	0.76		26	(40)	7	(13)	0.003	
	2	36	(30)	27	(31)	9	(29)			20	(31)	16	(30)		
	3	13	(11)	8	(9.1)	5	(16)			4	(6.2)	9	(17)		
	4	37	(31)	28	(32)	9	(29)			15	(23)	22	(41)		
N-stage	0	78	(66)	17	(55)	61	(69)	0.31		52	(80)	26	(48)	0.001	
	1	18	(15)	7	(23)	11	(13)			5	(7.7)	13	(24)		
	2	21	(18)	7	(23)	14	(16)			8	(12)	13	(24)		
	3	2	(1.7)	0	(0)	2	(2.3)			0	(0)	2	(3.7)		
M-stage	0	113	(95)	87	(99)	27	(87)	0.016		65	(100)	49	(91)	0.017	
	1	2	(5.0)	1	(1.1)	4	(13)			0	(0)	5	(9.3)		
HPV	Positive*	12	(10)	10	(11)	2	(6.5)	0.73		8	(12)	4	(7.4)	0.54	
	Negative	107	(90)	78	(89)	29	(94)			57	(88)	50	(93)		
EBV	Positive†	3	(2.5)	87	(99)	2	(6.5)	0.17		1	(1.5)	2	(3.7)	0.59	
	Negative	116	(98)	1	(1.1)	29	(94)			64	(99)	52	(96)		
p16	No overexpression	15	(13)	10	(11)	5	(16)	0.53		57	(88)	47	(87)	1.0	
	Overexpression	104	(87)	78	(89)	26	(84)			8	(12)	7	(13)		
p53	0-mutant type	27	(23)	19	(22)	8	(26)	0.80		11	(17)	16	(30)	0.25	
	Wild-type	39	(33)	30	(34)	9	(29)			22	(34)	17	(32)		
pRb	Mutant-type	53	(45)	39	(44)	14	(45)			32	(49)	21	(39)		
	Loss	30	(25)	21	(24)	9	(29)	0.63		14	(22)	16	(30)	0.40	
	Positive	89	(75)	67	(76)	22	(71)			51	(79)	38	(70)		

DFS: disease free survival; OS: overall survival; HNSCC: head and neck squamous cell carcinoma; IQR: inter quartile range; *: using at least two detection methods (RNAscope ISH, COBAS PCR, and/or p16 IHC); †: using EBER-ISH

Supplementary table 2. Predictors for 5-year DFS and OS in non-smokers and non-drinkers with virus negative OSCC

Clinical characteristics		Total (n=94)		5-year DFS (n=70)		Recurrence (n=24)		p-value		5-year OS (n=53)		Death (n=41)		p-value	
Age (years)	Median (IQR)	n	(%)	n	(%)	n	(%)			n	(%)	n	(%)		
Sex	Female	16	(17)	59	(84)	19	(79)	0.54		9	(17)	7	(17)	1.0	
	Male	78	(83)	11	(16)	5	(21)			44	(83)	34	(83)		
T-stage	1	28	(29)	21	(30)	7	(29)	0.98		22	(42)	6	(15)	0.025	
	2	32	(34)	23	(33)	9	(38)			17	(32)	15	(37)		
	3	7	(7.4)	5	(7.1)	2	(8.3)			3	(5.7)	4	(9.8)		
	4	27	(29)	21	(30)	6	(25)			11	(21)	16	(39)		
N-stage	0	64	(68)	51	(73)	13	(54)	0.22		45	(85)	19	(46)	<0.001	
	1	17	(18)	10	(14)	7	(29)			4	(7.5)	13	(32)		
	2	11	(12)	7	(10)	4	(17)			4	(7.5)	7	(17)		
	3	2	(2.1)	2	(2.9)	0	(0)			0	(0)	2	(4.9)		
M-stage	0	90	(96)	69	(99)	21	(88)	0.050		53	(100)	37	(90)	0.033	
	1	4	(4.3)	1	(1.4)	3	(13)			0	(0)	4	(9.8)		
p16	No overexpression	91	(97)	70	(100)	21	(88)	0.015		53	(100)	38	(93)	0.080	
	Overexpression	3	(3.2)	0	(0)	3	(13)			0	(0)	3	(7.3)		
p53	0-mutant type	19	(20)	14	(20)	5	(21)	0.95		9	(17)	10	(24)	0.67	
	Wild-type	26	(28)	20	(29)	6	(25)			15	(29)	11	(27)		
	Mutant-type	49	(52)	36	(51)	13	(54)			29	(55)	20	(49)		
pRb	Loss	19	(20)	13	(19)	6	(25)	0.56		8	(15)	11	(27)	0.20	
	Positive	75	(80)	57	(81)	18	(75)			45	(85)	30	(73)		

DFS: disease free survival; OS: overall survival; OSCC: oral squamous cell carcinoma; IQR: inter quartile range

7

CHAPTER 7

General discussion

Cancer is still a crucial public health concern accounting for nearly 9.6 million deaths worldwide in 2018 ¹. The overall cancer death rate remains high and this reflects to several risk factors including the increase of the population and the life expectancy ¹. However, the etiology of many cancers is still unknown. To date, it is estimated that up to 20% of the world's cancer cases burden is attributed to infectious pathogens ¹⁻⁴. In the past years, studies have shown that viral infection plays a significant role in the carcinogenesis of ~10-15% of all human cancers worldwide ⁵⁻⁷. In the context of cancer patient management, the discovery of novel virus-associated human cancers provides an important aspect in understanding the mechanism of tumorigenesis, which will result in the improvement of knowledge about prevention and treatment. Given the potentially serious implications of human viruses in tumorigenesis, it is important to elucidate further evidence for the involvement of additional viruses in human carcinogenesis.

Infection with polyomaviruses (PyVs) comes with a long history of controversy about its role in human oncogenesis. PyVs and papillomaviruses (HPVs) used to belong to the same family of *Papovaviridae* since both viruses are small non-enveloped with double-stranded DNA genomes and sharing some genetic organization and viral replication mechanisms ⁸⁻¹⁰. According to the ICTV 7th Report in 2000, *Papovaviridae* has been split into two new families, PyVs assigned to *Polyomaviridae* family while HPVs assigned to *Papillomaviridae* ¹¹. Thus, it is speculated that PyVs are possibly involved in human tumorigenesis as an -analogous to HPVs. Until 2007, BK polyomavirus (BKPyV) and JC polyomavirus (JCPyV) were the only known HPyVs, both revealing a high seroprevalence in healthy individuals and possibly causing serious diseases in immunocompromised subjects ^{12, 13}. Between 2007 and 2019, 13 new PyVs have been isolated from human specimens as outlined in **chapter 1** (Table 2) and 9 of them have been confirmed to be HPyVs ^{10, 14-16}. In general, it seems that the infection of HPyVs is asymptomatic, but might reactivate and lead to diseases in immunocompromised individuals. Moreover, there are strong evidence suggesting that some of HPyVs have the ability to transform cells in animal models. For instance, epithelium tumor developed in transgenic mice after injecting them with the early region gene (tumor antigen region) of BKPyV, while the injection of JCPyV early region gene initiated neural tumor formation in these mice ^{17, 18}. In addition, JCPyV showed to be able to induce brain tumors in rats and hamsters ¹⁹⁻²². Nevertheless, except for MCPyV, none of the new HPyVs has yet been implicated in human tumorigenesis.

MCPyV is the only HPyV that has been categorized as a “Group 2A carcinogen: probably carcinogenic to humans” by the International Agency for Research on Cancer (IARC) in 2012 ²³. These days, almost over 10 years later of additional evidence of finding MCPyV-DNA clonally integrated into highly malignant non-melanoma skin cancer Merkel cell carcinoma (MCC) tumor host genome with no UV mutational signature, it

seems very likely that MCPyV would be classified as “Group 1 carcinogen: Carcinogenic to humans”²⁴⁻²⁸. MCPyV-positive MCC have been reported to have a relatively favorable clinical outcome, drug response, and survival rate compared to MCPyV-negative MCC. The etiopathogenesis of MCPyV-negative MCC is closely linked to UV damage and is characterized by a very high tumor mutational burden²⁹⁻³³. The rapidly expanding number of HPyV members raises the question whether these viruses may also be involved in the etiopathogenesis of human diseases, including malignant tumors. The discovery of MCPyV and its role as a major etiopathogenetic agent of MCC broadly renewed the research interest in HPyV-associated tumorigenesis. To this end, researchers throughout the world have studied the prevalence of HPyVs in a variety of diseases^{10, 34-39}.

This thesis aims to gain more insight into the possible involvement of known HPyVs in the tumorigenesis of a variety of human cancers. The presence of HPyVs and their association with human malignancies have commonly been tested by PCR, and only a very limited number of studies showed the presence of these viruses at the single-cell level within the histomorphological context **chapter 2**. The detection of viral-DNA in tissue extracts does not prove a possible role of HPyVs in cancer, because it cannot be interpreted in which cellular compartment the viral DNA is present. Viral protein expression as assessed by IHC in FFPE tissues is mostly hampered by the limited number of virus-specific, commercially available antibodies. Based on these limitations we tried to utilize other molecular techniques such as FISH, RISH, and IHC to show the viral DNA, RNA, and protein expression of the respective HPyV in a histomorphological context as demonstrated in **chapter 3, 5, and 6**. By doing so, we have added novel data about the potential role of the tested HPyVs and their involvement in human diseases, including their latency. Recently, next-generation sequencing (NGS) technology has helped in discovering novel members of PyVs such as QPyV^{40, 41}. NGS is not only an important approach to determine the presence and the sequences of the virus but also to identify their sites of integration in the human tumor genome. In the nearby future, employing new emerging approaches combining NGS technology with bioinformatics will be beneficial for analyzing FFPE archival tissue for virus integration⁴².

To date, five HPyVs have been isolated from the human skin of asymptomatic individuals (i.e., MCPyV, HPyV6, HPyV7, TSPyV, and HPyV9) **chapter 1**. Although it has been reported that HPyV6 and 7 appear to be associated with pruritic dermal dyskeratotic dermatoses^{43, 44}, further associations of these viruses with clinical diseases have not been studied intensely. In **chapter 2** we conducted the first comprehensive review to provide an insight into the emerging role of these two closely related HPyVs in human cancers. The frequent prevalence of HPyV6 and 7 DNA in non-neoplastic and neoplastic tissues along with their high seropositivity in the normal population indicates that both viruses have long-term latency in humans. Therefore, HPyV6 and 7 are putative candidates that

may contribute to the etiopathogenesis of human diseases, especially skin tumors. It could be concluded that the detection frequency of HPyV6 and 7 is higher in malignant tissues than in non-neoplastic tissues. Interestingly, HPyV6 prevalence was especially high in cutaneous malignancies, whereas only a low frequency of these tumors harbored HPyV7. In contrast, HPyV7 was more frequently detected in other malignancies such as cholangiocarcinoma, which is described in **chapter 3**. In addition, by summarizing the age-specific sero-epidemiological studies of HPyV6 and 7, it was found that both viruses infect individuals at an early age, and HPyV6 seroprevalence was found to be slightly higher than HPyV7 and increased with age^{16, 45-52}. Very limited studies reported the seroprevalence of HPyV6 and 7 in specimens of cancer patients, and the seropositivity was found to be comparable to those in healthy population age groups^{51, 52}.

Cholangiocarcinoma (CCA) is rare biliary duct neoplasia with a very poor prognosis and the etiology of CCA remains largely unknown⁵³. Few infectious agents have been identified to be associated with the etiopathogenesis of CCA such as hepatitis B and C viruses⁵³⁻⁵⁵. Interestingly, a recent study by Chan *et al.* research group investigated the presence of HPyVs in the bile fluid of patients with a variety of hepatobiliary malignancies and they reported that HPyV6 but not HPyV7 was detected in bile specimens from CCA patients⁵⁶. Therefore, we investigated the prevalence and possible pathological associations of HPyVs with patients diagnosed with CCA in **chapter 3**. The FFPE tissues obtained from the CCA cohort provided a unique opportunity to examine the prevalence of HPyVs not only in CCA tumor tissues, but also the adjacent non-neoplastic bile duct epithelium, and non-lesional hepatocytes⁵⁷. In addition, a sensitive degenerated PCR primer set was designed to simultaneously detect highly conserved sequences in the LTA_g gene of all currently known HPyVs. As a result, HPyV7, HPyV6, and MCPyV were commonly detected in the FFPE of malignant, premalignant, and non-lesional CCA using degenerated PCR and subsequent sequencing. In summary, HPyV7 (69%) was the most prevalent HPyV in the tested cohort, followed by MCPyV (24%), and HPyV6 (14%). Interestingly, co-detection of two HPyVs was common, i.e. eight cases showed coinfection with HPyV7 and MCPyV, and two cases with HPyV6 and HPyV7. These findings are slightly different than those from the previous study by Chan *et al.*⁵⁶. The most likely explanation for this difference between high HPyV7 prevalence in CCA found in this thesis and high HPyV6 prevalence found by Chan *et al.* is the primary source of the DNA, i.e. primary CCA tissues versus bile fluids of CCA patients, respectively. In addition, the findings might reflect geographical differences in the prevalence of HPyV6 and 7. In conclusion, the review presented in **chapter 2** reveals that many screening studies for HPyVs in FFPE and fresh frozen tissue material show an elevated prevalence of HPyV6 and/or 7 compared to those using liquid biopsy specimens.

Assessing the possible role of HPyVs in CCA carcinogenesis solely based on PCR results is insufficient. Additional molecular techniques are therefore indicated in **chapter 2** to also show which cells harbor the respective HPyV. Upon the start of this study, the presence of known HPyVs at the single-cell level in either non-neoplastic or malignant cells of CCA and liver tissues had not been shown previously. This study thus is the first to localize HPyV6, HPyV7, and MCPyV in the bile duct epithelium and hepatocytes using FISH, IHC, and HPyV6 transcripts by RNA-ISH. Altogether, the results strongly indicate that three HPyVs (HPyV7, HPyV6, and MCPyV) are hepatotropic viruses and may infect non-neoplastic human hepatocytes, bile duct epithelium, and CCA lesions. Yet, it remains unclear if and how HPyVs might contribute to the tumorigenesis of CCA. The frequent finding of HPyVs, especially HPyV7 in CCA and adjacent peritumoral hepatocytes tissues, suggests an indirect role of HPyV7 in CCA carcinogenesis, which is possibly initiated by inflammation. For instance, hepatitis B and C viruses are known to induce neoplasms indirectly and are found in the non-malignant hepatocytes adjacent to the hepatocellular carcinoma as discussed in **chapter 2**.

BKPyV is the first identified HPyV and is known to be latently present in more than 50% of the population at very low levels within the epithelium of the proximal tubules of the kidney, the urothelial cell layer of the bladder, the ureters, and the renal pelvis^{58, 59}. But so far, BKPyV has only been found latently in the urogenital tract. BKPyV is reported to be reactivated following immunosuppression, especially in the context of kidney and solid organ transplantations, with the risk of developing BKPyV-associated nephropathy (PyVAN) and hemorrhagic cystitis diseases^{60, 61}. Therefore, BKPyV has been suspected as a putative oncogenic virus in the development of urothelial cell carcinomas (UCC) in immunocompromised patients^{23, 62}. In **chapter 4**, we focused on elucidating further evidence for the involvement of BKPyV in the UCC of the urinary bladder. In this study, the detection of BKPyV-positive urine cytology specimens (UCS) was evaluated in the UCC of the urinary bladder. Additionally, we assessed the relation of BKPyV-positive UCS to a previously given intravesicular BCG or mitomycin treatment UCC patients.⁶³ Interestingly, the overall cytology results revealed a very low detection rate (0.2%) of Decoy cells (urothelial cells often infected with viruses, including BKPyV) within the urine cytology samples from the database of Maastricht university medical center+ institution over a 15-year period, which is consistent with previous findings^{64, 65}. The presence of Decoy cells in the urine specimens is an indication for the reactivation of BKPyV⁶⁶. Although BKPyV-IHC revealed positivity in the urine of all (0.2%) specimens with Decoy cells. In addition, PCR detected BKPyV-DNA in the urine samples of patients diagnosed with either in situ or invasive UCC of the urinary bladder, while BKPyV-IHC and PCR were negative in FFPE tissues level of primary UCCs and metastases. In summary, these results strongly indicate that the reactivation of BKPyV in urine cytology is not associated with prior resected (range 1 -13 years) UCC⁶³. Furthermore, these

results revealed that BKPyV-reactivation is not restricted to immunosuppression since the virus could also be detected in the urine of immunocompetent patients without any history of transplantation, malignancy, or chronic diseases. A prominent role for BKPyV in the etiopathogenesis of the conventional type UCC is very unlikely because one would expect a high expression of tumor-viral proteins in every tumor cell, which is not the case in the cohort subset described in **chapter 3**. Recently, it has been suggested that BKPyV-DNA might be lost after inducing bladder cancer development through a ‘hit-and-run’ mechanism similar to the model of HPV inducing skin cancer in mice ^{67, 68}. However, such a mechanism proposed by Skinner in 1976 still remains speculation and lacks substantial evidence **chapter 2** ⁶⁹.

Thymoma is a rare intrathoracic malignancy of unknown etiology ⁷⁰. The major capsid protein VP-1 of murine polyomavirus was reported to induce thymomas when injected into neonatal mice ⁷¹. In addition, a few years ago our research group demonstrated the high prevalence of HPyV7 in thymic epithelial tumors ⁷². In **chapter 5** we aimed to uncover whether MCPyV also plays a role in the etiopathogenesis of human epithelial thymic tumors. This was pursued by applying diverse molecular techniques to screen for MCPyV prevalence in a clinicopathologically well-characterized thymoma cohort ⁷³. The presence of MCPyV-DNA was identified in 19.4% of the 36 thymomas FFPE tissues by PCR in this study. Additionally, we have reported the presence of MCPyV in epithelial thymoma tissues by FISH, RISH, and IHC. The most likely conclusion is that the contribution of MCPyV to the etiopathogenesis of thymoma is unlikely at least based on our findings data which revealed less detection and expression of viral DNA, RNA, and proteins in tumor cells. As we indicated in **chapter 3**, one would expect to observe a pattern of high expression of viral proteins in every tumor cell as has been reported e.g. Merkel cell carcinoma positive-MCPyV ⁷⁴. Since this is not the case despite the presence of MCPyV-DNA, the virus might be present in a latent form in thymus tissue. Furthermore, the presence of MCPyV in thymoma tumors is not enough, further investigation is needed to find if MCPyV is integrated into the tumor genome and possibly carries tumor-specific mutations within the viral helicase of LTag in analogy to MCC positive-MCPyV.

Human papillomavirus (HPV) is the primary cause of uterine cervical cancer but has also been shown to be involved in the development of head and neck squamous cell carcinoma (HNSCC), especially tumors originating in the oropharynx ¹. In addition, Epstein-Barr virus (EBV) has been found to be associated with nasopharyngeal carcinoma ¹, and the presence of MCPyV-DNA has been reported in oral tongue SCC and tonsillar carcinomas ⁷⁵⁻⁷⁷. Generally, HPV-related HNSCC has a more favorable overall and disease-free survival than HPV-negative HNSCC ⁷⁸. Similarly, MCPyV-positive MCC was demonstrated to have a better prognosis than MCPyV-negative MCC ²⁹⁻³³. It is

proposed that the frequency of human tumor viruses might be increased in HNSCC of non-smokers and non-drinkers⁷⁹⁻⁸¹. In **chapter 6**, we examined the frequency of three different tumor viruses, i.e. HPV, EBV, and MCPyV, in a unique large cohort of non-smokers and non-drinkers with HNSCC⁸². Data in **chapter 6** demonstrate the presence of HPV and EBV in patients with HNSCC, but MCPyV-LTag expression was not detected by both RNA-ISH and IHC. Early results by others indicated that MCPyV-DNA was detectable in 28.6% of oral tongue SCC⁷⁵. Additionally, the study of Saláková et. al. reported that MCPyV-DNA prevalence is significantly higher in tonsillar carcinomas compared to non-malignant tonsils (35.7% versus 10.2%, respectively)⁷⁶. In contrast, an earlier study from 2009 revealed a low prevalence of MCPyV (3.5%) in the tissue of non-malignant tonsils, which is not in agreement with the other two studies described above⁷⁷. The discrepancy of the results described in **chapter 6** with that from others is most likely due to the detection method because in **chapter 6** the presence of MCPyV-LTag expression evaluated in a single-cell level using RNA-ISH and IHC, while the other studies only tested their cohort by PCR. Moreover, a previous study revealed that the mean of MCPyV viral load was low in both malignant and non-malignant tonsils, which is different from the high MCPyV load detected in MCC⁷⁶. Thus, also tonsillar tissues might be the site of MCPyV latency as speculated for BKPyV and JCPyV^{77, 83}. As a result, the findings in this thesis implicate that is unlikely MCPyV plays an etiological role in HNSCC tumorigenesis.

Finally, the overall results presented in this thesis lead to the conclusion that much remains to be done to elucidate the role of HPyVs as human tumor viruses. MCPyV is classified as a tumor virus for the majority of MCC cases. Also, the frequent detection of HPyVs in diverse tumor tissues indicates that HPyVs remain important putative candidates possibly contributing to human tumorigenesis. The association of HPyVs with human cancers usually has been assessed by PCR. However, the sheer presence of viral DNA is insufficient to conclude an etiopathogenic role for HPyVs. In this thesis the presence of some HPyVs was tested in the histomorphological context of selected cancer types by using diverse molecular techniques. Nevertheless, future work needs to address if these viruses are integrated into the human tumor genome and carry tumor-specific mutations as discussed in **Chapter 2**. Therefore, additional molecular techniques are needed to unravel if these viruses have specific interactions with the cell cycles. In particular to characterize the interaction of HPyVs with proteins such as p53 and pRb to possibly understand the contribution of these viruses to human cancers.

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ADDENDUM

Summary

Although the incidence of cancer is rapidly growing worldwide, the etiology of many malignant neoplasms remains largely unknown and thus also their cure. The link between the development of cancer and viral infections has been established since long. Globally, viruses have been demonstrated to be the causative agents of up to 15% of all human malignancies. During the recent two decades, the invention of new molecular techniques such as Next Generation Sequencing technology has marked a turning point by helping to discover new viruses, including novel members of human polyomaviruses (HPyVs). However, only very few HPyVs have been involved in neoplastic human diseases, which is mainly MCPyV, which was identified to be the major etiological agent of 80% of Merkel cell carcinomas (MCC). Much remains to be investigated concerning the putative role of HPyVs in human carcinogenesis. The main aim of the research described in this thesis was to screen and test for the presence of novel HPyVs in diverse human cancer tissues to get more insight into the role and possible involvement of these viruses in human tumorigenesis. For this purpose, we used FFPE tissues and applied a diverse sensitive, and specific set of molecular techniques to detect HPyV's at a single-cell level within the histomorphological context.

Chapter one is a general introduction summarizing the direct or indirect role of DNA and RNA tumor viruses in carcinogenesis. Furthermore, this chapter focused more on describing the polyomaviruses in general, then a brief background of each known HPyV. In addition, the outline and the aim of the research works in this thesis are explained. In **chapter two**, the current knowledge on the emerging role of the skin-associated HPyV6 and 7 in human cancers was comprehensively reviewed. Notably, previous studies revealed that the seropositivity of HPyV6 was found to be higher than HPyV7 and increased with age. Although an interesting finding, HPyV6 and 7 prevalence were higher in malignancy tissues than in non-neoplastic tissues. Furthermore, HPyV6 was detected more frequently in skin malignancies while HPyV7 was found to be more frequent in other cancers such as thymoma and cholangiocarcinoma. Based on the reviewed studies, we concluded that HPyV6 and 7 remain important putative candidates possibly contributing to the etiopathogenesis of human disease especially skin tumors.

In **chapter three**, the presence of novel HPyVs in cholangiocarcinomas (CCA) was assessed to evaluate their possible contribution to the etiology of this human malignancy. Only recently, the presence of HPyV6 DNA (27%) has been reported by a Chinese research group in the bile fluid of CCA patients by PCR. Here, we aimed to investigate the prevalence of HPyVs in CCA tissues to elucidate possible clinicopathological correlations between HPyVs and CCA. Interestingly, HPyV7 (69%) was highly prevalent in the CCA cohort, the next most frequent was MCPyV (24%) followed by HPyV6 (14%). An important finding of this study was that HPyV7, HPyV6, and MCPyV are hepatotropic viruses and able to infect non-neoplastic human hepatocytes, bile duct

epithelium, and CCA tumor cells. Yet, it remains unclear if and how HPyVs might contribute to the tumorigenesis of CCA. An interpretation of these results could be that the frequent finding of HPyV's in the adjacent peritumoral hepatocytes might suggest a robust indirect role of these viruses by leading to chronic inflammation which ends up inducing CCA transformation.

BKPyV is well known to reactivate under immunosuppression, especially in the context of solid organ transplantations, e.g. kidney transplantations. BKPyV has been suspected to be a putative oncogenic virus in the development of urothelial cell carcinomas (UCC) in immunocompromised patients. In **chapter four** we assessed whether reactivation of BKPyV in Decoy-positive urine cytology specimens (UCS) predicts or plays a role in the development of UCC of the urinary bladder. We showed that BKPyV detection was not restricted to the urine samples of patients who were diagnosed with UCC of the urinary bladder but also detected in the urine of non-UCC patients. In contrast to the UCC tissue specimens, all tested FFPE tissues were BKPyV-negative by IHC and PCR. Therefore, BKPyV reactivation is not restricted to immunosuppression but also can be found in the UCC of the immunocompetent patients. Therefore, the role of BKPyV in the oncogenesis of the majority of urothelial cell carcinoma of the bladder is rather unlikely.

In **chapter five** we investigated the prevalence of MCPyV in human thymoma, which is a rare malignancy of unknown etiology. In animal models, Murine polyomavirus (MuPyV) had been reported to induce thymomas after injecting MuPyV capsid protein (VP1) in neonatal mice. In addition, a few years ago our research group demonstrated the high prevalence of HPyV7 in thymic epithelial tumors. However, we aimed to uncover if the recent tumor virus identified MCPyV is playing a role in the etiopathogenesis in human epithelial thymic tumors. The presence of MCPyV-DNA was detected in 19.4% of the cohort and the DNA, RNA, and protein of MCPyV were shown in the single-cell level of epithelial thymoma. The most likely conclusion for these findings is that MCPyV expression does not act the same mechanism as seen in MCC. Thus, the contribution of MCPyV to the etiopathogenesis of thymoma is unlikely at least based on our dataset which revealed less expression of viral DNA, RNA, and proteins in tumor cells.

In **chapter six**, tissues obtained from 119 patients with head and neck squamous cell carcinoma (HNSCC) who had no history of smoking tobacco or drinking alcohol were investigated for the presence of human papillomavirus (HPV), Epstein-Barr virus (EBV), and MCPyV. HPV and EBV have previously been shown to be important etiological factors in the development of HNSCC. Our data confirmed the presence of HPV and EBV in patients with HNSCC. In contrast, MCPyV-LTAg expression was not detected by both RNA-ISH and IHC. This is in contrast with previous studies which reported that MCPyV-DNA was detected in tongue SCC. The most likely explanation for this

overt contradiction is simply explained by the fact that we assessed the presence of MCPyV-LTag expression on the single-cell level using RNA-ISH and IHC while the other studies tested their cohort by DNA PCR only. Our findings strongly support that there is no etiological role for MCPyV in HNSCC tumorigenesis. In **chapter seven**, a general discussion of the major findings of this thesis is presented.

ADDENDUM

Impact

Social relevance

Cancer is a leading global cause of death, accounting for nearly 9.6 million deaths worldwide in 2018¹. The global burden of cancer is rapidly rising because of population aging and low socioeconomic communities^{1,2}. To date, the causative agent of many cancers remains unknown. Therefore, unraveling the underlying causes of diverse human cancers is crucial to mitigate the cancer burden by protecting the population from exposure to the respective carcinogens or at least limiting it by promoting novel prevention and therapeutic approaches.

Among all human cancers, approximately 10-15% of cancers are attributed to viral infection agents^{1,3-5}. Shedding light on the detection of novel human tumor viruses is an important opportunity for cancer research by promoting novel prevention, and as a potential tool, early detection of these viruses might contribute to preventing early-stage pre-malignant lesions and thus progression to cancer. Importantly, some of the known human cancers induced by viruses can be prevented by vaccination^{1,6,7}. Human papillomavirus (HPV) which is directly linked to the etiopathogenesis of cervical carcinoma is a successful example from the past. The incidence of cervical cancer has already markedly declined in countries implementing the HPV vaccinations^{1,6,8}. Additionally, the finding of HPV as an etiological factor of cervical carcinoma opened the gate to uncover the association of HPV with several other neoplasms including vagina, vulva, head, neck, anal, and penile carcinomas^{1,2}. Therefore, there is a global call to all researchers to elucidate if currently known viruses or still to be discovered ones are implicated in human tumorigenesis. There is a continuous ongoing scientific debate regarding the role of human polyomaviruses (HPyVs) in human tumorigenesis. Here we focused to explore and providing more information on the role of HPyVs in human malignancies.

Target group

This dissertation aims to broaden and deepen our understanding of the presence and the possible role of HPyVs in human tumorigenesis. The HPyVs genome organization shares many similarities with the HPVs genome. Based on this it has been speculated that HPyVs might be involved in human tumorigenesis analogous to HPV⁹⁻¹¹. Our datasets could be of interest to many researchers, especially in the field of viral-related tumorigenesis. Additionally, we hope the outcome of this work will serve as a beneficial reference for researchers and professionals in viruses related to cancers in the future¹²⁻¹⁶. Yet, the presence of HPyVs defined in this thesis is insufficient to be translated into preclinical or clinical practice until further confirmation of their role in the tumorigenesis. The recommendation of this thesis is that HPyV's remain candidates for human tumorigenesis, but more effort is needed to develop more advanced molecular techniques to elucidate the role of HPyV's in cancer.

Scientific impact

The promising results obtained from the studies in this thesis have added to scientific knowledge since all the outcomes were published in a peer-reviewed journal as an open-access manuscript to make them freely accessible online and presented at international and national congresses¹²⁻¹⁶. Additionally, the results of this thesis possibly provide the basis for relevant follow-up studies on HPyV's and human tumorigenesis.

The oncogenic potential role of the novel HPyVs in human tumorigenesis has not yet been completely uncovered except for MCPyV, which is the causative agent of 80% of MCC¹⁷⁻²⁰. It is important to note that MCPyV-positive MCC appears to have better clinical outcomes and drug responses and a favorable survival rate compared to MCPyV-negative MCC²¹⁻²⁵. Given the potential implications of HPyV's in tumorigenesis, much more needs to be done to broaden the understanding of the involvement of these viruses in human carcinogenesis. To meet the immediate needs of elaborating the role of HPyV's, we aimed to provide insight into the presence of these viruses in various human malignancies and assess their association with tumors using a broad spectrum of molecular techniques.

HPyV6 and 7 contributions to human tumorigenesis are not yet elucidated. Studies about HPyV6 and 7 which shown a high seroprevalence in the human population and the ability to infect newborns¹³. Therefore, HPyV6 and 7 remain putative oncogenic viruses in humans for e.g. skin tumors as we discussed in detail in **chapter 2**. Moreover, a lot remains to be elucidated about HPyV6 and 7 such as their oncogenic potential, replication, latency, and tropism. This information will eventually help to understand the possible role of HPyV6 and 7 in carcinogenesis. As a consequence, these findings will be of major interest to stimulate further studies by researchers to bring advances in this field.

The etiology of Cholangiocarcinoma (CCA) is still largely unknown HPyV7, HPyV6, and MCPyV are reported for the first time as hepatotropic viruses and infecting bile duct epithelium and hepatocytes as shown in **chapter 3**¹⁴. The contribution of HPyV's to CCA carcinogenesis has not been definitely elucidated, but it is speculated that HPyV7 might act through an indirect mechanism to induce (chronic) inflammation as has been convincingly shown for Hepatitis B and C viruses in leading to hepatocellular carcinoma. In addition, the results and knowledge obtained from the study in **chapter 3** will pave the way to study the possible role of HPyV's in the etiopathogenesis of other hepatobiliary diseases.

Early reports showed that that BK polyomavirus (BKPyV) is highly oncogenic in animal models³⁰. This raised the question of whether BK polyomavirus (BKPyV) is a potential oncogenic virus in the development of urothelial cell carcinomas (UCC) in the urinary bladder³¹. In 2012, the International Agency for Research on Cancer

(IARC) classified BKPyV as a group 2B possibly carcinogenic candidate to humans³². that the data in **chapter 4** described that the reactivation of BKPyV detected in urine cytology is not associated with UCC¹⁵. In addition, BKPyV-reactivation is not restricted to immunosuppression but is also found in the urine of immunocompetent patients. Therefore, those findings open a new direction for the research which is that the intravesical treatment of UCC is possibly causing BKPyV reactivation, there is a need to perform further research to elucidate this relationship.

Thymoma is a rare tumor of unknown etiology. Previously, Murine polyomavirus was shown to induce thymomas in an animal model³³. In addition, a high prevalence of HPyV7 in thymic epithelial tumors was reported³⁴. MCPyV is closely linked to the etiopathogenesis of MCC and yet the only human carcinogenic polyomavirus^{20, 35, 36}. Therefore, screening if MCPyV is involved in other human tumors is highly needed such as if MCPyV is playing a role in the etiopathogenesis in human thymoma¹⁶. The contribution of MCPyV to the etiopathogenesis of thymoma is unlikely as described in **chapter 5**. Altogether, HPyVs remain suspected as a putative oncogenic virus in the development of thymomas. Therefore, further research is needed to investigate other HPyV's in thymomas.

In 2018, about 453,000 deaths of head and neck squamous cell carcinoma (HNSCC) and 888,000 new cases were diagnosed¹. Importantly, HPV is linked to the etiopathogenesis of HNSCC, particularly the oropharynx tumor. Given the potential role of involvement of HPV in HNSCC, other important factors correlated to the tumorigenesis such as aging, smoking, alcohol consumption, and gender^{1, 2}. A unique large cohort of non-smokers and non-drinkers with HNSCC is investigated for the presence of three known human tumor viruses (HPV, EBV, and MCPyV) in **chapter 6**¹². the results confirmed the presence of HPV and EBV in patients with HNSCC and no MCPyV was detected in this subset. This indicating that no etiological role of MCPyV in HNSCC tumorigenesis. Therefore, screening for other HPyVs in a cohort diagnosed with HNSCC without a history of smoking and alcohol drinking is needed,

Finally, the presence of HPyV's DNA and their association with human malignancies have commonly been tested by PCR alone which is insufficient to prove causality. Very few studies have shown the presence of these viruses on the single-cell level in the histomorphological context of the specific disease. In this thesis, advanced sensitive and specific molecular techniques were implemented such as FISH, RISH, and IHC to accurately determine the presence of HPyVs on a single cell level as we have shown in **chapters 3 to 6**. Even though, the molecular techniques we used (FISH, RISH, and IHC) for detecting the DNA, RNA, and protein of HPyVs are valuable assessment methods but remain insufficient to confirm the role of the respective virus in tumorigenesis.

Implementing new powerful molecular technologies such as NGS to study the role of these viruses as potential oncogenic will seem to be a promising interesting avenue for further research. Therefore, there is an urgent need to apply additional molecular techniques to unravel the molecular basis of virus-host interactions and assess a possible role of viral integration or mutation of the LTA_g in cancer development.

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ADDENDUM

List of abbreviations

AChR	Anti-acetylcholine receptor
AJCC	American Joint Committee on Cancer
ALHE	Angiolymphoid hyperplasia with eosinophilia
ATL	Aggressive T-cell lymphoma
BCC	Basal cell carcinoma
BCG	<i>bacillus</i> Calmette–Guerin
BKPyV	BK polyomavirus
bp	Base pair
CCA	Cholangiocarcinoma
ChPyV	Chimpanzee polyomavirus
CLL	Chronic lymphocytic leukemia
CMV	Cytomegalovirus
CTCL	Cutaneous T-cell lymphoma
DapB	Dihydrodipicolinate reductase gene
dCCA	Distal cholangiocarcinoma
DFS	Disease free survival
DNA	Deoxy ribonucleic acid
EBER	EBV encoded RNA
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assays
FF	Fresh frozen tissue
FFPE	Formalin fixed paraffin embedded
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GST	Glutathione-S-transferase
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV-1	Human immunodeficiency virus 1
HNSCC	Head and neck squamous cell carcinoma
HPF	High power field
HPV	Human papillomavirus
HPyV's	Human polyomaviruses
HPyV 6	Human polyomavirus 6
HPyV 7	Human polyomavirus 7
HPyV 9	Human polyomavirus 9
HPyV 10	Human polyomavirus 10
HPyV 12	Human polyomavirus 12
Hs-PPIB	Human peptidylprolyl isomerase B

HTLV-1	Human T-lymphotropic virus-1
IARC	International Agency on Research for Cancer
ICC	Immunocytochemistry
iCCA	Intrahepatic cholangiocarcinoma
ICTV	International Committee on Taxonomy of Viruses
IHC	Immunohistochemistry
ISH	In situ hybridization
JCPyV	JC polyomavirus
KS	Kilobase
KIPyV	Karolinska institute polyomavirus
KS	Kaposi sarcoma
KSHV	Kaposi's sarcoma herpesvirus
LIPyV	Lyon IARC polyomavirus
LTA _g	large tumor antigen
MCC	Merkel cell carcinoma
MCPyV	Merkel cell polyomavirus
MG	Myasthenia gravis
MM	Malignant melanoma
mRNA	Messenger ribonucleic acid
MuPyV	Murine polyomavirus
MWPyV	Malawi polyomavirus
NGS	Next generation sequencing
NJPyV	New jersey polyomavirus
NPA	Nasopharyngeal aspirates
NPSCC	Nasopharyngeal squamous cell carcinoma
NSND	Non-smokers and non-drinkers
OPSCC	Oropharyngeal squamous cell carcinoma
OS	Overall survival
OSCC	Oral squamous cell carcinoma
pCCA	Perihilar cholangiocarcinoma
PCR	polymerase chain reaction
PML	progressive multifocal leukoencephalopathy
PNA	Peptide nucleic acid
PP2A	Protein phosphatase 2A
pRB	Retinoblastoma protein
PVAN	Polyomavirus-associated nephropathy
PyVNA	BKPyV-associated nephropathy
PyVs	Polyomaviruses
QPyV	Quebec polyomavirus

RCA	Rolling circle amplification
RISH	RNA in situ hybridization
SCS	Specimen control size
SSC	Squamous cell carcinoma
sTAg	Small tumor antigen
StLPyV	St. louis polyomavirus
SV40	Simian virus 40
TAg	Tumor antigen
TMA _s	Tissue microarray
TNM	Tumor-node-metastasis
TS	Trichodysplasia spinulosa
TSPyV	Trichodysplasia spinulosa associated polyomavirus
UCC	Urothelial cell carcinoma
UCS	Urine cytology specimens
VLP	Virus-like particles
VP1	Viral protein 1
WHIM	Warts, hypogammaglobulinemia, infections, and myelokathexis
WUPyV	Washington university polyomavirus

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ADDENDUM

Acknowledgments

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Curriculum Vitae

Faisal Mahdi Klufah was born on October 26th, 1984, in Jazan, Saudi Arabia. His interest in medical laboratory sciences began after he completed his three-years associate degree in medical laboratory technology at King Khalid University in 2006. In the same year, he started working as a medical lab technician at Sharurah General Hospital, Sharurah, Saudi Arabia. In September 2007, he received a scholarship from the King Abdullah Scholarship Program to pursue his bachelor's and master's studies in the United States. In 2012, he obtained his bachelor's degree in clinical laboratory science at Northern Illinois University in DeKalb, Illinois states. Also, he received the opportunity to perform a one-year clinical training as medical lab scientist at Rockford Memorial Hospital in Rockford, Illinois states. In June 2012, he passed the board exam of the American Society for Clinical Pathology (ASCP) and was certified as Medical Laboratory Scientist (MLS). In May 2014, he completed his master's degree in medical laboratory science with pathology subspecialty at Quinnipiac University in Hamden, Connecticut state. Afterward, he went back to Saudi Arabia and began working as a director of diagnostic laboratory services in Sharurah General Hospital. In December 2014, he moved to work as a lecturer at Albaha University in the department of laboratory medicine in the faculty of applied medical sciences besides working as a medical laboratory specialist at Albaha University Medical Center. In April 2017, he carried out his PhD trajectory in the Pathology Department, Maastricht University Medical Centre+ within the School for Oncology and Developmental Biology (GROW) at the faculty of Health, Medicine, and life sciences at Maastricht University. He worked under the supervision of prof. Dr. Axel zur Hausen, prof. Dr. Ernst Jan M. Speel, and Dr. Véronique Winnepenninckx for the project entitled "Towards Uncovering Polyomavirus-Carrying Human Cancers: Pathology and Implications for Tumorigenesis". After finishing this PhD thesis, Faisal is going back to continue his work at Albaha University.

ADDENDUM

List of publications and congresses participation

Publications:

Klufah F, Mobaraki G, Chteinberg E, Alharbi RA, Winnepeninckx V, Speel EJM, et al. High Prevalence of Human Polyomavirus 7 in Cholangiocarcinomas and Adjacent Peritumoral Hepatocytes: Preliminary Findings. *Microorganisms*. 2020;8(8).

Klufah F, Mobaraki G, Hausen AZ, Samarska IV. Reactivation of BK Polyomavirus in Urine Cytology Is Not Associated with Urothelial Cell Carcinoma. *Viruses*. 2020;12(12):1412.

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Chteinberg E, **Klufah F**, Rennspiess D, Mannheims MF, Abdul-Hamid MA, Losen M, et al. Low prevalence of Merkel cell polyomavirus in human epithelial thymic tumors. *Thorac Cancer*. 2019;10(3):445-51.

Oral presentations at scientific congresses:

F. Klufah, E. Chteinberg, R. A. Alharbi, A. K. Kurz, V. Winnepeninckx, E.-J. Speel, A. zur Hausen
“Detection of human polyomavirus 6 and 7 in the human cholangiocarcinoma tissues”
103. Jahrestagung der Deutschen Gesellschaft für Pathologie (German Society for Pathology), Frankfurt Am Main, Germany, 2019 June 13–15

F. Klufah, G. Mobaraki, E. Chteinberg, R. A. Alharbi, A. K. Kurz, V. Winnepeninckx, E.-J. Speel, I. Samarska, A. zur Hausen
“Detection of Human Polyomavirus 6 and 7 in the Human Cholangiocarcinoma Tissues”
ICGEB DNA Tumour Virus Meeting – 50th Anniversary, Trieste, Italy, 2019 July 9th–14th

Poster presentations at scientific congresses:

F. Klufah, E. Chteinberg, G. Mobaraki, R.A. Alharbi, A.K. Kurz, V. Winnepenninckx, E. Speel, A. zur Hausen

“Detection of human polyomavirus 6 and 7 in the human cholangiocarcinoma tissues”
31st European Congress of Pathology, Nice, France from 7–11 September 2019

F. Klufah, G. Mobaraki, E. Chteinberg, R.A. Alharbi, A.K. Kurz, V. Winnepenninckx, E. Speel, I. Samarska, A. zur Hausen

“Human Polyomaviruses 6 and 7 and Merkel Cell Polyomavirus are Hepatotropic viruses”
The United States and Canadian Academy of Pathology (USCAP) 2020, Los Angeles Convention Center – Los Angeles, CA, February 28-March 5, 2020

F. Klufah, G. Mobaraki, A. zur Hausen, I. Samarska

“Detection of Human Polyomavirus JCPyV DNA in Urothelial Carcinomas of Urinary Bladder”
33rd European Congress of Pathology, 29–31 August 2021

I. Samarska, **F. Klufah**, G. Mobaraki, A. zur Hausen

“Relation of BK virus-positive urinary cytology to urothelial cell carcinoma of the urinary bladder”
The United States and Canadian Academy of Pathology (USCAP) 2020, Los Angeles Convention Center – Los Angeles, CA, February 28-March 5, 2020

F. J. Mulder, **F. Klufah**, F. M. E. Janssen, F. Farshadpour, S. M. Willems, R. de Bree, A. zur Hausen, M. F. C. M. van den Hout, B. Kremer, and E. M. Speel 2 “The usual viral suspects in head and neck cancer of non-smokers and non-drinkers: an analysis of HPV, EBV, and MCPyV”

7th WORLD CONGRESS of the International Academy of Oral Oncology, IAOO 2019, ROME Italy 31 August to 3 September

ADDENDUM

Summary in Arabic

واللوزتين، إلا أنه لم يثبت وجود علاقة سببية بين وجود هذا الفيروس في تكوّن هذه السرطانات إلى الآن. يعتبر سرطان الخلايا الحشفية في الرأس والرقبة (HNSCC) من السرطانات الخبيثة التي يمكن أن تصيب تجاويف الفم، الحلق، الأنف، البلعوم، أو الحنجرة، لذلك في **الفصل السادس** من هذه الرسالة، تم فحص أنسجة ورمية أخذت من ١١٩ مريضاً مصاباً بسرطان الخلايا الحشفية في الرأس والرقبة (HNSCC) ليس لديهم أي من عوامل الخطورة المعروفة وهي تدخين التبغ أو شرب الكحول لاختبار احتمال وجود فيروس الورم الحليمي البشري (HPV)، وفيروس إبشتاين بار (EBV)، أو فيروس ميركل الورمي المتعدد (MCPyV) في هؤلاء المرضى. إجمالاً، أكدت النتائج في هذا الفصل وجود فيروس الورم الحليمي البشري وفيروس إبشتاين بار في المرضى الذين يعانون من سرطان الرأس والرقبة. حيث أظهرت النتائج أن جميع أورام البلعوم الفموي في المجموعة التي فحصناها إيجابية لفيروس الورم الحليمي البشري بنسبة (١٠٠٪) بينما كان ورم فموي واحد إيجابي يمثل (١٪) وورم أنفي بلعومي واحد إيجابي ويمثل (٢٥٪) من المجموعة البحثية، بالإضافة إلى ذلك كانت ثلاث عينات لأورام الأنف البلعومي إيجابية لفيروس إبشتاين بار بنسبة (٧٥٪)، والنتائج في هذا الفصل متوافقة مع الدراسات السابقة في نفس المجال. ومن المثير للاهتمام أن جميع العينات النسيجية من المرضى في هذه الدراسة تم فحصها للكشف عن وجود الحمض النووي الريبي وبروتين فيروس ميركل الورمي المتعدد وكانت جميعها سلبية. وهذا على النقيض من الدراسات التي وجدت أن الحمض النووي لهذا الفيروس في أورام اللسان واللوزتين. يمكن تفسير هذا التناقض بأن الدراسات السابقة استخدمت فحص تفاعل البلمرة المتسلسل (PCR) وهو اختبار لا يمكن منه تحديد أي من الخلايا مصابة بالفيروس، بينما في دراستنا استخدمنا تقنيات حيوية جزيئية لتحديد وجود الفيروس في الخلايا السرطانية. والنتائج التي توصلنا إليها تدعم بقوة إلى أنه لا يوجد دور رئيسي لفيروس ميركل الورمي المتعدد (MCPyV) في تكوّن أورام الرأس والرقبة.

أخيراً في **الفصل السابع** من هذه الرسالة، قمت بعرض مناقشة عامة لأهم النتائج التي توصلت إليها هذه الأطروحة البحثية.

عينات بول مرضى سرطان المثانة البولية، بل تم اكتشاف الفيروس أيضاً في عينات بول مرضى يعانون من أمراض غير سرطانية، على سبيل المثال مرضى زراعة الكلى أو الأمراض المزمنة التي تتطلب تثبيط المناعة. على النقيض من عينات البول، بينت النتائج في هذا الفصل أن جميع عينات الأنسجة السرطانية الموجودة في ورم المثانة البولية سلبية للفيروس للمرضى المصابين بسرطان المثانة البولية باستخدام فحص تفاعل البلمرة المتسلسل (PCR) والكيمياء النسيجية المناعية (IHC). علاوة على ذلك، نتائج هذا الفصل تشير إلى أن إعادة تنشيط الفيروس الورمي المتعدد الأول (BKPyV) لا يقتصر على تثبيط المناعة فحسب، بل يمكن أن يعيد الفيروس نشاطه في مرضى سرطان المثانة للمرضى غير المثبطين مناعياً. لذلك خلص هذا الفصل إلى أنه من الأرجح لا يوجد دور للفيروس الورمي المتعدد الأول (BKPyV) في تكوّن الأورام لغالبية سرطان خلايا الظهارة البولية في المثانة.

من الأورام التي ما زال سبب تكونها مجهول هو ورم الغدة الصعترية (Thymoma)، والذي يطلق عليه ورم التوتة، يعتبر من الأورام النادرة التي تحدث في الغالب عند الأشخاص الذين تجاوزوا الخمسين عاماً. من الدراسات التي قام بها الباحثون لمعرفة إن كان للفيروسات دور في هذا الورم، هي دراسة أول فيروس ورمي متعدد اكتشف عام ١٩٥٠م وهو فيروس مورين (MuPyV). حيث قام الباحثون بحقن جزء من بروتين فيروس مورين في فئران حديثة الولادة فوجدوا أنها سببت ورم الغدة الصعترية في الفئران. بالإضافة إلى ذلك، أظهر فريقنا البحثي قبل بضع سنوات عن وجود الفيروس الورمي المتعدد السابع (HPyV7) بنسبة عالية في أورام الغدة الصعترية. لذلك هدفنا في **الفصل الخامس** إلى عمل دراسة للكشف عما إذا كان سرطان الغدة الصعترية (ورم التوتة) قد ينشأ بسبب فيروس ميركل الورمي المتعدد (MCPyV) وهو فيروس معروف بأنه يسبب ورم جلدي خبيث. ومن خلال فحص الأنسجة السرطانية للغدة الصعترية باستخدام تفاعل البلمرة المتسلسل (PCR) وجدت الدراسة أن فيروس ميركل الورمي المتعدد موجود بنسبة ١٩،٤٪. كما أظهرت البيانات أن وجود الحمض النووي، والحمض النووي الريبي، وبروتين فيروس ميركل على مستوى الخلية أقل في الخلايا السرطانية لورم التوتة، وبالتالي؛ من غير المحتمل أن يساهم فيروس ميركل في تكوّن ورم التوتة.

في الأونة الأخيرة، كشفت الدراسات إلى أنه يوجد عوامل عديدة قد تزيد من خطورة سرطانات الرأس والرقبة، ومن بينها على سبيل المثال التدخين والمشروبات الكحولية أو مسببات أخرى وراثية أو بيئية. بالإضافة لهذه العوامل أثبتت الدراسات أن التعرض لفيروس الورم الحليمي البشري (HPV) أو فيروس إبشتاين بار (EBV) لهما علاقة مباشرة في الإصابة ببعض أنواع سرطانات الرأس والرقبة. وكشف باحثون مؤخراً عن وجود فيروس ميركل الورمي المتعدد (MCPyV) في عينات سرطانات اللسان

الفصل الثالث من هذه الرسالة تم تقييم وجود الفيروسات الورمية المتعددة البشرية الجديدة في أنسجة سرطان القنوات الصفراوية من أجل التحقق من مساهمتها المحتملة في تكوّن هذا الورم الخبيث. الهدف من هذا البحث هو استكشاف نسبة انتشار الفيروسات الورمية المتعددة على مستوى أنسجة وخلايا سرطان القنوات الصفراوية بالإضافة لدراسة أي ارتباطات سريرية مرضية محتملة بين الفيروس والورم. فمن خلال فحص الأنسجة السرطانية باستخدام تفاعل البلمرة المتسلسل (PCR) وجدنا نسبة انتشار واسع للفيروس الورمي المتعدد السابع (HPyV7) تصل إلى ٦٩٪، بينما ٢٤٪ من الأنسجة المفحوصة كانت إيجابية لفيروس خلايا ميركل الورمي المتعدد (MCPyV)، وكان الفيروس الورمي المتعدد السادس (HPyV6) أقل انتشاراً بنسبة ١٤٪. كما تم تأكيد وجود هذه الفيروسات في الأنسجة الورمية وأنسجة الكبد المجاورة لها باستخدام تقنيات حيوية جزيئية متقدمة مثل التهجين الموضعي المتألق للحمض النووي (FISH) وللحمض النووي الريبي (RISH) واستخدام كيمياء المناعة النسيجية لتحديد بروتين الفيروس (IHC). من النتائج المهمة التي توصلنا إليها في هذه الدراسة، أن هذه الفيروسات الورمية المتعددة الثلاثة (السابع، والسادس، وميركل) تعتبر فيروسات كبدية أي قادرة على إصابة خلايا الكبد غير الورمية بالإضافة إلى إصابة خلايا القنوات الصفراوية الطبيعية والورمية معاً. ومع ذلك، إلا أنه لا يزال من غير الواضح ما إذا كانت هذه الفيروسات تساهم في تكوّن أورام القناة الصفراوية بشكل مباشر أو لا. قد يكون تفسير هذه النتائج هو أن الاكتشاف المتكرر للفيروسات الورمية المتعددة خاصة الفيروس السابع (HPyV7) في الخلايا الكبدية الصفافية المجاورة للقنوات الصفراوية يشير إلى دور قوي غير مباشر لهذه الفيروسات من خلال التسبب في التهاب مزمن ينتهي بتحفيز تكوّن سرطان القناة الصفراوية. إلا أنه ما تزال الحاجة إلى إجراء المزيد من الأبحاث للوصول إلى نتائج حاسمة.

يعتبر سرطان المثانة (Urothelial Cell Carcinoma) نوع شائع من الأورام وغالباً ما يبدأ سرطان المثانة في خلايا الظهارة البولية التي تبطن المثانة من الداخل، كما أنه لا يعرف السبب الرئيس لسرطان المثانة، لكن هناك عوامل قد تكون مرتبطة بهذا الورم مثل الوراثة أو التعرض للمواد الكيميائية. مؤخراً اشتبه الباحثون من دول مختلفة في أن الفيروس الورمي المتعدد الأول (BKPyV) يحتمل أن يكون مسبب لأورام سرطان خلايا الظهارة البولية (UCC) في المرضى الذين يعانون من نقص المناعة. كما بات من المعروف أن الفيروس الورمي المتعدد الأول (BKPyV) يكون كامناً في الخلايا الطلائية الأنوبية الكلوية ويعاود نشاطه عند تثبيط المناعة، خصوصاً بعد عمليات زراعة الأعضاء كزراعة الكلى. لذلك في **الفصل الرابع** من الرسالة، قمنا بفحص الخلايا الطلائية المتواجدة في عينات بول من مرضى تم تشخيصهم بسرطان المثانة البولية لتقييم ما إذا كان الفيروس الورمي المتعدد الأول أعيد تنشيطه في هؤلاء المرضى. أظهرت النتائج أن وجود الفيروس الورمي المتعدد الأول (BKPyV) في حالته النشطة لم يقتصر على

الرئيسي من الأبحاث في هذه الرسالة هو تسليط النظر على الآثار الخطيرة المحتملة عن تواجد الفيروسات الورمية المتعددة الجديدة في الخلايا والأنسجة السرطانية البشرية المتنوعة، بالإضافة إلى تحري احتمالية دور هذه الفيروسات في عملية تكوّن الأورام وذلك من خلال استخدام تقنيات جزيئية متنوعة وحساسة لفحص وجود هذا النوع من الفيروسات في الأنسجة والخلايا السرطانية.

اشتمل **الفصل الأول** من هذه الرسالة على مقدمة عامة تلخص الدور المباشر أو غير المباشر للفيروسات التي تسبب أورام بشرية. حيث تم اكتشاف ثماني فيروسات مسببة للسرطان إلى الآن، وأيضاً يشتهر في أنه مازال هناك فيروسات تسبب الأورام لم تكتشف بعد. في هذا الفصل تم التركيز بشكل موسع على الوصف العام لكل فيروس ورمي متعدد (HPyV) يصيب الإنسان بالإضافة لدورها في الأمراض. فيوجد خمس فيروسات ورمية متعددة لها علاقة بأمراض مختلفة غير سرطانية وفيروس واحد فقط له علاقة بتكون ورم جلدي. في **الفصل الثاني** تضمن مراجعة شاملة لما هو معروف حالياً حول ما إذا كان هناك دور لفيروسين مستجدين في الأورام، وهما الفيروس الورمي المتعدد السادس (HPyV6) والسابع (HPyV7) من الفيروسات الورمية المتعددة البشرية المرتبطة بالأمراض الجلدية. الجدير بالذكر أن الدراسات الطبية السابقة كشفت أن الإصابة بالفيروس السادس تعتبر أكثر شيوعاً من الفيروس السابع وتزيد نسبة الإصابة بالفيروسين مع التقدم في العمر. من المثير للاهتمام في هذا الفصل، وجدنا أن الفيروسين السادس والسابع يتواجدان بكثرة في الأنسجة والخلايا السرطانية مقارنة بالأنسجة والخلايا الغير سرطانية. علاوة على ذلك، لوحظ أن وجود الفيروس السادس أكثر في السرطانات الجلدية بينما وجود الفيروس السابع كان أكثر انتشاراً في سرطانات أخرى مثل سرطان القنوات الصفراوية وورم الغدة الصعترية. وبناءً على الدراسات التي تمت مراجعتها، خلص **الفصل الثاني** إلى احتمالية أن الفيروسين السادس والسابع من عائلة الفيروسات الورمية المتعددة يظان مرشحين في المساهمة في الأمراض التي تصيب الإنسان خصوصاً الأورام الجلدية.

يعتبر سرطان القنوات الصفراوية (CCA) ورم نادر وشديد الضراوة ويتشكل في الأنابيب التي تحمل سائل الصفراء الهضمي، ومازالت أسباب هذا السرطان غامضة إلى حد كبير. حيث يوجد عوامل قد تزيد من خطر الإصابة بهذا السرطان وتشمل التهاب القنوات الصفراوية المصلب الابتدائي، وحصوات الكبد، والتهاب الأمعاء، وتليف الكبد، ومرض السكري، وتكيسات القنوات الصفراوية. وتعد العدوى بالميكروبات عاملاً خطيراً للإصابة بهذا الورم أيضاً، مثل الطفيليات الكبدية، وفيروسات التهاب الكبد الوبائي (ب) و (ج). في الآونة الأخيرة، قام باحثون من الصين بالكشف عن وجود ٢٧٪ من الحمض النووي للفيروس الورمي المتعدد السادس (HPyV6) في السائل الصفراوي لمرضى سرطان القنوات الصفراوية. لذلك في

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نبذة مختصرة عن الرسالة

يعد السرطان من أحد الأسباب الرئيسية للوفيات في جميع أنحاء العالم ويعتبر مصدر قلق رئيسي للصحة العامة. فحسب الوكالة العالمية لبحوث السرطان أنه تم تشخيص ما يقارب من ١٨ مليون حالة سرطان جديدة في عام ٢٠١٨م، كما تسبب السرطان في وفاة ١٠ مليون حالة تقريباً في ذات العام. لا يزال معدل الإصابة والوفيات بالسرطان يتزايد بسرعة في جميع أنحاء العالم، إلا أن العديد من مسبباته لا تزال غير معروفة إلى حد كبير وبالتالي يصعب علاجها والحد من ازديادها. ومن ضمن العوامل الأكثر شيوعاً التي تساهم في خطر الإصابة بالسرطان التعرض للمواد الكيميائية، التدخين، الكحول، الإشعاع، أو وراثية الطفرات الجينية. من المثير للاهتمام أن الإصابة بالميكروبات قد تشكل سبباً في زيادة الخطر بالإصابة بالسرطان، حيث إنه منذ فترة طويلة تم إثبات أن الإصابة ببعض أنواع الفيروسات يؤدي إلى تكوّن بعض الأورام الخبيثة. تقدر الأبحاث العلمية أن الفيروسات تلعب دوراً مهماً في نشوء ما بين ١٠ إلى ١٥٪ من جميع الأورام الخبيثة التي تصيب الإنسان. ولقد تمت دراسة أهمية الفيروسات المسرطنة في تكوّن الأورام البشرية بشكل مكثف في العقد الماضي، مما ساهم في فهم مسببات بعض الأورام وتشخيصها وعلاجها. لذلك اكتشاف ودراسة دور الفيروسات المعدية الناشئة التي يحتمل أن تسبب أو تساهم في تكوّن الأورام البشرية يعد فرصة هامة في فهم آلية تكوّن الأورام التي من شأنها أن تساعد في الوقاية والحد من أضرار هذه الفيروسات بشكل فعال.

في هذه الرسالة، سيكون التركيز على الكشف عن الفيروسات الورمية المتعددة البشرية (HPyVs) في سرطانات مختلفة، وهي فيروسات تتميز بحمض نووي صغير. في خلال العقد الماضي زادت عائلة هذه الفيروسات إلى أكثر من ١٠٢ نوع قادرة على إصابة الثدييات والطيور والأسماك. خلال العقد الأخيرين، ساهم اختراع تقنيات حيوية جزيئية جديدة مثل تسلسل الجيل التالي (NGS) في المساعدة على اكتشاف فيروسات جديدة، ومنها اكتشاف أعضاء جدد من عائلة الفيروسات الورمية المتعددة التي تصيب الإنسان (HPyVs). تم اكتشاف اثني عشر فيروس ورمي متعدد يصيب الإنسان إلى الآن، ومنها فيروس واحد فقط وهو فيروس خلايا ميركل الورمي المتعدد (MCPyV) الذي تم إثباته كمسبب رئيس في تكوّن ٨٠٪ من حالات سرطان خبيث يصيب الجلد يسمى سرطان خلايا ميركل (MCC). إلا أنه لا يزال هناك الكثير لم يتم الكشف عنه فيما يتعلق بدور الفيروسات الورمية المتعددة في تكوّن الأورام البشرية. لذلك كان الهدف

شكر وتقدير

الحمد والشكر لله عز وجل على فضله وإليه ينسب الفضل كله بأن سخر لي أسباب الوصول لإتمام هذه الرسالة العلمية التي أرجو أن تكون خالصة لوجهه الكريم.

إلى من اشتاق إليها دوماً **جدتي** الحبيبة، لا تسعني الكلمات في شكرك على كرمك وحبك ودعائك المتواصل والمستمر لي بالتوفيق والسداد.

إلى قدوتي **أبي** وإلى **أمي** نبع المحبة والإيثار حفظكم الله، فمهما كتبت من عبارات الشكر ستظل عاجزة عن إيفائكم حقكم، لكم الفضل بعد الله فيما وصلت إليه فشكراً لكم من كل أعماق قلبي.

إلى رفيقتي في الكفاح وشريكتي في الحياة **زوجتي**، شكراً على وقوفك معي طوال تلك السنوات فكنتي دائماً الداعم الأول لي خلال مسيرتي العلمية. وشكراً لروحي ونبض **فؤادي أبنائي** وبناتي فوجودكم في حياتي هو مصدر التحفيز والسرور لي دائماً.

إلى **إخواني وأخواتي**، شكراً لكم فأنتم دائماً سندي وعضدي في الشدة والرخاء. وإلى **الأقارب والأصدقاء** وكل من دعا لي بالخير وساعدني بشكل أو بآخر لتحقيق هدي، جميعكم يستحق الشكر والثناء.

إلى **وطني** الغالي، لك عظيم الشكر والامتنان على الدعم السخي منذ بداية ابتعاثي للباكالوريوس والماجستير بالولايات المتحدة الأمريكية وإلى أن أنهيت هذه الرسالة بدولة هولندا، مهما قدمت من كلمات الشكر لا أستطيع أن أوافيك حقك.

كما أتوجه بالشكر لجامعة **الباحة والملحقية الثقافية بدولة هولندا** لكل ما قدموه لي من دعم واهتمام طوال فترة الابتعاث.

وأخيراً، لم يكن بإمكانني إكمال هذه الرسالة بدون دعم **المشرفين الدراسيين** بالمركز الطبي بجامعة ماسترخت، فهم من قدم لي النصيحة، والتوجيه لإنجاز هذا العمل فلكم مني كل الشكر والثناء والتقدير.

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